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Effects of physical activity during pregnancy on health of mother and child

- a PhD project based on results from the FitMum randomized controlled trial

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Effects of physical activity during pregnancy on health of mother and child
- a PhD project based on results from the FitMum randomized controlled trial

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Caroline Borup Roland, Copenhagen, February 2022

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Paper 1
Caroline Borup Roland*, Signe de Place Knudsen*, Saud Abdulaziz Alomairah*, Anne Dsane Andersen, Jane Bendix, Tine D. Clausen, Stig Molsted, Andreas Kryger Jensen, Grete Teilmann, Astrid Pernille Jespersen, Jakob Eg Larsen, Gerrit van Hall, Emil Andersen, Romain Barrès, Ole Hartvig Mortensen, Helle Terkildsen Maindal, Lise Tarnow, Ellen Christine Leth Løkkegaard, Bente Stallknecht. (2021). Structured supervised exercise training or motivational counselling during pregnancy on physical activity level and health of mother and offspring: FitMum study protocol. BMJ Open, 11, e043671. http://dx.doi.org/10.1136/bmjopen-2020-043671
*Contributed equally. This paper will also be included in the PhD theses of Signe de Place Knudsen and Saud Abdulaziz Alomairah.

Paper 2
Caroline B. Roland, Signe dP. Knudsen, Saud A. Alomairah, Anne D. Jessen, Ida K. B. Jensen, Nina Brændstrup, Stig Molsted, Andreas K. Jensen, Bente Stallknecht, Jane M. Bendix, Tine D. Clausen*, Ellen Løkkegaard*. Effects of prenatal exercise on gestational weight gain, obstetric and neonatal outcomes: FitMum randomized controlled trial. *In review in American Journal of Obstetrics and Gynecology.*
*Contributed equally.

Paper 3
Caroline B. Roland, Diego Hernandez-Saavedra, Adnan Khan, Kajetan Trost, Thomas Moritz, Nina Brændstrup, Signe dP. Knudsen, Anne D. Jessen, Stig Molsted, Tine D. Clausen, Saud A. Alomairah, Ellen Løkkegaard, Bente Stallknecht, Kristin I. Stanford, Jane M. Bendix. The effects of prenatal exercise interventions on breast milk composition in puerperal mothers. *Unsubmitted manuscript.*

In addition, the author has contributed to these publications during the PhD period (and other manuscripts not yet published):

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SUMMARY

Physical activity during pregnancy is well-established as a safe and beneficial lifestyle component and several mechanisms underlying the exercise-induced improvements of maternal and offspring health have been proposed. Danish and international recommendations prescribe physical activity at moderate intensity for 210 and 150 minutes per week, respectively, throughout pregnancy for all pregnant women with uncomplicated pregnancies. Nevertheless, high prevalence of insufficient physical activity during pregnancy, as well as in general, is a global health challenge and the efficacy of different physical activity intervention strategies needs to be compared to clarify how to increase physical activity level among pregnant women and improve maternal and offspring health most efficiently.

The main objective of this PhD thesis was to investigate the effects of two different physical activity interventions during pregnancy; structured supervised exercise training versus motivational counselling on physical activity, on clinical health outcomes in healthy inactive pregnant women and their offspring compared to standard care, and to explore possible underlying mechanisms for optimized offspring health. Secondary outcomes data from the FitMum study, which design is described in Paper 1, formed the basis for the analyses in the thesis and papers. The focus of this thesis was on gestational weight gain and obstetric and neonatal outcomes during pregnancy and delivery (Paper 2) as well as exercise-induced adaptations to breast milk as a possible underlying mechanism mediating improvements of offspring health (Paper 3). The FitMum study was a randomized controlled trial conducted at the Department of Gynecology and Obstetrics at Nordsjaellands Hospital, Hillerod, Denmark from 2018-2021. The FitMum study randomized 219 pregnant women to one of three study groups; structured supervised exercise training offered three times per week throughout pregnancy (n=87), motivational counselling on physical activity offered through four individual and three groups counselling sessions during pregnancy (n=87), or a control group receiving standard care (n=45).

In Paper 2, we investigated the effects of our two different physical activity interventions on gestational weight gain and obstetric and neonatal outcomes compared to standard care. Overall, we found no effect of any of our interventions on gestational weight gain or obstetric and neonatal outcomes compared to standard care. However, women with obesity in both intervention groups gained less weight compared to women with normal weight within the same intervention groups. Further, associations between physical activity measures and gestational weight gain differed between women with obesity and normal weight. This indicates that pregnant women with obesity may be more susceptible to exercise benefits compared to women with normal weight. One

explanation for the lack of no overall effect of the interventions on gestational weight gain and obstetric and neonatal outcomes could be a relatively low physical activity level in the intervention groups in our study.

The aim of Paper 3 was to investigate the effects of our two physical activity interventions on the human breast milk metabolome and lipidome by performing metabolomic and lipidomic analyses on human breast milk samples obtained 7-14 days after birth. We found no major metabolite or lipid changes with our interventions compared to standard care, possibly due to low physical activity level as well, and several other confounding factors that might blur the effect of prenatal exercise. However, our interventions changed some metabolites and lipids compared to standard care, and some of the metabolites and lipids correlated with physical activity measures. Thus, maternal prenatal exercise may induce changes to the human breast milk metabolome and lipidome, which could partly explain optimized offspring metabolic health.

In conclusion, our findings of no overall effect of physical activity on gestational weight gain and obstetric and neonatal outcomes contrast with previous studies showing beneficial effects of prenatal exercise on these outcomes, but other studies have also shown limited or no effect. The indication of women with obesity possibly being more susceptible to exercise benefits is in line with previous studies. Moreover, we found no major changes in the breast milk metabolome and lipidome, but found changes in some metabolites and lipids, supporting existing literature that propose exercise-induced adaptations to breast milk as an underlying mechanism that contributes to improved offspring health. A renewed effort to increase physical activity level during pregnancy to optimize maternal and offspring health, and subsequent long-term follow-up in human offspring, are warranted. Further, to expand our understanding of underlying mechanisms of improved offspring health, human studies designed to investigate such mechanisms, for example with focus on adaptations in breast milk, placenta, or epigenetic changes, are needed.

DANSK RESUMÉ (SUMMARY IN DANISH)

Fysisk aktivitet under graviditeten er sikkert og kan medføre gavnlige sundhedseffekter for mor og barn, og forskellige underliggende mekanismer er blevet foreslået at mediere sundhedseffekterne. Danske og internationale myndigheder og organisationer anbefaler alle gravide kvinder med en ukompliceret graviditet at være fysisk aktive ved moderat intensitet i henholdsvis 210 og 150 minutter om ugen gennem hele graviditeten. Ikke desto mindre er der høj prævalens af lavt fysisk aktivitetsniveau under graviditet og generelt, hvilket udgør en global sundhedsudfordring, og effektiviteten af forskellige fysisk aktivitets-interventionsstrategier bør sammenlignes for at afklare, hvordan man kan øge det fysiske aktivitetsniveau blandt gravide kvinder og forbedre mødre og børns sundhed mest effektivt.

Hovedformålet med denne ph.d.-afhandling var at undersøge effekterne af to forskellige fysisk aktivitets-interventioner under graviditeten; struktureret superviseret holdtræning versus motiverende vejledning om fysisk aktivitet, på kliniske sundhedsparametre hos raske inaktive gravide kvinder og deres børn i forhold til standardbehandling, samt at undersøge mulige underliggende mekanismer for forbedret sundhed hos børnene. Data fra sekundære endepunkter fra FitMum studiet, hvis design er beskrevet i Artikel 1, dannede grundlag for analyserne i afhandlingen og artiklerne. Fokusområderne for afhandlingen var vægtøgning under graviditeten og obstetriske og neonatale parametre under graviditet og fødsel (Artikel 2) samt ændringer i modermælkssammensætningen som følge af fysisk aktivitet som en mulig underliggende mekanisme, der medierer forbedringer af barnets sundhed (Artikel 3). FitMum studiet var et randomiseret kontrolleret studie, der blev udført på Gynækologisk Obstetrisk Afdeling på Nordsjællands Hospital, Hillerød, Danmark fra 2018-2021. FitMum studiet randomiserede 219 gravide kvinder til en af tre grupper; struktureret superviseret holdtræning, der blev tilbudt tre gange om ugen under hele graviditeten (n=87), motiverende vejledning om fysisk aktivitet, der blev tilbudt gennem fire individuelle og tre gruppevejledningssessioner under graviditeten (n=87), eller en kontrolgruppe, der modtog standardbehandling (n=45).

I Artikel 2 undersøgte vi effekterne af vores to forskellige fysisk aktivitets-interventioner på vægtøgning under graviditeten og obstetriske og neonatale parametre i forhold til en kontrolgruppe der modtog standardbehandling. Samlet set fandt vi ingen effekt af nogen af vores interventioner på vægtøgning under graviditeten eller obstetriske og neonatale parametre i forhold til kontrolgruppen. I begge interventionsgrupper havde svært overvægtige kvinder dog mindre vægtøgning sammenlignet med normalvægtige kvinder inden for de samme interventionsgrupper. Derudover var associationer mellem fysisk aktivitet og vægtøgning under graviditeten forskellige

mellem svært overvægtige og normalvægtige kvinder. Dette indikerer, at svært overvægtige kvinder måske i højere grad opnår gavnlige effekter af fysisk aktivitet under graviditeten i forhold til normalvægtige kvinder. Et forholdsvist lavt fysisk aktivitetsniveau i interventionsgrupperne kan være en forklaring på, at vi overordnet set ikke fandt nogen effekt af interventionerne på vægtøgning under graviditeten og obstetriske og neonatale parametre.

Formålet med Artikel 3 var at undersøge effekterne af vores to fysisk aktivitets-interventioner på metabolomet og lipidomet i human modermælk. Dette blev undersøgt ved hjælp af metabolomics og lipidomics analyser på humane modermælksprøver, der blev indsamlet 7-14 dage efter fødslen. Vi fandt ingen store ændringer i metabolitter eller lipider i vores interventionsgrupper sammenlignet med kontrolgruppen, hvilket muligvis også skyldtes for lavt fysisk aktivitetsniveau i interventionsgrupperne, samt flere andre faktorer, der kunne påvirke modermælks-sammensætningen og dermed sløre en mulig effekt af fysisk aktivitet under graviditeten. Vores interventioner forårsagede imidlertid ændringer af flere metabolitter og lipider i forhold til kontrolgruppen, og nogle af metabolitterne og lipiderne korrelerede med fysisk aktivitet. Fysisk aktivitet under graviditeten kan dermed inducere ændringer i metabolomet og lipidomet i human modermælk, hvilket kan udgøre en underliggende mekanisme for de gavnlige effekter på barnets metaboliske sundhed.

Alt i alt fandt vi ingen overordnet effekt af fysisk aktivitet på vægtøgning under graviditeten eller på obstetriske og neonatale parametre. Dette er i kontrast til tidligere studier, der har vist gavnlige effekter af fysisk aktivitet under graviditeten på disse parametre, men andre studier har også vist begrænset eller ingen effekt. Indikationen af at svært overvægtige kvinder i højere grad opnår gavnlige effekter af fysisk aktivitet under graviditeten i forhold til normalvægtige kvinder er i tråd med tidligere studier. Vi fandt ingen store ændringer i metabolomet og lipidomet i human modermælk, men fandt ændringer i nogle metabolitter og lipider, hvilket understøtter eksisterende litteratur, der peger på ændringer i modermælkssammensætningen som en mulig underliggende mekanisme, der bidrager til forbedret sundhed hos barnet, efter at moderen har været fysisk aktiv under graviditeten. Der synes at være behov for en fornyet og styrket indsats for at øge det fysiske aktivitetsniveau blandt gravide kvinder med henblik på at forbedre sundheden hos mor og barn, samt efterfølgende langsigtet opfølgning af børnenes sundhed. For at udvide vores forståelse af mulige underliggende mekanismer for gavnlige sundhedseffekter hos barnet, er der tilmed behov for humane studier designet til at undersøge sådanne mekanismer, for eksempel med fokus på ændringer i modermælkssammensætning, moderkagen eller epigenetiske forandringer.

ABBREVIATIONS

3’-SL	3’-sialyllactose
12,13-diHOME	12,13-dihydroxy-9Z-octadecenoic acid
BMI	Body mass index
BW	Birth weight
CON	Control/standard care
DXA	Dual-energy X-ray absorptiometry
EXE	Structured supervised exercise training
FAHFA	Fatty acid hydroxy fatty acids
GA	Gestational age
GDM	Gestational diabetes mellitus
GWG	Gestational weight gain
HIT	High-intensity training
HMO	Human milk oligosaccharide
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IL-6	Interleukin-6
LGA	Large for gestational age
MET	Metabolic equivalent
MetS	Metabolic syndrome
min	Minute
mL	Milliliter
MOT	Motivational counselling on physical activity
MRI	Magnetic resonance imaging
MVPA	Moderate to vigorous intensity physical activity
n	Number of participants
OGTT	Oral glucose tolerance test
P	P-value
PA	Physical activity
PAHSA	Palmitic acid ester of hydroxystearic acid
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1 α
SD	Standard deviation
SGA	Small for gestational age
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

INTRODUCTION AND OBJECTIVES

The theme of this thesis is effects of physical activity (PA) during pregnancy on maternal and offspring health and potential underlying mechanisms for health-enhancing adaptations. PA during pregnancy is widely acknowledged as a beneficial and safe lifestyle component[1–3]. The World Health Organization (WHO) recommends all pregnant women without contraindications to be physically active at least 150 minutes (min) per week at moderate intensity throughout pregnancy[1] and in Denmark specifically, the recommendation from the Health Authorities is at least 30 min per day (210 min per week) at moderate intensity[4]. Nevertheless, insufficient PA is a worldwide global health challenge[5–7], and in Denmark, the prevalence of insufficient PA among pregnant women is more than 60%[8]. The reasons for the high prevalence of insufficient PA among pregnant women are complex and many, and some of the most frequently reported barriers for PA are nausea, tiredness and lack of time[9,10]. A considerable proportion of pregnant women are motivated to increase their PA level[11] so it is crucial to improve evidence-based guidance on how the society and health care system can support and promote pregnant women to implement PA in their everyday lives in a safe and effective way. This is also mentioned by WHO in their global action plan on PA 2018-2030 where it is recommended to integrate assessment, brief advise and, when needed, referral to opportunities for appropriate supervised support on PA into care of pregnant women[12].

The FitMum randomized controlled trial, on which this PhD thesis is based, was designed to investigate how pregnant women can increase their PA level. Therefore, we investigated the effects of two different exercise approaches compared to a control group receiving standard care (CON) on PA level measured in three different ways in pregnant women. The two interventions were structured supervised exercise training (EXE) and motivational counselling on PA (MOT), respectively. The primary outcome was min per week of moderate to vigorous intensity PA (MVPA) from randomization to gestational age (GA) of 28+0-6 weeks, measured by a Garmin activity tracker. In addition to the primary outcome, we investigated several secondary outcomes at different timepoints during the entire study period through a multidisciplinary approach combining different scientific methods and fields. The overall study design of the FitMum study is shown in Figure 1 and described in Paper 1.

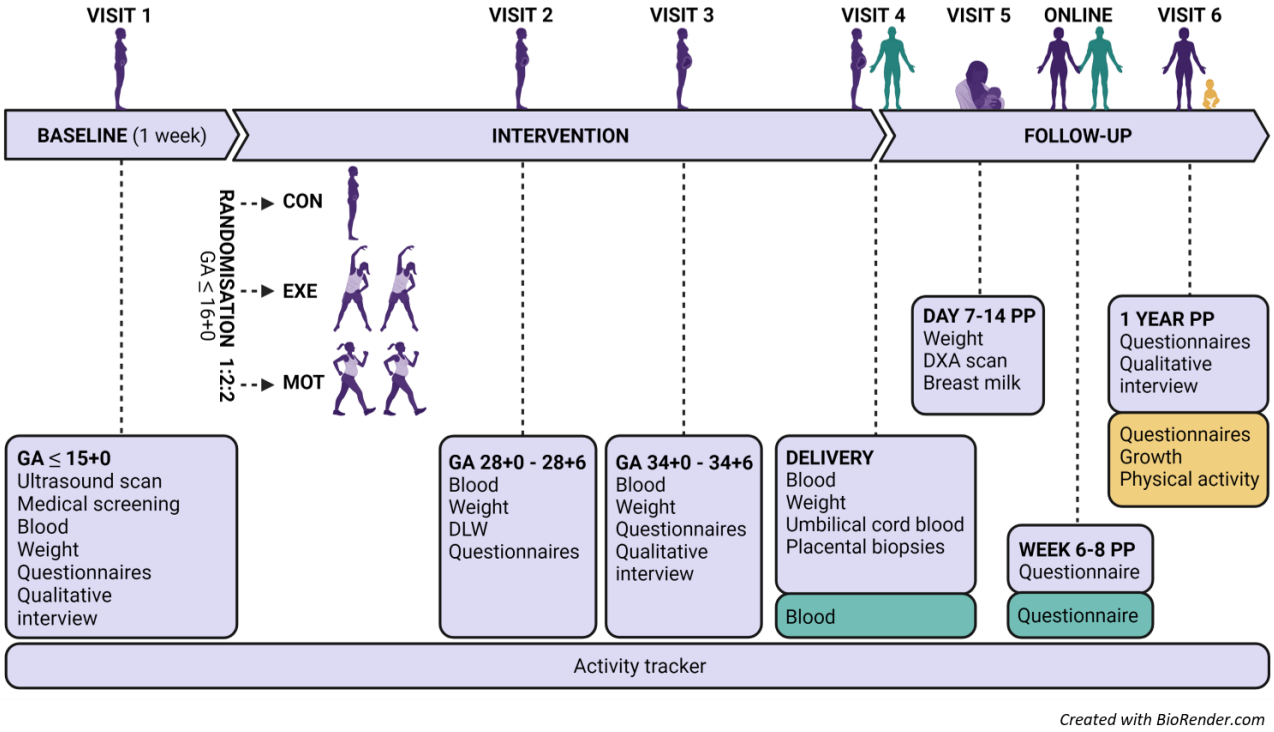

Figure 1. Study design of the FitMum study. Participants completed a one-week baseline period and were randomized (n=219) in the ratio 1:2:2 to CON, EXE, or MOT not later than at GA 16+0 weeks. Data were collected at the hospital three times during pregnancy (visit 1-3), at delivery (visit 4), and two times in the first year after delivery (visit 5 and 6). Data were also collected via online questionnaires and continuously throughout the study period by the activity tracker. Purple: participant, green: partner, yellow: offspring. GA; Gestational age, CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, DLW; Doubly labelled water, DXA; Dual-energy X-ray absorptiometry.

Some of the secondary outcomes in FitMum relate to how PA during pregnancy affects health outcomes in mother and child, and possible underlying mechanisms, which are research areas that call for further investigation. The focus in this thesis is to investigate health outcomes of the FitMum interventions on mother and child, and possible underlying mechanisms focusing on exercise-induced adaptations to breast milk (see objectives below). The outcomes included in this thesis are gestational weight gain (GWG) (self-reported prepregnancy weight and measured at visit 1-4), obstetric and neonatal outcomes from the medical records, and breast milk composition (samples obtained at visit 5).

The FitMum study was approved by the Danish National Committee on Health Research Ethics (#H-18011067) and the Danish Data Protection Agency (#P-2019-512). Written informed consent was obtained from all participants. The study was registered with clinicaltrials.gov (#NCT03679130).

Objectives and hypotheses

The main objective of this PhD thesis was to investigate the effects of PA during pregnancy (FitMum interventions) on clinical health outcomes in healthy inactive pregnant women and their offspring, and to explore possible underlying mechanisms for optimized offspring health, focusing on:

- GWG and obstetric and neonatal outcomes during pregnancy and delivery (Paper 2)
- Breast milk composition 7-14 days after delivery investigated by metabolomics and lipidomics analyses (Paper 3)

We had two predefined hypotheses for GWG (described in the statistical analysis plan for the study available with the trial registration at clinicaltrials.gov):

- Pregnant women in the EXE group gain less weight than those in the MOT group.
- Pregnant women in the MOT group gain less weight than those in the CON group.

The remaining investigations in this thesis were explorative.

The rest of the thesis constitutes 1) a background section describing this PhD project placed in the context of international state-of-the-art research within in the subject area, 2) three chapters summarizing and discussing the methods and results of the three papers included in the thesis in relation to international state-of-the-art research within the subject area, and 3) conclusions and perspectives for future research. The three papers that this thesis is based on (see page 7) can be found in the end after the references.

BACKGROUND

Impact of poor maternal prenatal lifestyle on maternal and offspring health in humans

The idea of in utero environmental influence on offspring metabolic health and risk of development of type 2 diabetes (T2D), metabolic syndrome (MetS) and cardiovascular disease later in life originated several decades ago with the Developmental Origins of Health and Disease Hypothesis[13,14] and has been confirmed more recently[15]. Epidemiologic insights gained from historical events of undernutrition, such as the Dutch famine in 1944-1945, have indicated that in utero exposure to maternal undernutrition increases the risk for development of obesity later in life[16,17]. Furthermore, low birth weight (BW) has been associated with increased risk of developing T2D later in life[18]. Other studies have indicated that maternal obesity and gestational diabetes mellitus (GDM) increase the risk for high BW and development of obesity, T2D and cardiovascular disease in adult offspring as well[19–21]. Thus, a U-shaped relationship between BW and long-term metabolic health seems to exist, where both low and high BW are associated with increased risk of obesity, metabolic and cardiovascular diseases later in life[22]. In 2016, the prevalence of overweight or obesity (Body mass index (BMI) $\geq$25 kg/m^2) among women in general was 54% in Europe[23] and above 60% in the United States[24]. Among pregnant women, the global prevalence of overweight and obesity in 2014 was estimated to be 38.9 million with 14.6 million pregnant women being obese (BMI >30 kg/m^2)[25].

Impact of maternal obesity and gestational diabetes mellitus on maternal health

Maternal overweight or obesity during pregnancy increases the risk of miscarriage, excessive GWG, GDM, and preeclampsia[19]. Prenatal overweight and obesity have also been associated with increased risk of caesarean section compared to normal weight women[26]. Moreover, GDM is, besides the immediate risks to pregnancy, associated with several long-term adverse metabolic health effects in mother, including increased risk of recurrent GDM, T2D, MetS, hyperlipidemia and obesity[19]. A Danish research group headed by Peter Damm investigated the long-term effects of GDM on both maternal[27,28] and offspring[20,21] metabolic health. To investigate maternal health outcomes, they applied oral glucose tolerance test (OGTT) or glucagon test in women with previous diet-treated GDM at a median of 10 years after pregnancy[27]. They found that 40% of the women had diabetes (89% of these had T2D) and 27% had impaired glucose tolerance[27], which respectively was a ten times higher and a two times higher incidence compared to the background population of 30-60-year-old females from the Inter99 study[29]. In the same cohort of women, a

three-fold higher prevalence of MetS was found among previously diet-treated GDM women compared to a control group that constituted of 1000 age-matched women randomly selected from the Inter99 study[30] and who had a normal weight BMI on average and were expected to have similar prevalence of GDM as the Danish background population[28]. Moreover, MetS was significantly more prevalent in obese women with previous GDM compared to normal weight women with previous GDM. Significantly higher prevalence of MetS was also found for control women with obesity compared to control women with normal weight[28].

Impact of maternal obesity and gestational diabetes mellitus on offspring health

Maternal overweight or obesity is associated with increased risk of macrosomia (BW>4000g), having a large for gestational age (LGA) infant, small for gestational age (SGA) infant, intrauterine growth restriction, and development of overweight and obesity later in life[19]. The transmission of obesity across generations was further demonstrated in a study of offspring born from obese women before and after a gastric bypass surgery[31]. The prevalence of obesity in offspring at seven years or older was markedly higher among offspring born before maternal gastric bypass surgery compared to offspring born after surgery when compared at the same age[31]. Besides maternal obesity, GWG above the Institute of Medicine guidelines[32] during pregnancy has also been associated with higher risk of macrosomia and LGA[33,34], as well as increased offspring BMI later in life[35]. Offspring exposed to GDM in utero has been associated with increased metabolic risk later in life[20,21]. The above-mentioned Danish research group exploring long-lasting effects of GDM on maternal and offspring metabolic health used an OGTT to investigate glucose tolerance in 18-27-year-old offspring of women with GDM compared to offspring of women from the background population, who were assumed to have a relatively low genetic risk of developing T2D. They found a higher prevalence of T2D and impaired glucose tolerance among offspring of women with GDM compared to offspring from the background population[20]. Being exposed to GDM in utero was also associated with a doubling of the risk of being overweight and a four-fold increase in the risk for developing MetS compared to background population offspring[21]. Similarly, follow-up on 10-14-year-old offspring of women with GDM showed increased risk of obesity and having fat percentage (measured by air displacement plethysmography), waist circumference and sum of skinfolds above 85th percentile, compared to offspring of women without GDM[36]. Lowe et al. also investigated influence of maternal blood glucose levels during pregnancy on offspring adiposity outcomes in the same cohort[37]. Higher HbA1c and maternal blood glucose concentrations after an OGTT performed around GA 28 weeks were associated with increased risk of being overweight/obese and having fat percentage, waist circumference and

skinfold thickness above 85th percentiles[37]. Higher maternal HbA1c level measured at GA 20-34 weeks has also been associated with higher fasting blood glucose concentration and lower insulin sensitivity in offspring at 4-7 years of age compared to offspring of women with lower HbA1c levels during pregnancy[38].

Exercise during pregnancy improves maternal health

It is well-established from research in humans that PA during pregnancy induces several positive health effects in mother[1–3]. In fact, aerobic exercise has been suggested to be more effective than metformin on reducing the risk of GDM and perhaps excessive GWG in pregnant women with overweight[39]. PA during pregnancy reduces GWG[40–43] and the incidence of several pregnancy and delivery related complications including GDM, gestational hypertension, preeclampsia, preterm delivery and caesarean section[42,44–46]. Looking at other delivery outcomes, the literature is more divergent. In a systematic review and meta-analysis, Davenport et al. reported a decreased risk of instrumental delivery in exercising versus non-exercising pregnant women, but no association of other delivery outcomes with exercise[47]. Some studies found shorter duration of labor among physically active women[48,49], whereas others found no effect of exercise[50]. Likewise, prenatal exercise has been indicated to reduce risk of induced labor[51] and pain during labor[52], but did not affect the use of epidural analgesia[52]. Focusing on mental health, exercise during pregnancy has been associated with reduction of maternal prenatal[53] and postpartum depressive symptoms[54–56]. Similar to observations of improved cardiovascular fitness with regular exercise training in non-pregnant populations[57–60], regular exercise training during pregnancy can also increase aerobic capacity by improving overall cardiovascular function[61]. Further, prenatal exercise programs may reduce the risk[62] and intensity[63] of low back pain during pregnancy.

Focusing on maternal glucose metabolism, prenatal exercise training has been indicated to reduce maternal fasting blood glucose, blood glucose response to an OGTT, insulin concentration, insulin resistance, incidence of GDM, and required amount of insulin for the management of GDM, compared to non-exercising controls in pregnant women with or without GDM[64,65]. Likewise, a recent review and meta-analysis of randomized controlled trials with supervised exercise training in pregnant women with overweight and obesity found that exercising groups had a lower increment in insulin resistance derived by the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and a lower post-prandial blood glucose concentration measured two hours after an OGTT compared to non-exercising controls[66]. Also, McDonald et al. investigated the effects of exercise in normal weight, overweight and slightly obese pregnant women and showed reduced insulin concentration and insulin resistance derived by HOMA-IR in late

pregnancy, as well as attenuated increase in insulin concentration from GA 16-36 weeks, compared to non-exercising pregnant women[67].

Regarding maternal lipid metabolism, blood lipid regulation seems to be improved with prenatal exercise training as well[61]. Both triglyceride and total cholesterol have been found to be inversely associated with PA[68]. However, a recent secondary analysis combining data from two longitudinal exercise intervention studies in pregnant women found no effect of exercise on fasting blood concentrations of triglyceride, total cholesterol, high-density lipoprotein or low-density lipoprotein in late pregnancy[67].

Moreover, maternal exercise has been indicated to influence cytokine levels[69,70]. Interleukin-6 (IL-6) concentration was increased with high amount of objectively measured MVPA compared to low MVPA in overweight and obese pregnant women[69] and regular exercise has been shown to decrease tumor necrosis factor alpha (TNF-α) levels compared to controls in normal weight women[70]. These exercise-induced changes in IL-6 and TNF-α levels might improve insulin sensitivity, since increased TNF-α is associated with insulin resistance, and IL-6 secreted from muscle tissue (which is likely the case when IL-6 is increased in response to exercise) is anti-inflammatory and associated with increased lipolysis and fat oxidation, as well as inhibition of TNF-α[71]. Considering maternal metabolic hormones, prenatal exercise has been associated with lower levels of leptin during pregnancy[68]. These findings support the positive impact of prenatal exercise on insulin sensitivity since leptin is a marker of obesity and has been associated with inhibition of insulin secretion from pancreatic beta cells[71].

In summary, a large body of evidence suggests that PA during pregnancy can improve maternal metabolic and cardiovascular health and reduce the risk of pregnancy and delivery related complications. As highlighted in a systematic review of meta-analyses by Hayes et al., investigating intervention components involved in the effectiveness of interventions are important to advance our understanding of how to optimize health benefits from prenatal exercise[44].

Effects of maternal exercise on offspring health

Focusing on effects of maternal exercise on human offspring health indicates several beneficial effects. Even grand-maternal lifestyle seems to influence obesity risk of grandchildren shown in a large study with 14,000 grandmother-mother-child triads[72]. In this study a lower risk of being overweight or obese in adolescence or young adulthood was found in grandchildren of women with the healthiest self-reported lifestyle during pregnancy, including being physically active, compared to grandchildren of women with the least healthy prenatal lifestyle[72]. The literature is inconsistent regarding the effects of maternal prenatal exercise on offspring anthropometric

parameters showing both optimization of BW into a healthy range with exercise[45,73] and no effect of exercise on mean (by far most reported) BW[46,74,75]. However, prenatal exercise has been shown to decrease the risk of macrosomia[74] and LGA[76]. Already 25 years ago, offspring body composition was suggested to be improved by prenatal exercise[77]. Clapp reported skinfold thicknesses at birth and five years after, and found reduced fat mass at birth in offspring of exercising women compared to offspring of pregnant women who were active before pregnancy but stopped exercise during pregnancy[77]. The results of a more favorable body composition among offspring born from exercising women were still present at five years of age[77]. Since then, more studies using measurements of skinfold thicknesses have supported these findings by indicating that prenatal exercise reduces offspring fat percentage at birth[78] and in 1-month old infants[79] compared to offspring of non-exercising controls. A recent study found increased lean mass measured with Dual-energy X-ray absorptiometry (DXA) scan within 48 hours of birth in offspring of women who participated in a lifestyle intervention including PA during pregnancy[80]. Likewise, objectively measured PA during pregnancy has been shown to be positively correlated with infant fat-free mass measured by air displacement plethysmography 11-19 weeks after birth[81]. Conflicting evidence exists regarding longer term follow-up on anthropometric outcomes in offspring born from mothers who participated in lifestyle interventions during pregnancy. A recent systematic review and meta-analysis showed no overall association between lifestyle interventions, including PA, and weight and BMI in offspring aged one month to seven years[82]. These findings were supported by a recent study that found no effect of a 12-week prenatal structured exercise program on iso-BMI (BMI adjusted for sex and age) and proportion of offspring with overweight at seven years of age compared to offspring of control group mothers[83]. In contrast, self-reported moderate dose of exercise during pregnancy was associated with lower risk of being overweight/obese among 5,125 eight-year-old offspring compared to offspring of mothers who reported to be sedentary during pregnancy[84].

Exercise during pregnancy has been shown to improve development of fetal cardiovascular autonomic control[85] and the improvement continued in infancy[86]. This was shown by May et al. in utero at GA 36 weeks[85] and in one-month-old infants[86] where heart rate variability was increased with exercise compared to non-exercising women. Recently, May et al. have also suggested that maternal aerobic exercise during pregnancy improves cardiac function and outflow parameters measured at GA 34-36 weeks[87]. Furthermore, prenatal exercise seems to improve neuromotor development indicated by increased neuromotor skills in one-month-old infants of exercising women compared to infants of non-exercising controls[88]. This improved capacity for movement suggests that offspring born from women who exercised during pregnancy may be more physically

active, thereby potentially reducing their risk of developing childhood obesity[88]. Objectively measured maternal MVPA has also been associated with improved offspring motor development in older offspring aged 12-30 months (22 months old on average)[89]. Additionally, objectively measured PA in 2974 one-year-old infants were positively associated with maternal PA during pregnancy, regardless of whether the infants were able to walk independently[90]. Some of the health benefits of exercise during pregnancy on mother and child are summarized in Figure 2[45] with kind permission from Elsevier (license # 5226961297745).

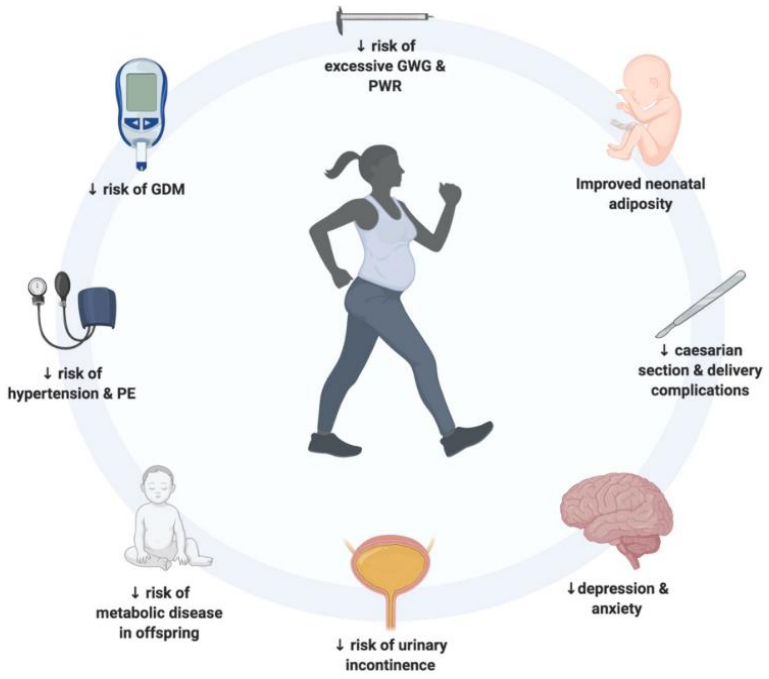

Figure 2. Health benefits of exercise during pregnancy on mother and child. GDM; gestational diabetes mellitus, GWG; gestational weight gain, PE; preeclampsia, PWR; postpartum weight retention. *Reprinted from Tissue and Cell, 72, Bhattacharjee, Mohammad and Adamo, Does exercise during pregnancy impact organs or structures of the maternal-fetal interface?, 101543, Copyright (2021),* ***with permission from Elsevier (license # 5226961297745)***[45].

As highlighted by Kusuyama and colleagues[91], the effects of maternal exercise on long-term metabolic effects in adult human offspring have not been studied due to the long human generation times that complicates follow-up during the entire lifespan. However, when investigating effects of lifestyle behaviors, including prenatal exercise, on human offspring metabolism and risk of development of obesity and lifestyle related diseases, long-term follow-up is important since these conditions typically occur later in life. Therefore, importantly, rodent models, which have a shorter generation timeline, have been used to investigate underlying mechanisms of optimized offspring metabolic health in adulthood after being exposed to prenatal exercise.

Animal studies have shown that maternal exercise during pregnancy effectively improves offspring metabolic health[22,91,92], which leads to the hypothesis that exercise during pregnancy can

improve metabolic health of human offspring as well and contribute to combat the obesity epidemic. Studies of maternal exercise in pregnant dams have shown increased glucose tolerance[93–96] and insulin sensitivity[95,96], and decreased insulin concentration[93,94] in both male and female adult offspring. Prenatal maternal exercise has also been shown to reduce offspring body weight and fat mass[93–95] and improve cardiac function[97,98]. Like the indications of increased offspring PA after maternal prenatal exercise in humans, a higher PA level in offspring of prenatally active dams compared to offspring of sedentary dams has been indicated in adult mice as well[99].

Potential mechanisms underlying prenatal exercise-induced optimization of offspring health

Adaptations in breast milk

Some of the beneficial effects of exercise on optimized offspring metabolism might be conferred via the breast milk. Several studies have investigated the influence of maternal postpartum exercise on human breast milk components[100–104] and shown improvements of breast milk composition after acute[100] and chronic[101] postpartum maternal exercise, whereas only a few studies have focused on the influence of maternal exercise during pregnancy[98,105].

Focusing on effects of prenatal maternal exercise on breast milk, Harris et al. have recently proposed exercise-induced adaptations to breast milk as an underlying mechanism for improved offspring metabolic health after maternal prenatal exercise. In this study, they applied voluntary wheel-running in mice two weeks prior to conception and throughout pregnancy (three weeks in mice) and a cross-fostering model to isolate the effects of exercise-trained milk on metabolic health and cardiac function in adult mouse offspring. Both male and female offspring of sedentary dams during pregnancy but cross-fostered immediately after birth with exercise-trained dams (SED-TRAIN) had lower body weight, percentage fat mass in adulthood compared to offspring of trained dams cross-fostered with sedentary dams (TRAIN-SED). Improved glucose tolerance and decreased insulin concentration were also found in adult male SED-TRAIN offspring compared to TRAIN-SED[98]. These findings highlight the importance of exercise-induced adaptations to breast milk as a mediator to confer benefits of maternal exercise to offspring metabolism.

Human breast milk is synthesized by lactocytes in the breast alveoli. The lactocytes produce the milk continuously from water, lactose, fat, amino acids, minerals, and vitamins from maternal blood and the milk is stored in the alveoli until endogenous oxytocin stimulates the muscle cells around the alveoli to contract and send milk into the milk ducts towards the nipple during breastfeeding[106]. When breastfeeding is well established, mature human milk contains of almost

90% water and on average 1g protein, 4g fat and 7g carbohydrates per 100 mL milk[107]. The carbohydrates in the milk contain up to 15% oligosaccharides in both humans and mice and there are more than 150 difference oligosaccharides present in human milk, including 3'-sialyllactose (3'-SL) and 6'-sialyllactose, whereas mouse milk only contains these two different oligosaccharides[108]. Despite similarities in human and mouse milk, comparison between animal and human studies needs to be done cautiously. Harris et al. also investigated which components of milk that mediate the beneficial effects of maternal exercise on offspring health. They identified an exercise-induced increase in 3′-SL in breast milk in mice and showed that 3'-SL in breast milk mediated the beneficial effects of maternal exercise on mouse offspring's metabolic health and cardiac function. They also showed a positive correlation between PA measured with accelerometry three times during pregnancy and 3′-SL concentration in human breast milk obtained two months postpartum[98]. Moreover, Ribeiro et al. investigated effects of maternal prenatal exercise in rats, and found changes in maternal milk composition, including lower total cholesterol concentration, after low intensity treadmill exercise three times per week throughout pregnancy and lactation compared to milk from sedentary dams[105]. Further, this study showed lower body weight, fat depots, fasting plasma glucose concentration, insulin concentration, and insulin resistance in offspring from exercise-trained dams[105].

Overall, these studies show improvements of offspring metabolic health after maternal prenatal exercise and propose exercise-induced adaptations to breast milk as an underlying mechanism to confer the beneficial effects. However, the effects of prenatal chronic maternal PA/exercise training on human breast milk composition, for example the breast milk metabolome and lipidome, are unknown.

Maternal postpartum exercise has no adverse effects on volume and macronutrient composition of breast milk[102,103], infant acceptance of breast milk[104], and infant growth[102,109] in humans. Thus, lactating mothers can be reassured to practice exercise of moderate to high intensity during their breastfeeding period and we hypothesize that the same applies to practicing exercise during pregnancy.

Other potential mechanisms

Other mechanisms that may potentially mediate the beneficial effects of maternal prenatal exercise on offspring metabolism include among others epigenetic changes in fetal tissues and fluids, and adaptations in the placenta. Secretion of cytokines from maternal skeletal muscle tissue (myokines), for example IL-6, in response to exercise has also been proposed to affect placental development and optimize fetal growth trajectories[110].

Regarding epigenetic changes, a recent review of human studies suggested that there may be an association between maternal lifestyle, diet and PA during pregnancy and epigenic changes in the offspring[111]. Epigenetic changes include DNA methylation, micro-RNA changes and histone modification and can alter expression of genes without changing the DNA sequence. DNA methylation can increase or reduce expression of a gene depending on the site of methylation, but in general increased DNA methylation results in decreased expression of target genes. The review included 16 human studies and four of these investigated the association between PA and epigenetic changes, more specifically DNA methylation in cord blood and infant blood spots, and found an overall association between PA and changes in offspring DNA methylation[111]. Two of these studies were randomized controlled studies that intervened on both diet and PA and found their interventions to be associated with changes in DNA methylation of offspring sites of genes, potentially involved in offspring growth and body composition, as well as attenuation of DNA methylation changes associated with exposure to GDM in utero[80,112]. Studies of adult offspring skeletal muscle tissue in mice have found that maternal exercise before and throughout pregnancy prevents the detrimental effects of high-fat-diet-induced hypermethylation of the peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) promoter and subsequently decreased expression of PGC-1α (plays a key role in mitochondrial biogenesis and oxidative metabolism) and some of its target genes[113,114]. Epigenetic modulations of genes involved in mitochondrial biogenesis in cardiac tissue in offspring mice after maternal prenatal exercise have also been suggested to be involved in maternally exercise-induced improvements in offspring health[22].

Moreover, maternal prenatal exercise has been indicated to alter expression of placental genes essential for adequate nutrient delivery to the fetus and optimal fetoplacental growth, and to improve placental morphology and vascularization in humans and rodents[45,91]. Further, maternal exercise-induced cytokine secretion by the placenta, called placentokines and exerkines as well because they are secreted in response to exercise, have also been proposed to be involved in improved fetal development and potentially long-term offspring metabolic health. Recent studies have shown increased placenta expression and circulating levels of the placentokines apelin[115] and superoxide dismutase 3[116] in response to maternal prenatal exercise. Maternal exercise-induced apelin has been indicated to improve offspring metabolic health by inducing fetal brown adipose tissue adipogenesis[115], whereas superoxide dismutase 3 seems to influence offspring metabolism by increasing expression of genes involved in metabolic regulation in fetal offspring liver[116].

Impact of genes and environmental factors after birth

Besides the impact of maternal prenatal lifestyle, poor as well as physically active, other factors such as genetic inheritance and environment after birth can also influence offspring health. A meta-analysis of twin studies showed that genetic factors had a strong effect on the variation in BMI at all ages from 1-18 years and that environmental factors affected variation in BMI in childhood but the effect disappeared in adolescence[117]. Moreover, the influence of genes and family environment on childhood obesity have been investigated in adoption studies that showed correlations between adoptees and adoptive parents but substantially stronger correlations between parents and their biological offspring. This supports an influence by the family environment, but at the same time highlights the importance of genes[117]. Interaction between environment and genes may also exist, meaning that the effect of the environment on offspring health can be modified by the genotype or the other way around. For example PA has been shown to reduce the genetic variance in BMI and waist circumference in early adulthood, which suggests that the function of genes predisposing to obesity is suppressed in persons who are physically active[117]. An example of an adoption study is a study by Stunkard et al. published in New England Journal of Medicine in 1986[118]. This study investigated 540 adult Danish adoptees divided into four weight classes (thin, medium weight, overweight, obese) and found a strong correlation between weight class of adoptees and mean BMI of their biological parents, while there was no correlation between weight class of adoptees and mean BMI of their adoptive parents. Thus, the authors concluded that genetic factors are important determinants of adult offspring weight, which on the other hand does not seem to be influenced by family environment alone[118].

In summary, impact of genes and environmental factors after birth on offspring health needs to be considered when designing and evaluating studies investigating the effect of maternal prenatal lifestyle, including PA behavior, on offspring health.

Exercise during pregnancy is safe for mother and offspring

A large body of evidence from human studies supports moderate intensity exercise during uncomplicated pregnancy as a safe lifestyle behavior for mother and child. Prenatal exercise does not increase risk of miscarriage (fetal mortality <GA20 weeks)[119], perinatal mortality including stillbirth (GA≥20 weeks) and infant mortality (before 28 days of life)[119], birth defects[120], low BW (<2500g)[121] or preterm delivery[44,121]. Some studies even show a decreased risk of low BW[76] and preterm delivery[76,122] with exercise. Additionally, exercise at vigorous intensity has also been indicated to be safe for healthy low-risk pregnant women in a meta-analysis showing no increased risk of low BW or SGA infants, as well as preterm delivery[123]. Traditionally, activity restriction

has been recommended for women with high-risk pregnancies, and Bendix et al. reported in 2015 that most Danish obstetricians and midwives prescribe activity restriction in high-risk pregnancies[124]. However, a recent review concluded that activity restriction do not significantly prevent preterm birth even among women with the highest risk of preterm birth, for example having short cervices or ruptured membranes, and that activity restriction should not be routinely recommended to prevent preterm birth[125]. Another systematic review of the evidence of harms of PA for women with pregnancy complications[126] found that there is no strong evidence to recommend pregnant women with most pregnancy complications to avoid being physically active. The authors proposed that 11 current contraindications for prenatal exercise (e.g. gestational hypertension, overweight/obesity and twin pregnancies) should no longer be defined as contraindications, since there is no strong evidence of harms and that pregnant women with these complications might even improve their conditions by being physically active. Further, they proposed to redefine 10 relative contraindications (e.g. mild preeclampsia and mild respiratory diseases) for which pregnant women in the future can be advised to do MVPA with or without modifications and maintain daily activities. They also support 10 absolute contraindications for PA (e.g. severe respiratory diseases and placental abruption) that should remain as absolute contraindications where MVPA should be avoided, however daily activity should be maintained[126].

In summary, exercise is safe and highly recommended during pregnancy for women with uncomplicated pregnancies. Recommendations of prenatal exercise at vigorous intensity and for pregnant women with different pregnancy complications need to be re-evaluated to strengthen the beneficial effects of PA on maternal and offspring health outcomes.

PAPER 1: INTERVENTIONS AND METHODS IN THE FITMUM STUDY

The FitMum study was designed as a single-site randomized controlled trial and conducted at the Department of Gynecology and Obstetrics at Nordsjaellands Hospital, Hillerod, Denmark. Participants in the study were recruited from October 2018 – October 2020 and the last participant gave birth in May 2021. The study was designed to explore strategies to increase PA during pregnancy among women with low PA, and to assess the effects of PA on health outcomes in mother and child. More specifically, the primary objective was to investigate the effects of two different PA approaches, structured supervised exercise training (EXE) versus motivational counselling on PA (MOT), compared to a control group receiving standard care (CON) on the primary outcome MVPA from randomization to GA 28+0-6 weeks. The FitMum study was a multidisciplinary study and in addition to the primary outcome, we investigated several secondary outcomes, for example how PA during pregnancy affected health outcomes in mother and child as well as possible underlying mechanisms, which are included in this thesis. Paper 1 is a protocol paper describing the overall study design and methods of the FitMum study.

Discussion of study design and findings of physical activity level and intervention adherence

Participants

We included healthy, inactive, pregnant women who were to give birth at Nordsjaellands Hospital, Hillerod. Numbers of women included, randomized, and analyzed in the study are shown in flow diagrams until delivery (Figure 1, Paper 2) and until visit 5 (7-14 days after delivery) (Figure 1, Paper 3). In our two-year inclusion period from October 1st, 2018 to September 30th, 2020, 8245 women attended first trimester scan at Nordsjaellands Hospital and were exposed to the FitMum study recruitment material. This number corresponds to around 7% of all women giving birth in Denmark over two years (currently around 60,000 births per year)[127]. Of these 8245 women exposed to the study recruitment material, 11% (n=872) were screened for eligibility in the study by completing an online questionnaire. Of these 33% (n=284) remained eligible for further assessment of whether they met the criteria for inclusion in the study and most of these women were included (n=220) and randomized (n=219) to one of the three study groups one week after inclusion (CON: n=45, EXE: n=87, MOT: n=87). Thus, 3% of the women exposed to the recruitment material were included in the study (220 out of 8245 women) and of women interested in participating in the study, hence screened for eligibility (n=872), 25% were included in the study

(n=220). Other randomized controlled trials with lifestyle interventions, including exercise training, in pregnant women have found similar inclusion rates ranging from 16-37% of women assessed for eligibility[63,128,129]. Further research is warranted in women who do not sign up for human clinical trials like FitMum, including the reasons for not signing up. In our study, 7373 women (89% of women giving birth in the study period) did not fill the online screening questionnaire.

Nordsjaellands Hospital has a diverse uptake of pregnant women from 12 municipalities in North Zealand that are comparable to large parts of Denmark with regards to education level, occupation level and ethnicity, but have higher average household income[130]. Thus, we expected our study population to be relatively representative compared to other regions of Denmark. However, it is unknown whether the outcomes of our study would differ if the study was carried out in pregnant populations in other regions of Denmark or in other countries with different socioeconomic status, circumstances for participating in interventions (different transportation time to gym/swimming pool etc.), distributions of prepregnancy BMI, distributions of women being nulli- versus multiparous etc. Also, as in all other intervention studies there is a risk of selection bias since women with more resources and motivation towards a healthy and physically active lifestyle might be more likely to sign up for a study like FitMum. Therefore, it is unknown whether our results are generalizable to pregnant women in other regions of Denmark, in other countries, or with different ethnic background than Danish, or obstetric or medical complications.

Prepregnancy BMI (Paper 2), smoking and alcohol consumption before pregnancy (unpublished data) in our study population of 219 randomized women (mean age 31.5 ± 4.3 years) were compared to data from Danish women aged 25-34 years in a national health survey carried out by the Danish Health Authorities in 2017[131]. In our study population, the prevalence of normal weight, overweight and obesity was 56%, 24%, and 21%, respectively. This corresponded to the women in general where the prevalence of normal weight, overweight and obesity was 59%, 23%, and 15%, respectively. The FitMum population consisted of a larger number of women who never smoked (71%) compared to 61% of 25-34-year-old women in general. We also observed lower alcohol consumption before pregnancy in our study population with 41%, 58% and 1% of our participants consuming 0, 1-7, and 8-14 servings per week, respectively, compared to 19%, 51%, and 10% in the background population. Thus, compared to 25-34-year-old Danish women in general, our study population seems similar with regards to weight status but healthier with regards to alcohol and smoking behaviors.

Sample size

The sample size required to detect an overall significant difference with a power of 80% and significance level of 5% was calculated for the primary outcome of the study; MVPA from randomization to GA 28+0-6 weeks. We choose to randomize the participants in a 1:2:2 ratio to CON, EXE, or MOT as this required the lowest number of participants and left the participants, who we assumed were motivated for PA and hence more interested in being randomized to one of the intervention groups, with a greater chance of being randomized to EXE or MOT. Sample size calculation and planned statistical analyses were described in the statistical analysis plan (clinicaltrials.gov, #NCT03679130). The sample size calculation is associated with a high degree of uncertainty because we used a novel commercial activity tracker (Garmin Vivosport)[132] to objectively measure the primary outcome. Thus, no obvious literature was available regarding which effect size and standard deviation (SD) to expect on PA measured by that specific tool. Therefore, we stipulated average weekly PA to be 60, 210 and 150 min per week in CON, EXE and MOT, respectively, and estimated SD based on a similar exercise intervention study in pregnant women that measured PA with accelerometers and had a SD of 116 min per week[133].

As shown in the flow diagram (Figure 1, Paper 2), the relative proportion of participants lost to follow-up until visit 2 (primary outcome measurement timepoint) was 15%, which is below 20% as estimated for the sample size calculation. Hence, the number of completers at visit 2 was 35, 77 and 74 in CON, EXE, and MOT, respectively, meaning that sufficient power for the primary outcome analysis should be reached if effect sizes and SD's in CON, EXE and MOT corresponded to the estimates from our sample size calculation.

The number of participants to be included in the study was decided based on the sample size calculation for the primary outcome. Therefore, it was unknown whether the power to detect effect sizes of relevance for secondary and additional outcomes (i.e. outcomes in Paper 2 and 3) in the study was sufficient. However, as described in the statistical analysis plan, a sample size calculation for the secondary outcome, GWG (measured at delivery), was performed after initiation of inclusion of participants in the study but before the interventions were completed. The sample size of participants needed to detect a significant difference in GWG at delivery between CON and the intervention groups was 33 women in CON and 66 women in each of the two intervention groups. At delivery, we had 34, 74 and 70 completers in CON, EXE, and MOT, respectively, meaning that sufficient power for the secondary outcome analysis should be reached if effect sizes and SD's in CON, EXE and MOT corresponded to the estimates from our sample size calculation.

Adherence and physical activity level

Since the primary objective of the study was to investigate the effects of structured supervised exercise training versus motivational counselling on MVPA from randomization to GA 28+0-6 weeks, no lower limit of adherence to our interventions was defined for participants to be included in the data analysis. From randomization to GA 28+0-6, average weekly MVPA was 32.7 min [95% confidence interval, 18.1;47.3] in CON, 49.7 min [39.2;60.2] in EXE and 40.2 min [29.7;50.7] in MOT (unpublished data). The average weekly MVPA from randomization to GA 28+0-6 was higher in EXE compared to CON, but did not differ between EXE and MOT or between MOT and CON (unpublished data), despite having more completers at GA 28+0-6 than needed to detect statistical difference between these groups according to our sample size calculation. SD's were within the estimated SD in our sample size calculation but the effect sizes on average weekly MVPA were lower in all three groups compared to the stipulated effect sizes in our sample size calculation.

From randomization to delivery, average weekly MVPA was 35.4 min [19.4;51.4] in CON, 53.5 min [42.0;65.0] in EXE and 43.1 min [31.6;54.6] in MOT (unpublished data) and hence still below one hour per week, which is markedly lower than the recommended level of PA during pregnancy[1,4]. Participants were stratified into five MVPA categories based on their average min of MVPA per week measured by the activity tracker from randomization to delivery to analyze if number of participants achieving MVPA below 30, 30-60, 60-150, 150-210, or above 210 min per week differed between the three groups (Table 1). Noteworthy, in both EXE and MOT less than 10% of participants achieved the international and Danish recommendations of 150 min per week[1] and 210 min per week[4], respectively. Differences in number and proportion of participants with MVPA below 30, 30-60, or 60-150 min per week were tested using Pearson's Chi-squared tests and showed no between-group differences. Due to low number of events (below 5) Fisher's Exact tests were performed to test differences in number and proportion of participants with MVPA between 150-210 min per week or above 210 min per week. Likewise, these tests showed no differences between the three groups in number of participants achieving MVPA 150-210 or above 210 min per week.

Table 1. Number and proportion of participants with average MVPA below 30 min per week, 30-60 min per week, 60-150 min/week, 150-210 min per week, and above 210 min per week from randomization to delivery

	CON (n=45)	EXE (n=87)	MOT (n=87)	p-value
MVPA < 30 min/week, n (%)	26 (58%)	38 (44%)	48 (55%)	0.192
MVPA 30-60 min/week, n (%)	13 (29%)	23 (26%)	22 (25%)	0.906
MVPA 60-150 min/week, n (%)	5 (11%)	20 (23%)	13 (15%)	0.174
MVPA 150-210 min/week, n (%)	0 (0%)	3 (3%)	2 (2%)	0.636
MVPA > 210 min/week, n (%)	1 (2%)	3 (3%)	2 (2%)	1.000

Manual registration of training sessions in EXE revealed that participants on average participated in 1.3 [1.1;1.5] out of three sessions offered per week from randomization to delivery. 40% of women in EXE participated in <1 session per week on average, 32% participated in 1-1.9 sessions per week, and 28% participated in 2-3 sessions per week. Women in MOT participated in on average 5.2 [4.7;5.7] out of seven (four individual and three group) counselling sessions offered during pregnancy (unpublished data). Thus, average adherence rates were moderate in MOT (with participants attending on average five out of seven counselling sessions) and among 60% of the participants in EXE (attending 1-3 sessions per week). The rather poor average adherence in our study and low PA levels measured by the tracker might have influenced the effects of our interventions on secondary and additional outcomes of the study. For example, it seems important to achieve a certain amount of PA to obtain beneficial effects on maternal and offspring health outcomes. The rather low PA level in our study is somewhat similar to the literature showing mixed effects of interventions to increase PA during pregnancy[133–135]. In a systematic review of systematic reviews, Heslehurst et al. concluded that PA interventions can increase metabolic equivalents (METs) and amount of oxygen used during maximal exercise, but the interventions did not seem to impact MVPA or steps during pregnancy[134]. However, some exercise intervention studies during pregnancy have shown relatively high adherence and PA levels[129,136–138]. An overall moderate effect of interventions on PA level among non-pregnant healthy adults has been found

in a review of diverse intervention studies designed to increase PA level (also including supervised exercise and motivational interviewing characteristics)[139]. Besides analyzing our data according to the randomized design in FitMum, we performed additional analyses to investigate if prenatal exercise per se independently of study group allocation was related to maternal and offspring health. We used a linear regression analysis to investigate associations between PA measures from the activity tracker (i.e. MVPA and active kilocalories) and secondary as well as additional maternal and offspring health outcomes (Paper 2 and 3).

A limitation of using a commercial wearable activity tracker to measure the primary outcome across all three groups is that we do not know to what extend the participants in CON interacted with the tracker and that participants in CON may have increased their PA level due to motivation from wearing the tracker. Studies have indicated that consumer-based wearable activity trackers can increase MVPA, number of steps, and energy expenditure among adults[140]. Whether the accumulated PA during pregnancy in CON would have been lower if not wearing an activity tracker is unknown since we did not include an additional control group not wearing activity tracker in our study. A control group without tracker would likely be more representative for the large amount of the pregnant population not engaged in wearing an activity tracker. The differences in MVPA between a control group without tracker and EXE or MOT would probably have been larger than what we found by comparing MVPA in EXE and MOT to the current tracker-wearing control group. Another limitation of using the Garmin Vivosport tracker for PA measurements in our study is that we do not know its validity. A review of the validity of other Garmin activity trackers indicated higher validity for steps but lower validity for heart rate and energy expenditure[141]. Other reviews and meta-analyses comparing the validity of Garmin activity trackers with other commercial wearable devices show inconsistent results[142–144]. Moreover, the activity tracker might not capture all PA. For example, only PA with a MET value of three or higher for bouts of at least ten consecutive minutes are reported as MVPA[132]. This may partly explain the relatively low MVPA in CON, EXE, and MOT in our study.

Influence of the COVID-19 pandemic

Lock-down periods because of the COVID-19 pandemic started on March 11th, 2020 (in Denmark) when the FitMum study had included participants for 17 months and was about halfway through. As described in the paper, intervention elements and test visits were during the lock-down period carried out online and we continued to include participants in the study. However, this means that the participants received different versions of the study. Either they 1) received exclusively physical interventions/test visits if they were included and gave birth before the occurrence of

COVID-19, 2) received a mix of physical and online study if they were included before, but gave birth during COVID-19, or 3) received exclusively the online version of the study if they were included after March 11th 2020. A sensitivity analysis of MVPA before versus during COVID-19 was performed including only participants exposed to version 1 and 3 of the study to investigate if the conversion to online interventions influenced MVPA. This analysis showed that MVPA did not differ during COVID-19 versus before COVID-19 in any of the three groups (unpublished data). Interestingly, sensitivity analyses of the influence of COVID-19 on the secondary outcome of the study showed higher total GWG in MOT among participants receiving online interventions compared to MOT participants receiving physical interventions. Total GWG did not differ in EXE or CON during COVID-19 versus before COVID-19 (Paper 2). The higher total GWG among MOT participants receiving online intervention could not be explained by differences in MVPA before versus during COVID-19, since MVPA did not differ with intervention mode. Other measures of PA from the activity tracker including PA at vigorous intensity and active minutes (everything beyond sedentary time) did not differ before versus during COVID-19 in any of the three study groups either ($p>0.05$). We have no other obvious explanations for this finding, but it might be important to keep in mind when analyzing additional outcomes of the study that there may be non-PA related differences within the groups between participants receiving online versus physical interventions.

Intensity in physical activity interventions

PA is defined as any bodily movement generated by skeletal muscles that results in increased energy expenditure[145]. Exercise is additionally defined as structured, planned, repetitive bodily movement with a purpose of improvement or maintenance of physical fitness[145]. The two interventions that we tested in FitMum, structured supervised exercise training versus motivational counselling on PA, constitute two different approaches to implement and maintain a physically active lifestyle. With both exercise approaches we aimed to accommodate motivators for PA such as 'advice and feedback from experts' and 'introduction to types of PA that are feasible to implement' and overcome barriers such as 'anxiety of overdoing exercise or exercising in an incorrect way' and increase the participants confidence with doing exercise. The rationale for choosing structured supervised exercise training as one of the intervention approaches was to test the effects of committing to exercise training at fixed time points with fixed type, duration and frequency of exercise sessions, carried out in a social environment together with other pregnant women, and being supervised by health professionals. The rationale for having motivational counselling on PA as the other intervention was to offer a more flexible approach for

implementation of PA with regards to type, duration and frequency of PA sessions to allow for more individualized PA programs, but still being followed closely with regular counselling and exercise action plans from health professionals. PA at moderate intensity was prescribed in both interventions in FitMum according to the current recommendations from the Danish Health Authorities[4].

Moholdt and Hawley have recently suggested exercise training at vigorous intensity, also called high-intensity training (HIT), to be a time-efficient intervention prior to and throughout pregnancy[146]. Prenatal exercise at vigorous intensity has been indicated to be safe for healthy low-risk pregnant women and their fetuses[123]. In fact, including elements of vigorous intensity exercise has also been associated with a markedly reduced incidence of GDM among overweight and obese women[129], and higher enjoyment of exercise[147], suggesting that HIT may have potential to increase exercise adherence among pregnant women. Currently, Danish pregnant women are advised against exercising at vigorous intensity during pregnancy if they were not used to exercise at vigorous intensity before pregnancy. However, pregnant women who normally exercise at vigorous intensity can continue their PA habits during pregnancy, but are advised against long-distance running and similar exhausting activities[4]. Likewise, WHO and the American College of Obstetricians and Gynecologists support that women who habitually engaged in PA at vigorous intensity prior to pregnancy can continue these activities during pregnancy[1,2]. Interestingly, pregnant women in Australia are now recommended to meet the general PA guidelines for adults, including vigorous intensity exercise[148].

Given that 'lack of time' is a barrier for PA often reported by pregnant women[149–151], it seems essential to investigate in future studies whether HIT can constitute a time-efficient beneficial exercise regime with high adherence prior to conception and during pregnancy.

Timing of intervention

The FitMum study was developed to intervene during pregnancy with the rationale of pregnancy possibly being a window of opportunity to implement a more physically active lifestyle. The study participants were enrolled as early in pregnancy as possible and maximum at GA 15 weeks and 0 days (GA 15+0 weeks). The inclusion period ended up ranging from GA 6+1 – 15+0 weeks and the median GA at inclusion was 12.9 (interquartile range, 9.4-13.9) weeks. Inclusion in early pregnancy has also been practiced in other exercise intervention studies in pregnant women[128,129,152–154]. Succeeding with implementation of lifestyle behavior changes can take long time and thus, it seems important to initiate interventions as early in pregnancy as possible to allow the intervention to last as long time as possible[155]. Moreover, initiation of PA prior to or in early

pregnancy might result in better results than starting later in pregnancy. A meta-analysis evaluating prepregnancy and early pregnancy PA reported a lower risk of developing GDM for women in the highest PA quantiles compared to women in the lowest PA quantiles[156]. Furthermore, optimized growth of placenta and fetus has been indicated with prenatal exercise initiated after only eight or nine weeks of gestation[157]. In fact, Moholdt and Hawley have proposed initiation of maternal lifestyle interventions already in the preconception period in the attempt of reducing obesity, which is present in about one-third of women in the reproductive age[146]. Higher prepregnancy PA level has been suggested as a predictor of higher PA level during pregnancy by several studies[158,159], which supports the proposal of establishing good exercise habits already prior to conception. Thus, further research focusing on the effects of initiating maternal lifestyle interventions preconceptionally on maternal PA and maternal and offspring health outcomes seems to be important.

PAPER 2: THE EFFECTS OF PRENATAL PHYSICAL ACTIVITY INTERVENTIONS ON GESTATIONAL WEIGHT GAIN AND OBSTETRIC AND NEONATAL OUTCOMES

The aim of Paper 2 was to investigate the effects of EXE versus MOT on GWG and obstetric and neonatal outcomes compared to CON. Further, we aimed to investigate if effects of prenatal exercise on GWG depended on prepregnancy BMI. We developed a novel method to estimate GWG at specific time points during pregnancy and to account for missing weight measurements and individual differences in GA at delivery. The estimation of GWG by this method was based on longitudinally observed body weights during pregnancy and at admission for delivery, which were fitted to a mixed effects model to predict maternal body weight and estimate GWG at different gestational ages. Obstetric and neonatal outcomes were obtained from medical records after delivery. Obstetric outcomes included incidence of GDM, gestational hypertensive disorders (including preeclampsia), induction of labor, use of epidural analgesia, oxytocin augmentation, duration of labor, mode of delivery, rupture degree 3 and 4, and postpartum hemorrhage. Neonatal outcomes included GA at delivery, preterm delivery, BW, birth length, birth weight z-score, SGA, LGA, and Apgar score at 5 min.

Overall, neither EXE nor MOT affected GWG or obstetric and neonatal outcomes compared to CON. However, women with obesity in both EXE and MOT gained less weight compared to women with normal weight within the same intervention groups. Further, associations between PA measures and GWG differed between women with obesity and normal weight. This indicates that pregnant women with obesity may be more susceptible to the beneficial effects of prenatal PA on GWG compared to women with normal weight.

Methodological considerations

Calculation of gestational weight gain by a novel method

In their reexamination of guidelines for weight gain during pregnancy in 2009, the Institute of Medicine presented ideal and practical methods for measurement and acquisition of body weight data required to determine GWG. Ideally, body weights used to calculate GWG should be prepregnancy weight measured at a preconceptional visit and the last measured available weight abstracted from clinical records. If not possible to measure maternal weight before conception and late in pregnancy (ideally at delivery) in practice, prepregnancy weight and last available weight can be recalled (self-reported) by the women as soon as possible, for example at the first prenatal visit, using standardized questions[32]. However, such data will most likely be less precise than

objectively measured weights during hospital visits. For practical reasons, most studies use weight measured at the last pregnancy visit to calculate GWG[41,42,160] and only few studies have measured weight at delivery and hence reported GWG for the entire pregnancy period[161,162]. In Paper 2, we aimed to estimate GWG for the entire pregnancy period and account for missing weight measurements and individual differences in GA at delivery. Thus, we obtained self-reported prepregnancy weight at visit 1, and measured the participants body weights at the hospital at visit 2, 3, and at delivery using two different, but calibrated, electronic scales. All these observed weights (self-reported and measured) were fitted to a mixed effects model to predict body weights at specific timepoints throughout pregnancy at the participant-level. The GWG at GA 40+0 weeks was subsequently estimated as the difference between the predicted weight at GA 40+0 weeks and predicted prepregnancy weight (predicted weight at GA 0 weeks). Using this model, we showed a good relationship between observed weights and predicted weights for all individuals in all three groups, and a complete-case analysis including only participants with weight measurements at delivery (n=131) showed similar results of GWG as the results of estimated GWG by the model (Paper 2). Thus, this novel method can be used to precisely estimate GWG at specific timepoints throughout pregnancy, for example GWG for the entire pregnancy period. Further, this method allowed us to take GA into account, which can vary up to five weeks within term-deliveries and thus likely influence GWG markedly. Moreover, missing data could be predicted by the model allowing us to report mean GWG at GA 40+0 weeks for all 219 study participants even though we only obtained weight data on 131 women at delivery.

Body weight measured on different scales

During COVID-19 lockdown periods, participants could not attend study visits at the hospital and were therefore weighed on a private scale at home. We performed a sensitivity analysis to investigate if weight measurements being obtained by the calibrated scale at the hospital versus on the participants' own scales at home influenced the GWG results. In this sensitivity analysis we included only participants, whose weight was exclusively measured at the hospital (n=167) and excluded participants who had at least one weight measurement obtained at home (n=52). The sensitivity analysis showed similar results of GWG at GA 40+0 weeks as the intention-to-treat analysis including all 219 participants independent of scale used to obtain weight data (Paper 2).

Weight gain rates during pregnancy

Weight gain rates during pregnancy were investigated as part of modelling the trajectories of observed body weights during pregnancy by the mixed effects model. Figure 5A shows the weight gain rates during pregnancy in the three groups and shows that the rates of the weight gain change at one point in all three groups. The weight gain rates change at GA 71 days [51;94] in MOT, at GA 93 days [76;105] in EXE, and at GA 107 days [77;132] in CON (Figure 5B). The probability that the weight gain rate changed earlier in MOT compared to CON was 97%. The probability that the weight gain rate changed earlier in EXE compared to CON was 81%, and the probability of an earlier weight gain rate change in MOT compared to EXE was 94%. This means that participants in both MOT and EXE will likely begin to gain weight at a higher rate before participants in CON and further, that participants in MOT will begin to gain weight at a higher rate before participants in EXE, given that the weight gain rate was lower in the beginning of pregnancy (Figure 5C) compared to later in pregnancy (Figure 5D). However, the weight gain rate change-points in MOT and EXE were earlier than median GA at randomization, which was at GA 97 and 95 days, respectively. In CON, the change-point was only 10 days after median randomization time at GA 97 days. This means that the different change-points in the three groups were likely independent of group allocation in the study.

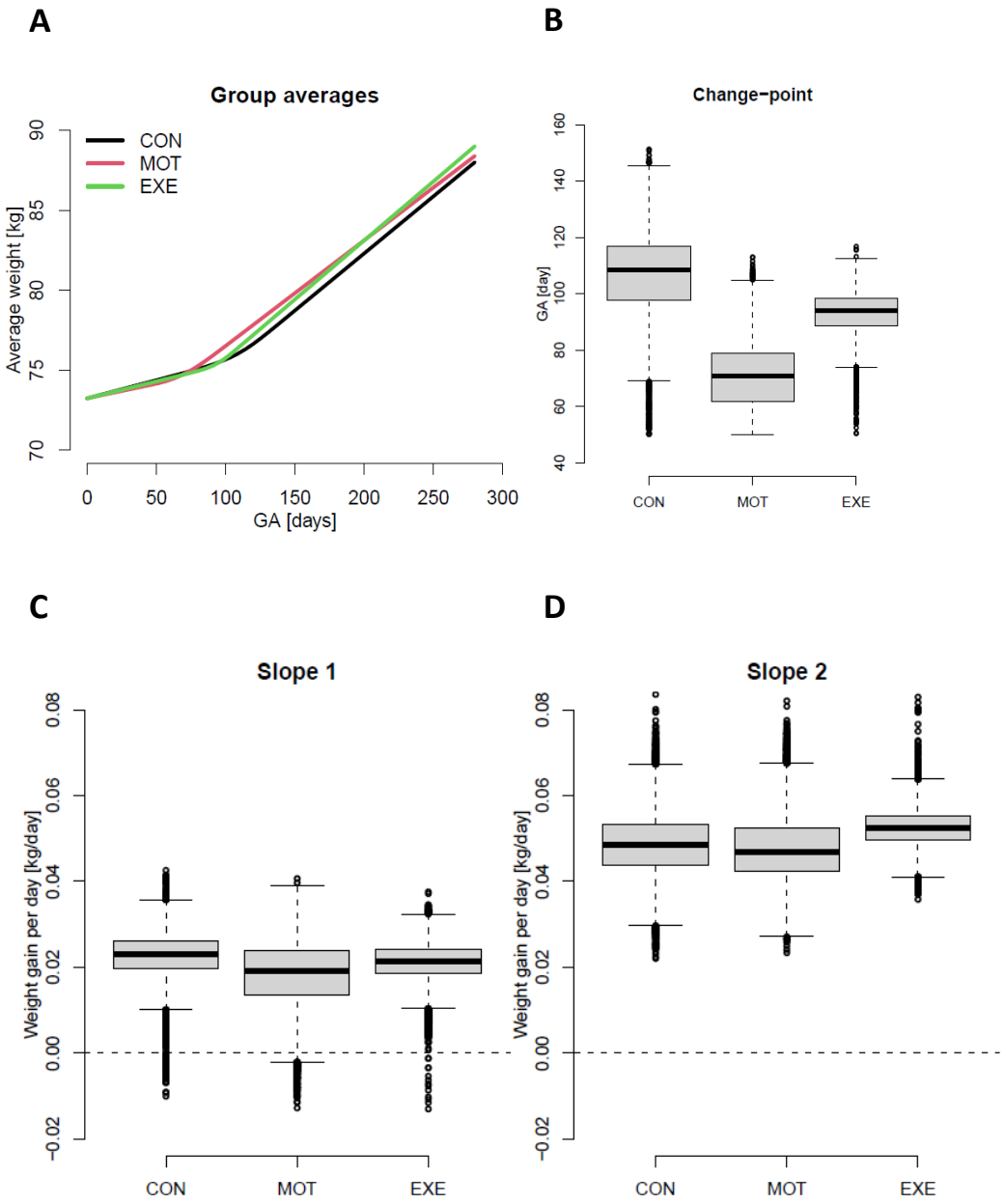

Figure 5. Average weight gain rates during pregnancy in CON (n=45), MOT (n=87), and EXE (n=87) **(A)**, gestational age change-point for weight gain rate in the three groups **(B)**, weight gain per day in CON, MOT and EXE before the change-point **(C)** and after the change-point **(D)**. CON; Control, MOT; Motivational counselling on physical activity, EXE; Structured supervised exercise training, GA; Gestational age.

Influence of prepregnancy body weight on gestational weight gain trajectory

Lastly, we investigated if the predicted slope 1 and 2 in CON, EXE and MOT in the model were associated with predicted weights at GA 0 weeks. As expected, we found a general tendency for smaller slopes with higher weights at GA 0 weeks, but no significant associations, meaning that estimated GWG for participants with higher prepregnancy BMI was not systematically lower due to higher weight at GA 0 weeks than estimated GWG for participants with lower prepregnancy BMI. If slopes were significantly associated with prepregnancy BMI, GWG would have to be adjusted for weight at GA 0 weeks to avoid systematic underestimation of GWG for women with obesity compared to women with lower prepregnancy BMI and subsequent overestimation of the difference in GWG between women with obesity and normal weight.

Discussion of findings in Paper 2

Sample size

As described in the discussion of Paper 1 and in our statistical analysis plan, our sample size calculation for GWG showed that 33 participants in CON and 66 in each of the two intervention groups were needed to detect a significant difference in GWG at delivery between CON and the intervention groups. The sample size calculation was based on a comparable study by Haakstad et al. with exercise training in pregnant women[163]. This study showed a difference in GWG of 2.8 kg between exercise and control groups, when including only participants in the exercise group who attended 24 sessions (twice per week), and SD's were 4 kg. Despite that we had 34, 74 and 70 completers at delivery in CON, EXE, and MOT, respectively, we did not find any differences in total GWG (estimated GWG at GA 40+0 weeks) between groups. This is likely due to lower effect sizes on GWG with less than 1 kg difference between the three groups, as well as higher SD of up to 6.4 kg (unpublished data), compared to the study by Haakstad et al. Further, the GWG difference of 2.8 kg between exercise and control groups in the study by Haakstad et al. were present when analyzing participants in the exercise group who participated twice per week. The exercise intervention in the study by Haakstad et al. consisted of supervised aerobic exercise training for one hour at least twice a week for 12 weeks during pregnancy and is comparable to the EXE intervention in our study. However, the average adherence of 1.3 sessions/week [1.1;1.5] in EXE in our study, is markedly lower than the attendance rate of 2 sessions/week in the study by Haakstad et al. Altogether, these factors probably explain why EXE and MOT did not have lower GWG compared to CON, even though we fulfilled the included number of participants needed to detect a significant difference according to our sample size calculation.

Physical activity level and influence on gestational weight gain and obstetric and neonatal outcomes

As mentioned in the discussion of Paper 1, the average MVPA measured by the activity tracker was below one hour in both EXE, MOT and CON, and the low PA level and rather poor average adherence might have influenced the effects of our interventions on GWG and obstetric and neonatal outcomes. Additionally, we performed a linear regression analysis to investigate if prenatal PA per se independent of study group allocation was associated with GWG and obstetric and neonatal outcomes. As shown in Figure S.3A-C in Paper 2 no associations were found between MVPA, steps or active kilocalories and total GWG.

Associations between MVPA, steps, active kilocalories and obstetric and neonatal outcomes were also investigated using linear and logistic regression for continuous and categorical outcomes, respectively (unpublished data). Most of these analyses included the 178 participants who were still enrolled in the study when they delivered. PA data used for regression analyses were average values from randomization to delivery day independent of GA at randomization and delivery, and missing PA data were imputed. For analyses of pregnancy complications, all 219 randomized participants were included, and PA data were average values from randomization to GA 40+0 weeks for the 41 participants who were lost to follow-up before delivery. Overall, no associations were found between any of the PA measures and obstetric pregnancy complications or delivery related outcomes, as well as neonatal outcomes.

To investigate the effects of obtaining higher amounts of tracker-measured MVPA in the intervention groups, per protocol analyses were made comparing total GWG for all women in CON independent of MVPA level with total GWG for EXE and MOT participants with average MVPA of 60-150 min per week, 150-210 min per week, above 210 min per week, and above 60 min per week. Similar to PA data used for associations between PA measures and total GWG at GA 40+0 weeks in Paper 2, PA data used for per protocol analyses had imputations for missing data and average values from randomization to delivery day for participants who delivered ≤GA 40+0 weeks, and from randomization to GA 40+0 weeks for participants who delivered >GA 40+0 weeks or were lost to follow-up before delivery. One-way ANOVAs were used to investigate differences between groups in total GWG for the different average MVPA levels. Compared to CON, EXE and MOT did not affect total GWG when including EXE and MOT participants having average MVPA of 60-150 min per week ($p=0.848$), 150-210 min per week ($p=0.221$), above 210 min per week ($p=0.234$), and above 60 min per week ($p=0.853$) (Figure 3).

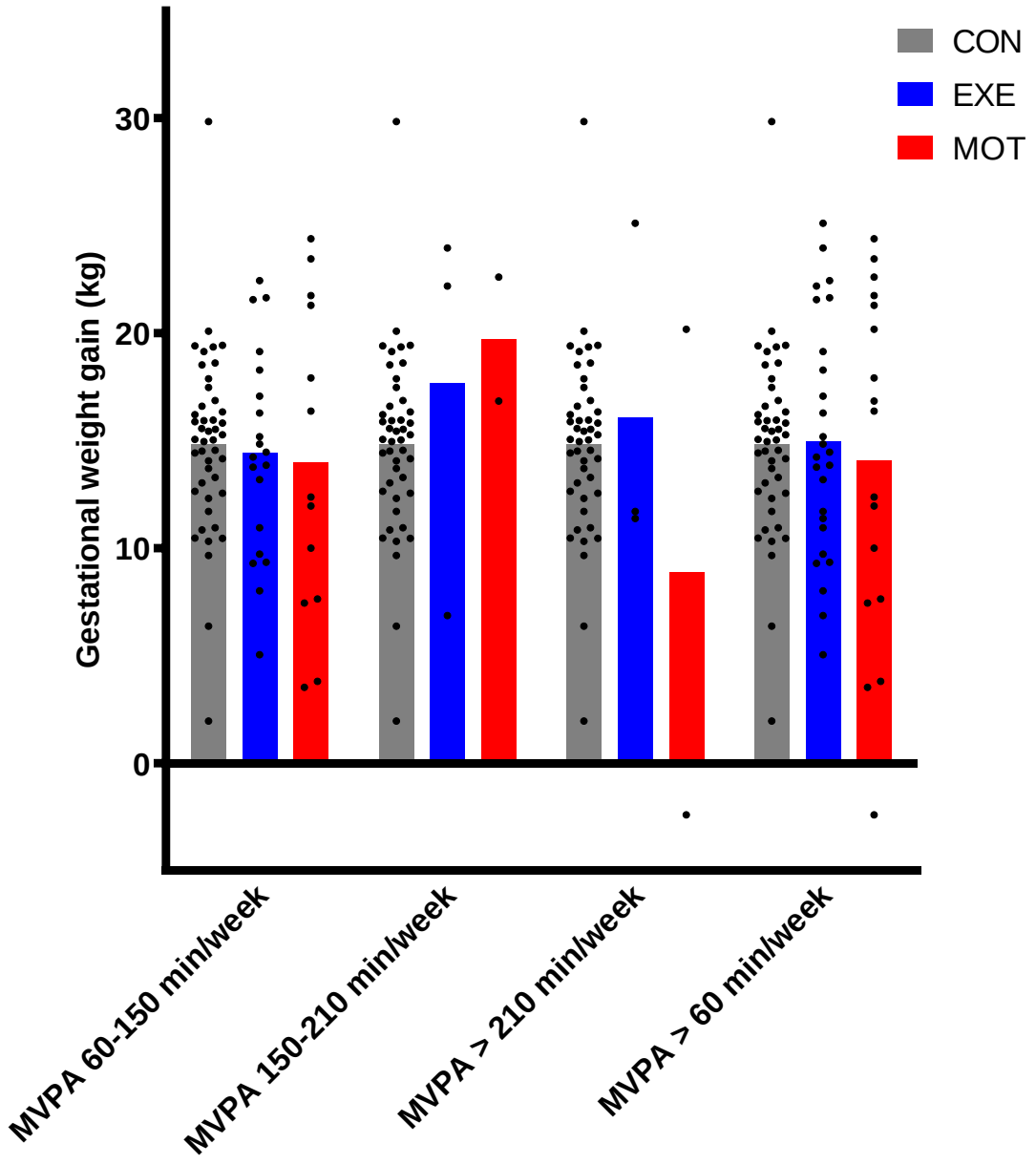

Figure 3. Total gestational weight gain for all CON participants independent of MVPA level (n=45) versus EXE and MOT participants with average MVPA of 60-150 min/week (EXE: n=20, MOT: n=13), 150-210 min/week (EXE: n=3, MOT: n=2), above 210 min/week (EXE: n=3, MOT: n=2), and above 60 min/week (EXE: n=26, MOT: n=17) from randomization to max. GA 40+0 weeks. Data are means and dots represent individual data points. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, MVPA; Moderate to vigorous intensity physical activity.

These analyses indicate that obtaining higher amounts of MVPA in EXE and MOT does not reduce total GWG compared to CON. However, very few participants had MVPA above 150 min per week and hence, low power constitute a challenge for the statistical per protocol analyses. Additional analyses were performed combining GWG data from EXE and MOT participants in one PA intervention group, in the attempt to increase power in EXE and MOT. Two-sample t-test analyses between all CON participants versus EXE and MOT participants combined showed no

differences in total GWG for neither EXE and MOT participants having an average MVPA of 60-150 min per week (p= 0.617), 150-210 min per week (p= 0.316), above 210 min per week (p= 0.743), or above 60 min per week (p= 0.847), similar to the results of the previous analyses.

Moreover, a per protocol analysis was performed based on manually registered adherence to the intervention in EXE to compare total GWG for all women in CON with total GWG for EXE participants who attended on average 2-3 exercise sessions per week from randomization to delivery. Two-sample t-test showed no difference in total GWG between CON and EXE participants who attended on average 2-3 exercise sessions per week (p= 0.374) (Figure 4). Thus, relatively high adherence to the EXE intervention did not reduce total GWG compared to CON.

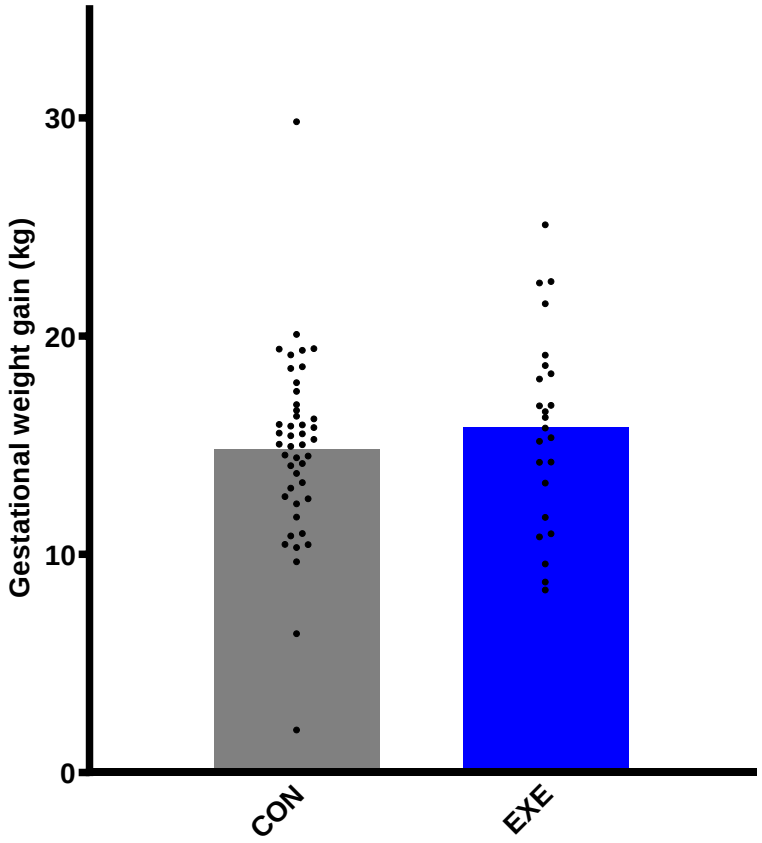

Figure 4. Total gestational weight gain for all CON participants (n=45) versus EXE participants who attended on average 2-3 exercise sessions per week from randomization to delivery (n=24). Data are means and dots represent individual data points. CON; Control, EXE; Structured supervised exercise training.

Altogether, tracker-measured PA was not associated with total GWG, obstetric or neonatal outcomes. Further, higher amounts of average weekly tracker measured MVPA in EXE and MOT did not reduce total GWG compared to CON. Likewise, attending on average 2-3 exercise sessions per week in EXE did not influence total GWG compared to CON. These findings are in contrast with the recent systematic reviews and meta-analyses of studies with exercise interventions during

pregnancy. Overall, they report reduced GWG with effect sizes of 1-2 kg with prenatal exercise interventions compared to control in both normal weight and overweight/obese women[41–43], and a 32% reduced risk of excessive GWG[41]. The methodology and quality of the studies in the meta-analyses vary, which likely influences the results, and risk for publication bias needs to be considered. However, all three meta-analyses assessed publication bias via funnel plots and found no evidence of publication bias[41–43]. Exercise characteristics including frequency, intensity, duration, and type of exercise for optimization of GWG were also reported in these meta-analyses. Wang et al. indicated that the most beneficial effect of prenatal exercise interventions on reducing GWG appeared to be when women exercised three times per week for 30-60 min each time[43]. Similarly, Ruchat et al. reported that to achieve at least 25% reduction in risk of excessive GWG, the pregnant women had to exercise at least two times per week for 35 min each time or accumulate at least 456 MET-min per week of moderate intensity exercise corresponding to exercising at moderate intensity for around 105 min per week[41]. In the meta-analysis by Diaz-Burrueco et al. that also found a protective effect of prenatal exercise on GWG, they reviewed various types of PA during pregnancy and reported a mean duration of 51.3 min per PA session and a frequency of three times per week[42]. Further, they reported that supervised exercise sessions including aerobic, strengthening, and stretching guided exercises, as well as cycling sessions were effective on reducing GWG.

Thus, it seems necessary to accumulate a certain amount of exercise during pregnancy of at least two weekly exercise sessions and minimum 100 min per week to achieve beneficial effects on GWG. This is more than the PA level obtained in our study with average weekly MVPA of 53.5 min [42.0;65.0] in EXE and 43.1 min [31.6;54.6] in MOT, and in average attendance in 1.3 [1.1;1.5] sessions per week in EXE from randomization to delivery, which may constitute a possible explanation for the lack of effect of EXE and MOT on total GWG and obstetric and neonatal outcomes. We experienced a moderate to high adherence to the MOT intervention with participants attending on average 5.2 [4.7;5.7] out of 7 counselling sessions during pregnancy, but this was not reflected in the MVPA level and GWG. In contrast, Haby et al. showed decreased GWG and a higher amount of women with GWG below 7 kg among obese pregnant women with higher adherence to a motivational counselling intervention on diet and PA compared to obese control women[164].

Other possible explanations for the lack of difference in total GWG and obstetric and neonatal outcomes in EXE and MOT compared to CON might include the health status of CON, health status of our overall study population, and that we intervened on exercise only. The participants in CON were likely relatively healthy, for example being normal weight on

average and overall non-smoking, which minimizes differences between CON and the intervention groups. Further, as previously mentioned in the discussion of Paper 1 we do not know whether participants in CON increased PA during the study period due to motivation from wearing the tracker.

However, reduced GWG after prenatal exercise interventions compared to controls has both been found in studies including women with overweight/obesity[129,165] and normal weight[136,137,154].

Regarding health status of our overall study population, the prevalence of overweight or obesity[24] and the level of insufficient PA[5] are lower among Danish women in general compared to women in other western countries such as the United States and the United Kingdom. This might reduce the potential for exercise to induce beneficial health effects on GWG and obstetric and neonatal outcomes in our study population compared to pregnant populations in other countries with a higher overweight and obesity burden among pregnant women, for example the United States[25]. Thus, intervention effects of EXE and MOT on reduced GWG and improved obstetric and neonatal outcomes would probably have been more evident among populations with overweight or obesity. In fact, stratification of our study participants into subgroups based on their prepregnancy BMI indicated that women with obesity in EXE and MOT gained less weight than participants with normal weight in EXE and MOT, respectively (Paper 2). These findings indicate that women with obesity may be more susceptible to beneficial effects of prenatal exercise, but we could not confirm an intervention effect of EXE or MOT compared to CON in the subgroup of obese women.

Further, we speculated whether the average GWG in FitMum was lower compared to other studies since a potentially lower average GWG in FitMum could reduce the potential for EXE and MOT to reduce GWG, and constitute a possible explanation for the lack of effect of EXE and MOT on GWG. However, the total GWG (GWG at GA at 40+0 weeks) in FitMum was 14.9 kg [13.6;16.1] in CON, 15.7 kg [14.7;16.7] in EXE and 15.0 kg [13.6;16.4] in MOT, whereas differences in GWG between intervention and control groups have been found in other studies that report lower average GWG of only 8-12 kg in the exercise intervention groups and 10-13 kg in the control groups[129,136,137,154]. Thus, average GWG in FitMum was higher compared to other studies reporting reduced GWG after exercise, so a reduced potential for reduction in GWG in FitMum could not explain the lack of intervention effects on GWG in our study.

Focusing on the effects of intervening on PA only versus on multiple lifestyle components, a three-armed randomized controlled study by Renault et al. included Danish pregnant women with obesity and compared a PA plus dietary intervention, a PA intervention only, and a control group. The authors found reduced GWG in both intervention groups compared to control but no difference in GWG between the two intervention groups[153]. Other reviews and studies have shown

that it might be more effective for reduction of GWG and improvement of obstetric and neonatal outcomes to intervene on both diet and PA[40,44,128], in contrast to our interventions that focused on PA only.

Maternal and infant body composition

Measuring maternal body weight and GWG reflect changes in several maternal and fetal components such as fat mass, fat-free mass, total body water and placenta[32]. However, total weight measurements do not reveal the contribution from each component and hence, adding measurements of maternal body composition during pregnancy seems important to gain a more detailed understanding of alterations in metabolic health profile. Several studies in non-pregnant populations have shown exercise-induced beneficial changes in body composition with increased fat free mass[57,166] and reduced fat mass[57,58,166,167] after exercise. Computed tomography and magnetic resonance imaging (MRI) are nowadays recognized as gold standard methods for measurements of body composition[168]. DXA scan is considered gold standard for measurement of bone mineral density but can also estimate total and regional fat and lean mass and show similar accuracy with MRI[168]. Computed tomography and DXA methodologies are not recommended during pregnancy due to ionizing radiation exposure[169]. In contrast, MRI is considered safe for the pregnant woman and fetus and is therefore the only method available for in vivo measurements of masses and distributions of fat, skeletal muscle, and organ tissue[169]. However, MRI has several limitations including expensive costs, technician expertise needed, and being unsuitable for field-based settings[169]. Thus, investigation of body composition during pregnancy are most of the time limited to use of less robust methods such as air displacement plethysmography, anthropometric measurements such as skinfold thicknesses and upper arm circumferences to estimate fat mass, and estimation of body composition based on total body water measured by DLW[169]. Furthermore, challenges of measuring body composition during pregnancy include that the methods available cannot distinguish between maternal and fetal depots, and that pregnancy-induced body composition changes, for example increase in total body water, can violate assumptions inherent in many of the currently available methods that do not have pregnancy specific corrections[169]. Body composition in response to exercise among pregnant women are seldomly reported, probably due to complicated and costly methods compared to measuring body weight, which is an easy measure routinely obtained as part of prenatal care. Ferrari et al. measured fat mass using anthropometry in humans at GA 36 weeks and reported lower fat mass among exercising pregnant women compared to controls[75]. On the other hand, Cavalcante et al. showed no differences between a prenatal water aerobics exercise group and a control group in estimated body fat mass

and fat-free mass based on skinfold thicknesses measured three times during pregnancy[170]. In Paper 2, we only report data on GWG. However, in FitMum we have also investigated maternal body composition during pregnancy using DLW, and 7-14 days after delivery using DXA scan. These data are planned to be reported in a future paper and will add important information to data in the current paper regarding GWG. The lack of difference in GWG between groups could be due to an increase in lean mass and decrease in fat mass in EXE and MOT compared to CON that were not reflected in the GWG but would be a metabolically healthier weight gain.

Regarding infant anthropometry, we report infant weight and length measured at birth in Paper 2. For the same reasons as described above, it seems important to investigate infant body composition. Several studies have estimated infant body composition using skinfold thicknesses and found reduced fat mass in early infancy after maternal prenatal exercise[77–79]. Studies using more advanced methods to measure infant body composition, including DXA and PEA POD (air displacement plethysmography), in early infancy also indicate maternal exercise to be associated with reduced offspring fat mass[171] and increased lean mass[80,81]. In FitMum, we obtain data on infant body weight and length as well as head circumference several times during the first year of life, and we have recently been granted funding to perform follow-up measurements in the offspring at three years of age using DXA and a BOD POD body composition system (air displacement plethysmography).

PAPER 3: THE EFFECTS OF PRENATAL PHYSICAL ACTIVITY INTERVENTIONS ON THE HUMAN BREAST MILK METABOLOME AND LIPIDOME

In Paper 3, we aimed to investigate the effects of EXE versus MOT on breast milk composition compared to CON. Breast milk samples were obtained from 99 participants from a single feed 7-14 days after birth at the first feeding after 6:00 AM. Ultrahigh Performance Liquid Chromatography mass spectrometry untargeted metabolomics and lipidomics analyses were used to analyze the breast milk metabolome and lipidome. Overall, we found no major metabolite or lipid changes with EXE or MOT compared to CON, but our interventions changed some metabolites and lipids compared to CON, and metabolites and lipids correlated with PA measures. Thus, maternal prenatal PA may induce changes to the human breast milk metabolome and lipidome, which in part could explain improved offspring metabolic health.

Methodological considerations

Metabolomic and lipidomic profiling

Use of metabolomic and lipidomic profiling techniques have been expanded during the past decade. Metabolomics is defined as the comprehensive analysis of the metabolome in a biological system, for example biofluid or tissue[172]. The metabolome is the entire collection of metabolites within a biological system. The metabolites include low molecular weight (<1500 Daltons) chemical substrates, intermediates or end products of enzyme-mediated reactions[172]. Lipidomics is a subfield of metabolomics and includes the study of the lipidome, for example total lipid content, within a given biological system[172]. Omics approaches are generally described as unbiased global analyses to identify the largest possible amount of compounds within a biological system[172]. However, different approaches including untargeted and targeted metabolomics and lipidomics analyses are available. In Paper 3 we used untargeted metabolomics/lipidomics analyses, which aim to detect as many metabolites/lipids as possible and provide the relative abundances, and not absolute concentrations, of metabolites/lipids[172].

Potentials and challenges of metabolomic and lipidomic profiling

Untargeted metabolomics and lipidomics are considered hypothesis-generating approaches that provide tremendous potential for identification of previously unknown metabolic targets, which can be validated using the targeted omics approach[172]. This offers outstanding possibilities to expand and accelerate our understanding of mechanisms involved in exercise-induced changes of

complex biological systems, since metabolites lie downstream of all other biological regulations and therefore reflect changes that occur as a result of several processes involving the genome, transcriptome and proteome[172]. As mentioned in Paper 3, metabolic profiling provides a metabolic phenotype snapshot and allows for rapid indications of metabolic perturbations in response to exercise. Since metabolomic and lipidomic profiling is suitable for investigations in both preclinical and clinical settings, it can be used for drug discovery and development in the pharmaceutical industry as well[173]. Discovery of previously unknown metabolites and lipids possibly involved in optimization of offspring metabolic health may also provide possibilities for supplementation of these during lactation, as tested in mice by Harris et al.[98], who suggest supplementation of 3'-SL as a potential therapeutic tool to prevent development of offspring obesity, T2D and cardiovascular disease later in life.

Further, only small sample volumes of typically 10-100 ul are needed for analysis, which makes sample collection feasible, and metabolomic and lipidomic profiling analyses have been performed in several human and animal bodily fluids and tissues[172]. Biofluids, such as blood, urine, saliva and sweat, are far more used in exercise metabolomics and lipidomics studies in humans, but metabolomic/lipidomic profiling can also be performed in tissue biopsies from for example liver and skeletal muscle. In contrary, metabolomics and lipidomics studies in animal models have predominantly used tissues relative to biofluids[172]. Metabolomics and lipidomics analyses have also been performed in human breast milk[100,174–177], but to my knowledge only one study has investigated effects of maternal exercise on human breast milk using metabolomics and lipidomics analyses[100]. Dried blood spots are also considered interesting with regards to advancement of metabolomics/lipidomics techniques. Dried blood spots are obtained with finger prick for adults and heel prick for infants and are therefore less invasive compared to blood sampling by venepuncture[172]. In Denmark, all newborn infants are offered a screening for a number of congenital diseases via a heel prick test performed 48-72 hours after birth[178]. Implementing metabolomics/lipidomics analyses of dried blood spots from these tests could offer a great possibility to gain insight into the metabolic profile in early infancy and investigate whether the metabolome or lipidome is associated with for example maternal GWG as well as diet and exercise during pregnancy.

Metabolomic and lipidomic profiling data also have the potential to be used for predictions of diseases, based on specific metabolites or lipids known to be involved in specific diseases. In a recent study, Ooi et al. performed lipidomics analysis and indicated moderate to strong correlations between several lipids in liver and plasma in severe or morbidly obese patients undergoing bariatric surgery[179]. This suggests that the plasma lipidome can reflect pathological changes in the

liver and hence constitute a less invasive method than liver biopsies to predict liver disease[179]. If the breast milk metabolome/lipidome is also associated with for example liver metabolome/lipidome, utility of breast milk metabolites/lipids revealed by metabolomics/lipidomics analysis could carry a potential to non-invasively investigate biomarkers of disease conditions in mother and maybe also indirectly in the child. Use of metabolite/lipid data for predictions can facilitate the development of personalized medicine, including personalized treatment strategies such as personalized training interventions.

In untargeted analysis, data processing includes identification/annotation of detected metabolites/lipids via comparison to in-house libraries and available databases, for example the Human Metabolome Database. Identification of metabolites and lipids constitutes a main challenge of the untargeted metabolomic and lipidomic approaches[172]. In our study metabolomic and lipidomic analyses detected 219 annotated metabolites and 172 annotated lipids, respectively, and 21,434 and 14,278 non-annotated metabolite and lipid mass features, respectively (Paper 3). This is a normal output of untargeted metabolomic/lipidomic profiling analysis. Besides the need for expansion of libraries to allow for better identification of unknown metabolites and lipids, other challenges using metabolomic and lipidomic profiling analyses include large interstudy variation, meaning that comparison of findings between studies needs to be done cautiously[172]. Large variation between studies using metabolomics/lipidomics approaches may be partly caused by high interindividual variation in confounding factors such as nutritional status (fasted versus fed), fitness (trained versus untrained) and medication use, which seem important to report or control for[172]. If investigating effects of exercise training on the metabolome/lipidome it will most likely also be important to control for exercise prior to obtaining the sample. As described in Paper 3, a limitation of our study is that we did not control for these confounding factors. Further, differences in experimental factors including use of different analytical platforms and data acquisition modes also contribute to the large variation between studies. In our study we used the analytical platform liquid chromatography mass spectrometry for data acquisition, which is currently the most widely used metabolomic profiling technique[172].

Discussion of findings in Paper 3

Influence of physical activity level on human breast milk composition

Overall, in Paper 3 we found no major differences in relative abundances of metabolites and lipids in EXE and MOT compared to CON. However, EXE and MOT seemed to slightly increase relative abundances of oxoglutarate and caffeine, respectively, and decrease fatty acid hydroxy fatty acids (FAHFA)(36:3) and some phospholipids. The average weekly MVPA from randomization to delivery was 36.5 min [26.5;46.6] in CON, 60.7 min [44.2;77.3] in EXE and 45.8 min [28.2;63.4] in MOT among the 99 participants included in the breast milk analyses (unpublished data). Thus, our findings of overall no major differences in metabolites and lipids between interventions and CON could be due to rather low MVPA in EXE and MOT. Comparing the MVPA level in our study with other studies is challenged by the sparse number of studies focusing on the influence of maternal exercise during pregnancy on breast milk composition[98,105]. Moreover, these studies do not provide measures of PA that are comparable with our study because they use animal models or report correlations between PA and human breast milk components. In our study we found positive correlations of some metabolites and several phospholipids with PA measures from the activity tracker, indicating that higher levels of PA increase the relative abundances of these metabolites and lipids.

Previous studies have shown changes in 3'-SL and 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) in human breast milk with prenatal and postpartum maternal exercise, respectively[98,100], but none of these metabolites differed in EXE or MOT compared to CON. Again, this could be due to rather low MVPA in EXE and MOT. Moreover, correlograms, which were performed for a global overview of all correlations between metabolites/lipids and PA measures, GA at delivery, age, prepregnancy BMI, GWG, as well as maternal weight 7-14 days after delivery, showed no strong correlations between any of these measures and 3'-SL or 12,13-diHOME.

Despite rather low weekly MVPA in EXE and MOT, we found some changed metabolites and lipids in EXE and MOT, for example caffeine and oxoglutarate. However, it is uncertain whether these changes were caused by lasting effects of the interventions or might be confounded by other factors, for example medication use in relation to the delivery, that possibly influence the dynamically changing metabolome and lipidome[172] in the period between termination of interventions (at latest at delivery) and breast milk sampling 7-14 days postpartum. Mature breast milk cannot be pumped before around one week after delivery, so if the aim is to investigate effects of exercise training performed during pregnancy on mature breast milk composition, this intermediate period of several days cannot be avoided. Nevertheless, there might be long-lasting

exercise-induced adaptations in the breast milk metabolome and lipidome after prenatal exercise training. As to my knowledge, only one study has investigated the effects of maternal exercise on the human breast milk metabolome and lipidome and this study focused on effects of maternal acute exercise in the postpartum lactating period[100]. Therefore, studies with the primary objective of investigating effects of prenatal exercise training on the human breast milk metabolome and lipidome are warranted. Such studies should perform sample size calculations and strictly control adherence to interventions to hopefully obtain high PA levels among participants during pregnancy. Further, confounding factors such as nutritional status prior to sampling should be controlled for, as seen in other studies investigating the effects of acute exercise on the human metabolome and lipidome[100,180–182]. In our study, the investigation of breast milk composition was exploratory and a secondary outcome and thus, no sample size calculation was performed for this outcome. We analyzed the data according to both the randomized controlled design to investigate differences in relative abundances of metabolites and lipids between the three groups, and by using an observational design to investigate if PA levels independent of study group allocation were associated with relative abundances of metabolites/lipids.

Other factors can affect human breast milk composition

The design of the current study (Paper 3) allowed us to investigate the influence of maternal prenatal PA on the human breast milk metabolome and lipidome in a real-life setting 7-14 days after delivery. However, we did not control for other factors such as maternal obesity, nutritional status, or medication use prior to obtaining the breast milk sample, that might influence breast milk composition and possibly blur potential effects of prenatal PA. A recent systematic review and meta-regression analysis including 66 studies indicated a positive association between maternal BMI and fat concentration in human breast milk 1-6 months postpartum[183]. De la Garza Puentes et al. investigated the influence of maternal prepregnancy BMI on fatty acid concentration in human colostrum and mature breast milk sampled 2-4 and 28-32 days postpartum, respectively[184]. Mothers with overweight and obesity had changes in several fatty acids in both colostrum and mature milk compared to mothers with normal weight[184]. Further, in a study by Brezinova et al. maternal obesity was indicated to decrease levels of FAHFA in human breast milk collected 72 hours after delivery[177]. FAHFA is a lipid class that includes 5-palmitic acid ester of hydroxystearic acid (5-PAHSA), 9-PAHSA, and 13-DHAHLA and act as lipokines[185]. Lipokines are lipid compounds that are predominantly secreted from adipose tissue and can act as signaling molecules and influence systemic metabolism[185,186]. FAHFA was discovered in 2014 and has been indicated to confer anti-diabetic and anti-inflammatory effects since it correlates strongly with insulin

sensitivity, measured by gold standard euglycemic hyperinsulinemic clamp, and is reduced in serum and adipose tissue from insulin-resistant humans[187]. The study by Brezinova et al. showed lower total PAHSA level in milk from mothers with obesity compared to mothers with normal weight[177]. Moreover, other studies have found maternal obesity to be associated with changes in the human milk metabolome[175,176]. One study found that 10 and 20 breast milk metabolites differed between women with overweight/obesity compared to lean mothers at one and six months postpartum, respectively[175]. Another study assessed maternal adiposity by prepregnancy or early pregnancy BMI as well as fat mass in early pregnancy determined by air displacement plethysmography. They collected breast milk at a half, two, and six months postpartum and showed that 23, 17 and 10 metabolites, respectively, were associated with maternal adiposity[176]. Finally, Sims et al. showed that concentrations of insulin, leptin and C-reactive protein were higher in breast milk from mothers with overweight/obesity compared to mothers with normal weight during several time points throughout lactation (one, two, three, four, and nine months postpartum)[188]. Altogether, several studies point towards an effect of maternal weight status on different components of breast milk composition in humans.

Other factors, including maternal nutrition and geographic location, have also been shown to influence breast milk composition. A systematic review included studies that assessed maternal usual diet, maternal diet during pregnancy or postpartum, by food-frequency questionnaires, dietary records or dietary recalls[189]. The review included studies from well-nourished populations in developed countries only, and concluded that the findings within this area are limited and conflicting, but presented some studies showing that maternal nutrition seems to relate to some extent to breast milk components including total protein, total fat, different types of fatty acids and vitamin C[189]. Moreover, the human milk oligosaccharide (HMO) concentration has been indicated to vary with geographic location in a study showing different concentrations of different HMO's in healthy women living in different parts of the world, including cohorts in countries from North America, South America, Europe and Africa. For example, concentration of the HMO 3-fucosyllactose was higher in breast milk collected from a Swedish population compared to in women from rural Gambia[190]. The study also showed differences in concentrations of several HMO's between women of the same ethnic origin, and therefore likely genetically similar, but living in different environments, as evidenced by differences in HMO concentrations between women living in rural versus urban sites of Ethiopia and Gambia[190].

Other factors such as medication use in relation to delivery, as well as sleep quantity and quality, may also influence breast milk composition, as indicated by studies of other components of breastfeeding. Use of epidural analgesia for pain relief in relation to delivery has been associated

with delayed initiation of breastfeeding, breastfeeding challenges, negative breastfeeding experiences, and early cessation of breastfeeding[191–194]. Moreover, oxytocin administration during delivery has been shown to reduce endogenous oxytocin concentration during breastfeeding two days after delivery in a dose-dependent manner, meaning that the more syntocinon (synthetic produced oxytocin) the women received during delivery, the lower postpartum oxytocin concentration during breastfeeding[195]. The same study showed that those women who received both syntocinon and epidural analgesia during delivery had the lowest median endogenous oxytocin concentration during breastfeeding[195]. Intrapartum oxytocin administration has also been indicated to reduce the expression of several primitive neonatal reflexes associated with breastfeeding[196,197]. Moreover, caesarean section may affect early breastfeeding initiation negatively and induce more breastfeeding difficulties compared to vaginal delivery[198,199] and hence, delivery mode may also influence breast milk composition.

CONCLUSIONS AND PERSPECTIVES FOR FUTURE RESEARCH

The main objective of this thesis was to investigate the effects of structured supervised exercise training versus motivational counselling on PA during pregnancy on GWG and obstetric and neonatal outcomes during pregnancy and at delivery in healthy inactive pregnant women and their offspring compared to standard care (Paper 2). Moreover, the thesis aimed to explore possible underlying mechanisms for exercise-induced improvements in offspring health, focusing on changes in breast milk composition 7-14 days after delivery investigated by metabolomics and lipidomics analyses (Paper 3). In contrast to previous studies, we found no overall effect of our interventions on GWG or obstetric and neonatal outcomes compared to standard care. Hence, our two predefined hypotheses for GWG that participants in EXE would gain less weight compared to those in MOT, and that participants in MOT would gain less weight compared to those in CON, were rejected, which might be explained by a relatively low PA level in EXE and MOT. We also found that women with obesity in both EXE and MOT gained less weight compared to women with normal weight within the same intervention groups, and that associations between PA measures and GWG differed between women with obesity and normal weight. This indicated that women with obesity might be more susceptible to the beneficial effects of PA compared to women with normal weight, which is in line with findings from previous studies (Paper 2).

During the explorative investigations in relation to underlying mechanisms of exercise-induced improvements in offspring health, we found no major changes in the breast milk metabolome and lipidome in EXE and MOT compared to CON. This might be explained by the relatively low PA level in EXE and MOT as well, and several other confounding factors that might blur the effect of prenatal exercise and which we did not control for, for example delivery mode, nutritional status, exercise or medication use prior to breast milk sampling. However, we found changes in some metabolites and lipids, which supports the existing literature that propose exercise-induced adaptations to breast milk as a possible underlying mechanism contributing to improved offspring health (Paper 3).

The conclusions on these secondary outcomes from the FitMum study are connected to a high degree of uncertainty, since the study might be underpowered to investigate these specific outcomes and sample size calculations were only made for the outcomes moderate to vigorous intensity PA and GWG.

For future research, studies with the primary objective of investigating effects of human prenatal PA interventions on these maternal and offspring health outcomes and possible underlying mechanisms for exercise-induced improvements of offspring health, for example with focus on adaptations in breast milk, are warranted. Adaptations in breast milk composition constitute only one possible mechanism mediating beneficial effects of maternal prenatal PA on offspring health. Other possible mechanisms might be epigenetic changes and adaptations in the placenta. In the FitMum study we have obtained umbilical cord blood and placenta samples at delivery as well, and papers regarding epigenetic changes (DNA methylation) and placenta adaptations are in preparation. These may contribute to expand our understanding of mechanisms mediating exercise-induced optimization of offspring health. Moreover, a renewed effort to increase PA during pregnancy to optimize maternal and offspring health is needed, as well as future research focusing on the effects of initiating maternal PA interventions preconceptionally on maternal PA before and during pregnancy and on maternal and offspring health outcomes. Furthermore, subsequent long-term follow-up in human offspring is warranted. Long-term follow-up is important when investigating effects of maternal prenatal PA on human offspring metabolism and risk of development of obesity and lifestyle related diseases, since these conditions typically occur later in life. Until now, long-term follow-up on adult offspring health after exposure to maternal prenatal PA has primarily been carried out in rodent models, which have a shorter generation timeline compared to humans. The gap of studies with long-term follow-up on human offspring health outcomes might explain the conflicting evidence regarding effects of lifestyle interventions during pregnancy, including maternal PA, on risk of childhood obesity. In the FitMum study, we currently collect follow-up data on offspring throughout the first year of life and we have recently been granted funding to perform follow-up measurements in the children at three years of age as well.

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Paper 1

BMJ Open

Structured supervised exercise training or motivational counselling during pregnancy on physical activity level and health of mother and offspring: FitMum study protocol

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ABSTRACT

Introduction A physically active lifestyle during pregnancy improves maternal and offspring health but can be difficult to follow. In Denmark, less than 40% of pregnant women meet physical activity (PA) recommendations. The FitMum study aims to explore strategies to increase PA during pregnancy among women with low PA and assess the health effects of PA. This paper presents the FitMum protocol, which evaluates the effects of structured supervised exercise training or motivational counselling supported by health technology during pregnancy on PA level and health of mother and offspring.

Methods and analysis A single-site three-arm randomised controlled trial that aims to recruit 220 healthy, pregnant women with gestational age (GA) no later than week 15 and whose PA level does not exceed one hour/week. Participants are randomised to one of three groups: structured supervised exercise training consisting of three weekly exercise sessions, motivational counselling supported by health technology or a control group receiving standard care. The interventions take place from randomisation until delivery. The primary outcome is min/week of moderate-to-vigorous intensity PA (MVPA) as determined by a commercial activity tracker, collected from randomisation until GA of 28 weeks and 0-6 days, and the secondary outcome is gestational weight gain (GWG). Additional outcomes are complementary measures of PA; clinical and psychological health parameters in participant, partner and offspring; analyses of blood, placenta and breastmilk samples; process evaluation of interventions; and personal understandings of PA.

Ethics and dissemination The study is approved by the Danish National Committee on Health Research Ethics (# H-18011067) and the Danish Data Protection Agency (# P-2019-512). Findings will be disseminated via peer-reviewed publications, at conferences, and to health professionals via science theatre performances.

Trial registration number NCT03679130.

Strengths and limitations of this study

- The efficacy of structured supervised exercise training and motivational counselling supported by health technology to improve physical activity and reduce weight gain of pregnant women is directly compared in a randomised controlled trial.
- The trial involves complex interventions and is held in one site only, so generalisability and fidelity might be a concern. Yet, as one of the additional outcomes, a process evaluation is conducted alongside the trial to explore how the interventions are carried out and adapted.
- The study is comprehensive and multidisciplinary in its design. Many different methodologies are used, and mother, partner and offspring are studied.
- Activity trackers can increase physical activity level and are feasible tools in everyday life, but commercial activity trackers have limited validity for the quantification of physical activity.
- Physical activity is extensively measured using three different methods: commercial activity trackers, gold standard doubly labelled water and the validated Pregnancy Physical Activity Questionnaire.

Protocol version This paper was written per the study protocol version 8 dated 28 August 2019.

INTRODUCTION

Although the health effects of PA are widely acknowledged, the means of how to best implement and maintain PA in everyday life are lacking.[1] Pregnancy can be regarded as a window of opportunity to implement good habits of PA as pregnant women are in regular contact with health professionals and are likely

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motivated to adopt healthy behaviours, as illustrated by reduced alcohol consumption and smoking cessation.[2–4] However, pregnancy can be seen as an opportunity to be exempt from fitness demands and bodily ideals and can be experienced as a troublesome time due to fatigue and discomfort.[5 6] Moreover, pregnancy is a relatively short period of time in regards to forming new habits[6] and that may affect the motivations and challenges in being physically active. Furthermore, differences in work status, social relations and family situations, as well as varying material and structural conditions, may contribute to the implementation of PA.[7]

Insufficient PA is a global problem[8] that occurs also during pregnancy.[8–12] It is a significant public health issue, as increasing evidence suggests that lifestyle during pregnancy influences health in the mother and her offspring.[4 13] Regular PA during pregnancy promotes clinical and metabolic health in both mother and offspring and reduces the number of complications during pregnancy and delivery.[14–19] PA reduces GWG,[20–26] the risk of gestational diabetes mellitus,[27–32] the intensity of low back pain[33] and the risk of caesarean delivery[22 29 34–37] and improves maternal body composition.[38] Additionally, a physically active pregnancy improves the health of the offspring by normalising birth weight,[22] reducing the risk of preterm delivery[39 40] and improving neonatal body composition[41 42] as well as placental function,[43 44] which results in optimised intrauterine growth conditions.

The Danish Health Authorities recommend that healthy pregnant women are physically active for at least 30 min/day at moderate intensity,[45] but only 38% of Danish pregnant women achieve this recommended level.[46] Several barriers to PA during pregnancy are addressed in the literature,[47] including anxiety about overdoing exercise, low motivation to adopt an active lifestyle during pregnancy, changing energy levels throughout the pregnancy and lack of time to be physically active.[48] The latest recommendations on lifestyle interventions during pregnancy support individualised advice on how to increase the PA level rather than a generic approach,[6] as pregnant women prefer personalised information.[49] Consequently, policymakers, healthcare professionals and pregnant women advocate for evidence-based guidance on how to implement PA in everyday life during pregnancy safely and effectively, with approaches that meet the needs, preferences and choices of the pregnant woman.

During the past decades, many PA intervention studies in pregnant women have been conducted on overweight and obese populations[23 24 26 28 50–57] as well as in healthy normal-weight pregnant women.[20 21 32 33 58–61] Still, none of these studies have focused primarily on investigating the effect of the exercise interventions on actual PA level in pregnant women nor have they used novel objective methods to measure actual PA levels. Structured, supervised exercise training and motivational counselling have been applied separately in pregnant women,[20 21 23 24 26 28 32 33 50–55 58–63] but the relative efficacy of these interventions has not been compared; this hampers the evidence-based implementation of effective exercise programmes into everyday life.

Objective

This paper describes the protocol of the FitMum study, which is a randomised controlled trial (RCT). The FitMum RCT aims to evaluate the effects of structured supervised exercise training (EXE) and motivational counselling supported by health technology (MOT) compared with standard care (CON) on PA level and GWG during pregnancy. Additional aims of the study are to investigate the effects of EXE and MOT on clinical and metabolic health parameters in both mother and offspring. We will also explore how the FitMum exercise programmes are carried out and adopted by conducting a process evaluation. In addition, we explore the personal attribution of meaning to the experiences and practices of PA among participants. Furthermore, we investigate how social, structural and cultural factors facilitate or hinder the successful implementation of exercise during pregnancy.

METHODS

Study design

The FitMum RCT is a single-site, three-arm randomised controlled trial study.

Setting

The study is carried out at the Department of Gynaecology and Obstetrics, Nordsjaellands Hospital (NOH), Hillerod, in the Capital region of Denmark, where approximately 4000 women give birth per year. NOH is a public hospital, and participation in FitMum is free of charge.

Participants

This study aims to include 220 healthy, pregnant women. Inclusion criteria are obtained written informed consent, maternal age of 18 years or older, gestational age (GA) of maximum 15 weeks, ultrasonic-confirmed viable intrauterine pregnancy, body mass index of 18.5–45 kg/m^2 and body weight <150 kg (prepregnancy weight or first measured weight in pregnancy), ability to wear a wrist-worn activity tracker 24/7 until one year postpartum and having a smartphone. Exclusion criteria are structured exercise at moderate-to-vigorous intensity for more than one hour/week during early pregnancy, previous preterm delivery, obstetric or medical complications, multiple pregnancies, inability to speak Danish, or alcohol or drug abuse.

Recruitment and inclusion

Participants are recruited: (1) *via* booking confirmation of a first-trimester scan, (2) at face-to-face meetings during the first-trimester scan and (3) through posters, flyers and social media. Before inclusion, interested women answer an online, one-page prescreening questionnaire. Eligible participants and their partners are invited to the first

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visit at NOH as soon as possible and no later than GA of 14 weeks and 6 days. At visit 1, the woman is verbally informed about the study and screened according to inclusion and exclusion criteria. Women who have not had a first-trimester scan are vaginally scanned to confirm a singleton, viable intrauterine pregnancy. All eligible women are included, and written informed consent is obtained (online supplemental file 1). Written informed consent is also obtained from the partner as biological samples are collected from the offspring and from the partner (online supplemental file 2). After inclusion, we obtain anthropometric and demographic information, a blood sample as well as a short semistructured interview with the participant. The interview provides knowledge of the participant's thoughts on participating in a research project, knowledge of prior and current PA level, and experiences with health technologies.

At the end of visit 1, the participant receives a commercial activity tracker, Garmin Vivosport. The participant is instructed to wear the tracker continuously 24/7 from the one week baseline period until one year postpartum, except during charging. The activity tracker is water resistant and determines the frequency, duration and intensity of activity periods on a minute-to-minute basis. The data from the activity tracker are wirelessly synced to the associated app, Garmin Connect, provided by Garmin International, and the research platform Fitabase (Small Steps Labs LLC), through which the compliance of wearing and synchronising the data from the tracker are continuously monitored during the study.

Baseline period and randomisation

After inclusion, the baseline PA level of the participant is measured by the activity tracker for one week. After the baseline period, participants are randomised into the EXE, MOT and CON groups (figure 1). The target number of participants randomised to each group is 88, 88 and 44, respectively, in order to have more participants in the intervention groups. Randomisation is performed via a numbered randomisation list administered

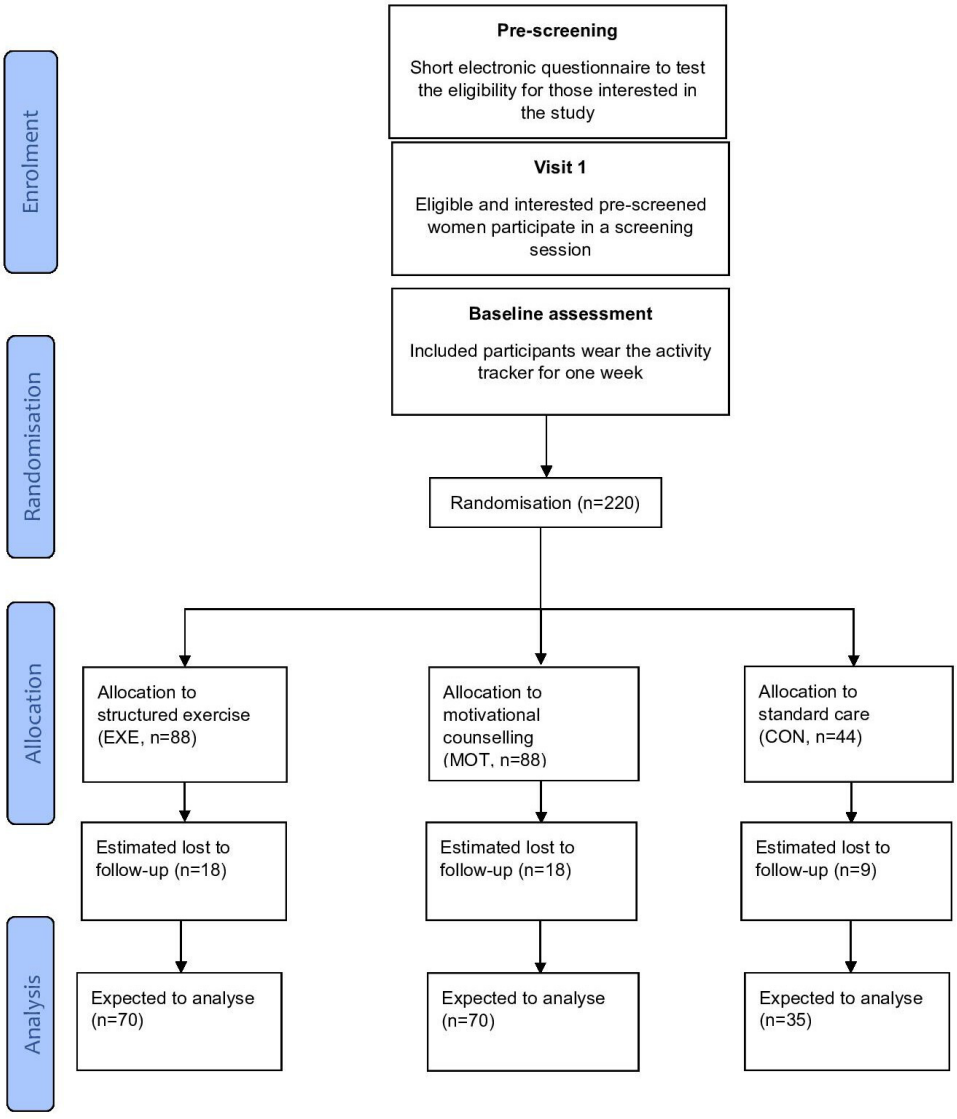

Figure 1 Flow diagram of the FitMum RCT.

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through the database Research Electronic Data Capture (REDCap), and the investigators are blinded to the procedure. Blinding of participants is considered impossible due to the inherent content of the exercise interventions. The participant is informed about the assigned group by email, and participants in EXE and MOT receive written information containing guidelines from the Danish Health Authorities about PA during pregnancy.

Patient and public involvement

Template for Intervention Description and Replication[64] was used as inspiration for the development and description of the study. As a part of the development phase, stakeholders in the field were involved in discussions and sharing of knowledge. Additionally, 27 semistructured interviews with Danish pregnant women, midwives and obstetricians were performed to explore the feasibility of such a study as well as the motivational factors and barriers to PA during pregnancy. Participants are not directly involved in the recruitment and conduct of the study, but a process evaluation is conducted, and personal understandings of the participants are obtained via interviews (see further). The insights from the study will be shared with the participants at an information meeting after the end of the study.

Interventions

Standard care at the hospital

All three groups are offered the standard care that applies to women giving birth at NOH. This consists of three appointments with their general practitioner (GA weeks 6–10, 25 and 32), five to six midwife consultations (GA weeks 14–17, 29, 36, 38, 40 and if still pregnant around week 41 as well) and ultrasonic scans at GA weeks 12 and 20.

Standard care control group (CON)

Participants in CON wear an activity tracker to determine their activity level. The face of the tracker looks like a normal watch showing only time and battery life.

Structured supervised exercise training intervention (EXE)

The targeted PA level for all participants in EXE and MOT is at least 30 min/day at a moderate intensity as recommended to healthy pregnant women,[6] and all participants are informed hereof if randomised to EXE or MOT. In EXE, exercise training takes place in teams and is supervised by health professionals (exercise physiologists, physiotherapists and public health scientists). It consists of three weekly 1-hour exercise sessions at moderate intensity, including two exercise sessions in a gym and one in a public swimming pool. The gym sessions consist of a combination of aerobic and resistance training with 30 min stationary bike training (a combination of hill climbing and high cadence intervals) and 30 min of other exercise, for example, elastic bands, exercise balls, mats, dumbbells or body weight. In the swimming pool, participants do 15 min of swimming and 45 min of water exercises with plates, balls, dumbbells or body weight. Moderate intensity during training sessions is assessed using both heart rate monitoring of 65%–80% of age-predicted maximal heart rate (from the activity tracker) and perceived exertion in the range of 12–14 on Borg's conventional 6–20 point scale,[64] as recommended by the American College of Obstetricians and Gynaecologists.[14] If a participant experiences any pain or needs to decrease intensity, the content of exercise sessions (repetitions and/or resistance) is individually adjusted accordingly. Special attention is paid to the newly recruited participants. Exercise sessions are offered at seven different times per week, and participants are recommended to sign up for three of these sessions. The sessions are held early mornings or late afternoons all weekdays and before noon on Fridays and Saturdays.

Motivational counselling supported by health technology (MOT)

This intervention is composed of four individual and three group counselling sessions as well as weekly SMS reminders. The overall focus of both the individual and group counselling sessions is based on what already motivates the participants to increase or maintain their PA level. The motivation technique applied is inspired by motivational interviewing,[65] self-determination theory[66] and behaviour change techniques.[67]

All four *individual sessions* last one hour and are led by professional health counsellors (exercise physiologists, physiotherapists and public health scientists). The sessions aim to discuss the participant's barriers, wishes, needs, knowledge and former PA experiences to identify individual characteristics and motivation towards a more physically active lifestyle. Aside from measuring the PA level, the activity trackers are also used as an intervention element to motivate the participants to increase their PA levels.[68] During individual sessions, feedback on recent PA performances is provided based on activity data acquired from the activity tracker, in order to give the participants insight into their PA level. The participants will, with guidance from the counsellor, set their own activity goals and make an individual action plan to increase the PA level, which may have a motivating effect on PA behaviour.[68 69] Individual sessions are scheduled during the daytime as conveniently for the participant as possible.

The first *group session* lasts one hour and aims to inform the participants about guidelines for PA, benefits associated with PA during pregnancy and possible ways to increase PA during pregnancy. In the following two 2-hour group sessions, the interaction between the participants is used to create meaningful group processes such as support, experience exchange, reflection, learning and development. These sessions focus on the discussion of relevant topics concerning PA during pregnancy, and the counsellor acts as a facilitator through the session, with the topics of conversation chosen by the participants. Issues like postpartum PA, the pelvic floor, uterine contractions, abdominal muscles and diastasis recti, and myths about pregnancy PA are discussed. Group sessions

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are held late afternoons or before noon for those on maternity leave.

The weekly SMS reminders have supportive and motivating content and are used to encourage the participants to achieve a moderate PA level. The texts are chosen based on every participant's PA level during the last week measured by the activity tracker. One example of the text: 'You have been exercising regularly for an extended period of time. Well done. Good habits make it easier for you to continue as your belly gets bigger and heavier'.

Outcome measures

The data collection procedures are illustrated in table 1.

Primary outcome: moderate-to-vigorous intensity physical activity

The primary outcome of FitMum RCT is min/week of MVPA measured continuously from randomisation to GA of 28 weeks and 0-6 days as determined by a wrist-worn activity tracker, Garmin Vivosport, with a built-in heart rate monitor and accelerometer.

Secondary outcome: gestational weight gain

Body weight of the participant before pregnancy is self-reported. The body weight during pregnancy is measured four times from inclusion until delivery on the same scale (Seca 799) with the participant in light clothes and without shoes.

Additional outcomes

Complementary measures of physical activity

Complementary measures of PA are obtained by the Danish version of 'Pregnancy Physical Activity Questionnaire' (PPAQ)[70] named PPAQ-DK and by the doubly labelled water technique.[71]

PPAQ is a semiquantitative and subjective instrument, which has been validated[70] and is considered one of the most valid and reliable questionnaires for the assessment of PA level in pregnant women.[72] Our research group has translated PPAQ to Danish and validated it in a Danish pregnant population.[73]

The doubly labelled water technique is the 'gold standard' technique to measure free-living energy expenditure objectively and is safe, even for pregnant women, as it relies on stable, non-radioactive isotopes.[74–77] The participants are administered a glass of water for oral intake containing 0.1 g of 99.8% $^{2}H_{2}O$ and 1.6 g of 10% ^{18}O per kg body weight. In total, five postdose urine samples are collected in the morning (not the first urine void of the day); on the day after oral water dosage; and after four, seven, 11 and 14 days. The urine samples are stored in the participant's freezer and later at −80°C.

In addition, the PA of the participants is determined from GA week 29 until delivery and in the first year postpartum by the activity tracker. The measures of PA include active calories, active time, steps, heart rate, moderate-intensity and vigorous-intensity activity, floors climbed, MET-min/week and type of activity, which is recognised automatically by the tracker.

Clinical and psychological health parameters in participant, partner and offspring

A variety of clinical and psychological health parameters are obtained from the participant, her partner and her offspring. *Clinical data* regarding pregnancy, delivery and neonatal outcomes are collected from medical records. *Health-related quality of life* is determined in the participant by the Danish version of the Medical Outcomes Study Short Form 36,[78 79] which has also been validated in pregnant women.[80] *Exercise self-efficacy* is determined by the Danish version of the Pregnancy Exercise Self-Efficacy Scale (P-ESES).[81] P-ESES has been translated into Danish and validated in a Danish pregnant population by our research group.[82] *PA motivation* is determined by the Danish version of the Behavioural Regulation in Exercise Questionnaire (BREQ-2),[83–85] which is the most widely used measure of the continuum of behavioural regulation in exercise psychology research. *Sleep quantity and quality* are assessed in the participant by the activity tracker and by the Danish version of the self-administered questionnaire Pittsburgh Sleep Quality Index (PSQI).[86 87] The PSQI is considered a valid and reliable tool to assess sleep metrics among pregnant women.[88] In addition, a validation of activity trackers to measure sleep will be conducted using polysomnography in a subgroup of women already participating in the FitMum study. *Sick leave and pelvic and low back pain* are registered by asking the participant whether she has been absent from work/study and on sick leave during her pregnancy and whether she has experienced pelvic and/or low back pain before and during her pregnancy. *Maternal body composition* is determined from total body water measured by doubly labelled water technique and by a postpartum dual-energy X-ray absorptiometry (DXA) scan. *Offspring growth:* head circumference, length and weight is measured at birth and by general practitioners at five weeks, five months and 12 months postpartum. Participants receive an electronic questionnaire and fill out the anthropometric data along with information on offspring dietary habits and vaccine status. *Parental mental well-being* is assessed six to eight weeks after birth. Both parents or holders of custody receive a questionnaire consisting of the Edinburgh Postnatal Depression Score and Gotland Depression Scale, which are combined as a screening tool for postnatal depression[89–92] in Danish postnatal care. *Psychomotor development of the offspring* is assessed by the validated Ages and Stages Questionnaire 3 (ASQ-3), which is administered electronically to participants 12 months after the due date. ASQ-3 pinpoints developmental progress in the fields of communication, gross motor, fine motor, problem solving and personal-social skills. The administration of ASQ-3 relative to due date and not to birth date aims to correct for variance in cognitive and motor skills due to premature birth. *Offspring physical activity* is assessed for seven days by an infant activity tracker (Actigraph GT3X+) 12 months after the due date. The tracker detects level, intensity and pattern of physical activity.

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Table 1 Procedures and measurements in FitMum RCT

Visit number	Visit 1	Email randomisation	Visit 2	Visit 3	Visit 4	Visit 5	
Gestational age (week+days)	Screening and baseline testing max. 15+0	One week after inclusion	Week 28+0–6	Week 34+0–6	Delivery Approximately week 40	7–14 days after delivery	One year after delivery
Ultrasound scan	×						
Oral information about the study	×						
Medical interview to assess inclusion and exclusion criteria	×						
Demographic, anthropometric, sickness absence and pelvic/low back pain data	×		×	×		×	
Medical history, concomitant disease and previous medication	×						
Demographic and anthropometric data of the participant's partner	×						
Written informed consent	×						
Activity tracker and associated oral and written information	×						
Randomisation		×					
Methodology for obtaining outcomes							
Activity tracker	Continuously during the trial and one year after delivery						
Maternal body weight	×		×	×	×	×	Six times at home during the first year postpartum
Doubly labelled water			×				
Questionnaires: PPAQ-DK, SF-36, PSQI, P-ESES, BREQ-2	×		×	×			×
Maternal blood samples	×		×	×	×		
Paternal blood sample					×		
Umbilical cord blood sample					×		
Placenta samples					×		
DXA scan						×	
Breastmilk sample						×	
Qualitative interview	×			×			×
Observation and autodocumentation		Recurring					
ASQ-3							×
Growth assessment at general practitioner							Five weeks, and five and 12 months
Parental mental well-being questionnaire							Six to eight weeks postpartum
7-day child accelerometer							×
Safety							
Record adverse events			×	×			
Symphysis-fundal height			×	×			

ASQ-3, Ages and Stages Questionnaire 3; BREQ-2, Behavioural Regulations Exercise Questionnaire; DXA, dual-energy X-ray absorptiometry; PA, physical activity; P-ESES, Pregnancy Exercise Self-efficacy Scale; PPAQ-DK, Pregnancy Physical Activity Questionnaire (Danish version); PSQI, Pittsburgh Sleep Quality Index; SF-36, The Medical Outcomes Study Short Form 36.

Analyses of blood, placenta and breastmilk samples

Plasma metabolites and hormones are assessed in maternal and paternal venous blood. The blood samples will be analysed for concentrations of glucose, cholesterol (total, high and low density), triglyceride, free fatty acids, amino acids, interleukin-6, and C reactive protein. Venous blood is obtained from the umbilical cord within 30 min after delivery of the placenta. The blood will be analysed for concentrations of glucose, cholesterol (total, high and low density), triglyceride, insulin, c-peptide, free fatty

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acids, amino acids, adiponectin and leptin. Furthermore, epigenetic profiling at the level of *DNA methylation* will be performed in maternal, paternal and umbilical cord blood mononuclear cells. Bioinformatic comparison of DNA methylomes from parents and offspring will infer on the DNA methylation marks that are modulated by maternal exercise and transmitted to the offspring. Information on DNA methylomes from each parent will allow us to distinguish between maternally and paternally epigenetic profiles transmitted to the offspring. Principal component analyses will be used to identify the specific metabolic or anthropometric features of the mother that are associated with a specific DNA methylation footprint transmitted to the offspring. *Placental function* is assessed from samples taken within 30 min after delivery of the placenta. The samples are immediately frozen on dry ice and stored at −80°C. Analyses will include RNA-seq, non-targeted metabolomics, RT-qPCR, Western blot, histology and immunohistochemistry. *Breastmilk* is obtained from a single feed at the day of visit 5 and stored at −80°C for later metabolomic and lipidomic analyses.

Process evaluation of interventions

A process evaluation is made using quantitative and qualitative methods to provide insight into mechanisms through which interventions bring about change, assess fidelity and quality of implementation, clarify causal mechanisms and identify contextual factors associated with variations in outcomes.[93–95] Integrating process evaluations alongside outcome data is recommended by the UK Medical Research Council guidelines in order to develop and evaluate complex interventions to improve the interpretation of the outcomes, design more effective interventions and apply interventions appropriately across groups and settings by understanding the implementation and functioning of interventions in a given context.[94 96] The Reach, Effectiveness, Adoption, Implementation, and Maintenance framework is used to improve reporting on key issues related to the implementation and external validity of FitMum RCT.[97]

Personal understandings of physical activity

The qualitative dataset is composed of 220 short standardised screening interviews, 30 semistructured interviews, 70 observations, five sets of autoethnographies, visual material, as well as drop-out and follow-up interviews. This subproject will explore the physical and mental health and well-being of the participants, their social relations, PA levels and their experience of pregnancy to identify the challenges and barriers of PA during pregnancy. Personal understandings of PA in the everyday life of participants are determined at inclusion, GA week 34 and one year postpartum, in approximately ten participants from each of the three study groups.

Changes during the COVID-19 pandemic

Due to the COVID-19 pandemic (present in Denmark from 11 March 2020), supplies of interventions (EXE and MOT) and visits are periodically changed. During the lockdown period in spring 2020, all visits (except birth) are converted into online versions using Zoom Cloud Meetings or telephone. From 11 March 2020, in EXE, the swimming pool sessions are replaced with online land exercises and all land exercise sessions consist of 30 min of aerobic exercise where the participants exercise on their own (eg, biking, power-walking, dancing and aerobics) followed by 30 min of supervised online group resistance training. All individual and group MOT sessions are held online.

As much data as possible are collected during the pandemic, but some clinical data have not been possible to obtain in all participants due to limitations on non-urgent visits to the hospital. No blood samples are obtained at the virtual 'visits', women are weighed at home and symphysis-fundal height measurements are not obtained. No doubly labelled water is administered at the virtual 'visit' 2. The participant's body weight at visit 4 is noted by the midwives on the day of giving birth, but biological samples are not collected. No DXA scans or breastmilk samples are collected at 'visit' 5.

Data management and analysis

Data management

The activity tracker data are collected by Fitabase, which regularly backs up the data. A participant who does not synchronise the tracker for seven days or more is reminded by email, text message or phone call. All tracker data are exported from Fitabase to R[98] for data analysis. Tracker data are used to calculate non-wear time; a week is included in the analysis if the week has four or more days with complete data. A day that has six hours or more of non-wear time is excluded and considered a missing day. An electronic case report form (e-CRF) is used to collect all clinical data related to the trial. Data are stored in coded form according to the rules of the Danish Data Protection Agency. Personal data processing complies with the Act on Processing of Personal Data. Data are owned by NOH and University of Copenhagen. Use of data generated in FitMum RCT in new contexts must be agreed and approved by the Steering group. Technical University of Denmark and Aarhus University must have access to the data they have collected and are free to use it in new contexts. The e-CRF is completed by the investigators at the time of the participant's visits at NOH so that it always reflects the latest observations of the participant. Data will be stored for ten years, after which they will be transferred to the Danish National Archives 'Rigsarkivet' in an anonymised format.

Sample size

FitMum RCT has been powered to detect an overall significant difference in the primary outcome between the three groups as well as a significant difference between the two intervention groups (EXE vs MOT) with average activity levels of 210 (EXE), 150 (MOT) and 60 (CON) min/week. The SD was set at 116 min/week and based

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on the results from Oostdam *et al*.[51] The required sample size is determined to obtain a power of 80% with a family-wise significance level of 5%. The sample size calculation showed that the required number of participants is 35 in CON and 70 in each of the two intervention groups due to the randomisation ratio of 1:2:2 to CON, EXE and MOT, respectively. Based on an expected lost to follow-up rate of 20%, as seen in similar exercise studies in pregnant women,[28 32 33 51] we plan to include 44 participants in CON and 88 participants in each of the two intervention groups, making a total of 220 participants.

Statistical methods

Data analyses of both primary and secondary outcomes will be performed using intention-to-treat analyses. In addition, a dose–response model will be estimated to quantify the relationship between adherence to the intervention (proportion of attendances in the planned EXE and MOT sessions, respectively) and the activity level. Moreover, analyses describing associations between the level of physical activity (as measured by the activity tracker) and the secondary and additional outcomes will be performed. Baseline data will be reported as averages and SDs (medians and IQRs) or frequencies and proportions as appropriate. No interim analyses will be performed on the primary and secondary outcomes. The analysis of the primary outcome will be performed using a linear model with the randomisation group as a categorical covariate and with adjustment for baseline PA level. Hypothesis tests will be performed using likelihood ratio tests. Statistical analysis will be conducted using R.[98] Analyses of the primary outcome will be performed by a statistician blinded from the intervention allocations. Investigators will perform analyses of baseline data and secondary and additional outcomes under the supervision of a statistician. A full statistical analysis plan is published in ClinicalTrials.gov.[99]

Trial status

The recruitment of participants began in September 2018 and ended in October 2020. Data collection of the primary outcome is completed in spring 2021. Full data collection is expected to be complete in 2022.

Ethics and dissemination

The FitMum study adheres to the principles of the Helsinki declaration. The study is approved by the Danish National Committee on Health Research Ethics (# H-18011067) and the Danish Data Protection Agency (# P-2019-512).

All participants consent in written form before inclusion and are informed that participation in the FitMum study is voluntary. Participants are informed that they may withdraw from the study at any time and that withdrawal of consent will not affect any subsequent pregnancy and delivery processes at NOH. The participant has time to ask questions and is allowed 24 hours to deliberate on study participation before the obtainment of written informed consent.

FitMum RCT is designed based on recommendations of appropriate PA during pregnancy,[14 45 100 101] and although anatomic and physiological changes occur during pregnancy, PA during an uncomplicated pregnancy is safe.[14 22 29 40 60 102–105] All information about adverse events and serious adverse events are documented consecutively and will be reported. Participants will be discontinued from the intervention if they are at risk of preterm birth, if a cervical length below 25 mm is measured, if serious obstetric or medical complications occur, if investigators' assessment reveals that continuation in the trial would be detrimental to the participant's well-being or if intolerable adverse events occur.

The FitMum study will provide evidence-based knowledge that can contribute to improving national and international recommendations of PA during pregnancy and to new, effective and simple guidance to implement health technology-supported exercise programmes to pregnant women. Based on the results and process evaluation, the knowledge and tools from the FitMum study can be transformed into initiatives in municipalities and hospitals to improve the health and quality of life for both mother and child and can be used for preventing the development of lifestyle-related diseases across generations.

Findings will be submitted for publication in peer-reviewed scientific journals and disseminated at national and international conferences. In addition, results will be disseminated to the public in relevant media and to health professionals via science theatre performances.

DISCUSSION

The FitMum study aims to evaluate the effects of structured supervised exercise training and motivational counselling supported by health technology on PA level during pregnancy to generate evidence about *how* to implement PA in everyday life in healthy pregnant women. Previous studies have investigated the effect of different lifestyle interventions on various health outcomes in normal weight,[23 24 26 28 50–57] overweight and obese pregnant women.[20 21 32 33 58–61] However, none of these studies have focused primarily on investigating the effect of PA interventions on actual PA level determined by novel objective methods. In addition, the FitMum study compares the effect of two very different PA interventions to explore strategies to implement PA programmes into pregnant women's everyday life. Moreover, offspring of FitMum participants will be studied for one year after birth, whereby knowledge on the effect of PA during pregnancy on offspring health will be obtained. A limitation of the study is that the true effect of motivational counselling is not identified, as technology is an integral part of the MOT intervention.

Consumer-based wearable activity trackers tend to increase PA level when they are used as an intervention tool or as part of an intervention.[106] Activity trackers are

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often relatively light weight, comfortable to wear and rechargeable.[107] In addition, using an activity tracker to measure PA during pregnancy is feasible, recommended[108] and has a reasonable compliance rate during pregnancy and after giving birth.[109] However, there are some challenges and limitations of using activity trackers in a long-term intervention study. First, the participants must recharge the device and synchronise their data approximately once per week, which burdens participants and challenges adherence and compliance. Second, we cannot control the interaction of CON participants with the tracker. Third, the main goal for the tracker's design is a comfortable wear, yet wearing the tracker for extended periods of time may cause skin irritation and discomfort.[110] Moreover, the unavailability of the raw data and algorithms used by the manufacturer creates a limitation in the validation of PA metrics.[107] Therefore, measuring PA by a variety of methods, and comparing these methods with the doubly labelled water technique (a gold standard method), will be used in order to obtain comprehensive measures of PA behaviours in FitMum participants.

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Contributors BS initiated the FitMum study together with LT and is the principal investigator of FitMum RCT. ECLL is the clinical trial manager. A steering group consisting of BS, ECLL, TDC, JEL and HTM oversees trial status and progression. CBR, SdPK and BS led the protocol development with contribution from SAA, ADA, JB, TDC, SM, AKJ, GT, APJ, JEL, GvH, EA, RB, OHM, HTM, LT and ECLL. CBR, SdPK, SAA, ADA, JB, TDC, SM, ECLL and BS constitute the clinical core group that guides the practical performance of FitMum RCT. CBR, SdPK and ADA conduct intervention activities together with research assistants and master's students. CBR, SdPK, SAA and ADA will perform most of the data analysis along with AKJ. Analyses of the primary outcome will be performed by AKJ. All authors read, contributed to and approved the final version of the manuscript.

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Paper 2

Effects of prenatal exercise on gestational weight gain, obstetric and neonatal outcomes: FitMum randomized controlled trial

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(http://dx.doi.org/10.1136/bmjopen-2020-043671) and a statistical analysis plan is available with the trial registration at clinicaltrials.gov.

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Condensation: Overall, two different prenatal exercise interventions did not affect gestational weight gain, obstetric or neonatal outcomes, but pregnant women with obesity may benefit from exercise.

Short Title: Roland et al. Prenatal exercise interventions for maternal health.

AJOG at a Glance:

Why was this study conducted?

Prenatal exercise improves maternal and neonatal outcomes, but efficacy of different types of intervention needs to be compared. We investigated the effects of two different exercise interventions on gestational weight gain and obstetric outcomes compared to standard care with special focus on prepregnancy body mass index.

What are the key findings?

None of the exercise interventions affected gestational weight gain, obstetric or neonatal outcomes compared to standard care. However, pregnant women with obesity may be more susceptible to exercise benefits.

What does this study add to what is already known?

We compared health effects of two different prenatal exercise interventions with standard care and developed a novel method to estimate gestational weight gain for the entire pregnancy period.

Abstract

***Background*:** Prenatal exercise might influence gestational weight gain (GWG) and obstetric and neonatal outcomes, but efficacy of different types of intervention needs to be compared.

***Objective*:** To investigate the effects of two different exercise interventions; structured supervised EXErcise training (EXE) versus MOTivational counselling on physical activity (PA) (MOT), on GWG and obstetric and neonatal outcomes compared to CONtrol (CON) receiving standard care. Additionally, we aimed to investigate if effects of prenatal exercise on GWG depended on prepregnancy body mass index (BMI).

***Study design*:** This study was a randomized controlled trial including 219 healthy inactive women at median gestational age (GA) 12.9 (9.4-13.9) weeks and randomized to one of three study groups consisting of EXE three times per week throughout pregnancy (n=87), MOT seven times during pregnancy (n=87), or CON (n=45). Uniquely, to investigate GWG at specific time points, we estimated GWG by a novel method based on longitudinally observed body weights during pregnancy and at admission for delivery. Observed weights were fitted to a mixed effects model that was used to predict maternal body weight and estimate GWG at different gestational ages. Obstetric and neonatal outcomes, among them gestational diabetes mellitus (GDM) and infants born large for gestational age (LGA), were obtained after delivery. The aims were investigated by both the randomized controlled trial design to investigate differences between groups, and an observational design to analyze the influence of prenatal PA per se.

***Results*:** In total 178 participants (81%) completed the study. Total GWG (estimated GWG at GA 40+0 weeks) did not differ between groups (CON: 14.9 kg [95% CI, 13.6;16.1]; EXE: 15.7 kg [14.7;16.7]; MOT: 15.0 kg [13.6;16.4], p=0.538), neither did obstetric nor neonatal outcomes. For example, there were no differences between groups in the proportions of participants developing GDM (CON: 4%, EXE: 6%, MOT: 7%, p=0.934) or having an LGA infant (CON: 6%, EXE: 10%, MOT: 13%, p=0.526). However, stratification of participants into subgroups based on their prepregnancy BMI showed that participants with obesity in MOT had lower total GWG compared to

participants with normal weight in MOT (-7.3 kg [95% CI, -11.4;-3.2], p<0.001). Likewise, participants with obesity in EXE had a lower total GWG compared to EXE participants with normal weight (-4.4 kg [-8.4;-0.3], p=0.023). In addition, analyzing all participants independent of group allocation in an observational design showed that associations between moderate to vigorous intensity PA (MVPA) and total GWG as well as active kilocalories and total GWG differed between women with obesity and normal weight (MVPA: difference between slopes -0.04 [-0.07;-0.03·10^{-1}], p=0.034; Active kilocalories: difference between slopes -0.01 [-0.02;-0.04·10^{-1}], p=0.003).

***Conclusion*:** Overall, during pregnancy neither structured supervised EXErcise training nor MOTivational counselling on PA affected GWG or obstetric and neonatal outcomes in healthy pregnant women compared to standard care. However, women with obesity in both intervention groups gained less weight compared to women with normal weight within the same intervention groups. Further, associations between PA measures and total GWG differed between women with obesity and normal weight.

***Key words*:** delivery, intervention, maternal exercise, moderate to vigorous intensity physical activity, obesity, pregnancy, obstetric outcomes

Introduction

Maternal prenatal exercise has been indicated in many studies to reduce GWG[1–3] and incidence of other pregnancy and delivery related complications including GDM, preeclampsia, gestational hypertension, preterm delivery, caesarean section and odds of instrumental delivery.[3–5] Further, prenatal exercise has been shown to reduce duration of labor in some pregnant populations[6] and to be associated with optimization of offspring birth weight into a healthy range,[5,7,8] but the literature is inconsistent.[9]

In order to optimize the health benefits from maternal exercise, it remains to be investigated which exercise approaches are more effective to improve health of mother and offspring.[4] Structured supervised exercise training and motivational counselling on PA constitute two exercise approaches being widely used in the literature.[3,10] Both approaches have been applied separately in pregnant women with normal weight[11–17] and overweight and obesity,[18–25] but a direct comparison of the effectiveness of the two approaches on improving GWG and obstetric and neonatal outcomes has not been conducted. Furthermore, GWG recommendations vary based on prepregnancy BMI,[26] measurement of GWG has not been standardized, and studies using comprehensive measures of PA are warranted to nuance the understanding of how prenatal exercise influences health.

In this randomized controlled trial[27] we aimed to investigate the effects of EXE or MOT during pregnancy on GWG and obstetric and neonatal outcomes compared to CON, and to investigate the influence of prepregnancy BMI. Our hypotheses were that GWG would be lower in EXE compared to MOT, and in MOT compared to CON.

Materials and Methods

Participants and study procedures

The FitMum study was a randomized controlled trial[27] conducted in 2018-2021 at Nordsjaellands Hospital, Hillerod, Denmark. Healthy pregnant inactive women (n=220) were enrolled in early pregnancy (GA≤15+0 weeks). The primary objective was to investigate the effect of the two different exercise interventions (EXE and MOT) on MVPA during pregnancy compared to CON (yet unpublished data), whereas this paper reports secondary outcomes of the study. Demographic information was obtained at inclusion and prepregnancy BMI (kg/m^2) was calculated based on self-reported prepregnancy weight and height. PA, including MVPA, steps, and active kilocalories, was measured continuously from inclusion to delivery by a wrist-worn activity tracker (Garmin Vivosport). Randomization (n=219) in a 1:2:2 pattern to either CON, EXE, or MOT, respectively, occurred after a one-week baseline period (GA≤16+0 weeks). Participants in the EXE intervention were offered supervised exercise training at moderate intensity three times per week, while the MOT intervention consisted of seven motivational counselling sessions on PA during pregnancy. During the COVID-19 pandemic, starting from March 11th, 2020 and throughout the intervention period, exercise training sessions, motivational counselling sessions and periodically test visits (except delivery) were conducted online using Zoom Cloud Meetings or telephone. The study was approved by the Danish National Committee on Health Research Ethics (#H-18011067) and the Danish Data Protection Agency (#P-2019-512). Written informed consent was obtained from all participants.

Outcome measurements

Gestational weight gain

Prepregnancy body weight was self-reported by the participants. From inclusion all weight measurements were recorded to the nearest 0.1 kg on calibrated electronic scales (SECA799) at baseline (GA≤15+0 weeks), GA 28+0-6 and 34+0-6 weeks (visit 2 and 3), and at delivery. During COVID-19, women were weighed at home on private scales. To estimate GWG for the entire pregnancy and account for missing measurements and individual differences in GA at delivery, all observed weights (self-reported and measured) were fitted to a mixed effects model to predict the weights at specific timepoints throughout pregnancy at the participant-level. GWG was estimated at GA 12+0, 28+0, and 40+0 weeks as the difference between the predicted weight and predicted prepregnancy weight (GA=0). The total GWG during pregnancy was defined as the estimated GWG at GA 40+0 weeks[26,28] and used in further stratification analyses of participants into subgroups based on their prepregnancy BMI and whether they had a total GWG below, within or above the Institute of Medicine's (IOM) recommendations.[26] IOM recommends a GWG of 11.5-16 kg, 7-11.5 kg and 5-9 kg for women with normal weight, overweight and obesity, respectively[26] and prepregnancy BMI were defined according to the World Health Organization's BMI categories.[29]

Obstetric and neonatal outcomes

Obstetric and neonatal outcomes were collected from medical records. Obstetric outcomes included pregnancy complications (GDM and gestational hypertensive disorders) and delivery related outcomes (induction of labor, epidural analgesia, oxytocin augmentation, duration of labor, mode of delivery, rupture degree 3 and 4, postpartum hemorrhage). Gestational hypertensive disorders were defined as gestational hypertension, preeclampsia, HELLP syndrome or eclampsia. Total duration of labor included the time from the active phase (starting when cervix was dilated 4 cm and the woman had regular contractions) until the baby was born. The active second stage was defined as the time of active pushing. Neonatal outcomes included GA at delivery, premature

delivery (GA<37+0 weeks), birth weight, birth length, birth weight z-score, small for gestational age (SGA), LGA and Apgar score (5 min). Birth weight was transformed to a z-score, and SGA (<10th percentile) and LGA (>90th percentile)[28] were defined for a Danish standard population and calculated from the Marsal formula,[30] which includes fetal sex, birth weight and GA.

Statistical analysis

Sample size calculations for the primary outcome of the study (MVPA from randomization to GA 28+0-6 weeks) and the secondary outcome GWG were performed (statistical analysis plan available with trial registration at clinicaltrials.gov). Data are presented as means ± SD for approximately symmetric distributions, median and interquartile ranges (IQR) for asymmetric distributions, and frequencies and proportions for categorical data. Estimated effect sizes are presented with 95% confidence intervals [95% CI]. Statistical analyses were performed using R[31] and statistical significance was defined as p-value below 5%.

Analysis of GWG was based on the intention-to-treat principle (ITT) including all randomized participants. Trajectories of observed gestational weights during pregnancy was modeled by a mixed effects model featuring an intercept constrained to be equal across groups due to the randomized design.[32] Group-specific change-points were included in the model to allow for a piece-wise linear relationship with two different slopes over time in each of the groups. This led to a total of ten fixed effects in the model consisting of the common intercept and two different slopes and the change-point for each of the three groups. Normal distributed random effects were included at the subject level as intercepts and the two slopes with an unstructured covariance matrix. The model was implemented in Stan[33] and estimated using Markov-Chain Monte Carlo in four parallel chains each running for 10,000 iterations with half of them used for warm-up. A uniform distribution between 50 and 250 days was used as priors for the change-points. The fitted model was subsequently applied to predict individual weights at predetermined timepoints.

We used the randomized controlled trial design to investigate differences between groups and an observational design combining all participants independent of group allocation to investigate the associations of prenatal PA per se. Between-group comparisons of estimated GWG after each trimester were performed using analysis of variance (ANOVA). A sensitivity analysis was conducted using linear regression to investigate total GWG in each group before versus during COVID-19, where the intervention groups received physical and online interventions, respectively. Another sensitivity analysis using ANOVA included only participants, whose weight were measured at the hospital, to investigate the influence of weight measurements being obtained by the calibrated scale at the hospital versus via the participant's own scales at home. For obstetric and neonatal outcomes, differences between groups were tested with Pearson's χ^2 test for categorical variables, ANOVA for symmetrically distributed variables, and Kruskal-Wallis test for asymmetrically distributed variables. We used a two-way ANOVA to investigate the effects of prepregnancy BMI and group allocation on total GWG. Fisher's Exact test was used to compare number of participants with total GWG within versus outside (below and above) IOM recommendations between groups. Post-hoc pairwise comparisons were performed using Pearson's χ^2 test with Holm-corrected p-values, Tukey's method, or Wilcoxon rank sum test with Holm-corrected p-values for categorical, symmetrically distributed, and asymmetrically distributed variables, respectively.

Associations between PA measures and total GWG among all participants were performed using linear regression. PA measures were the average values from randomization to delivery day for participants who delivered at GA ≤ 40+0 weeks, and from randomization to GA 40+0 weeks for participants who were lost to follow up before delivery or delivered at GA > 40+0 weeks.

Results

We included 220 participants from GA 6+1–15+0 weeks and randomized 219 to CON (n=45), EXE (n=87) and MOT (n=87) (Figure 1). Maternal baseline characteristics are presented in Table 1. All 219 participants were included in the analysis of GWG and pregnancy complications. From randomization to delivery, 19% of the participants were lost to follow up, thus data from 178 participants (CON: n=34; EXE: n=74; MOT: n=70) were included in the analysis of delivery related and neonatal outcomes. Lost to follow up rate did not differ between groups. Adverse events and serious adverse events did not differ between groups and the interventions did not seem to influence mother or offspring negatively. Adherence to EXE and MOT was on average 1.3 sessions per week [1.1;1.5] and 5.2 [4.7;5.7] out of seven pregnancy counselling sessions, respectively.

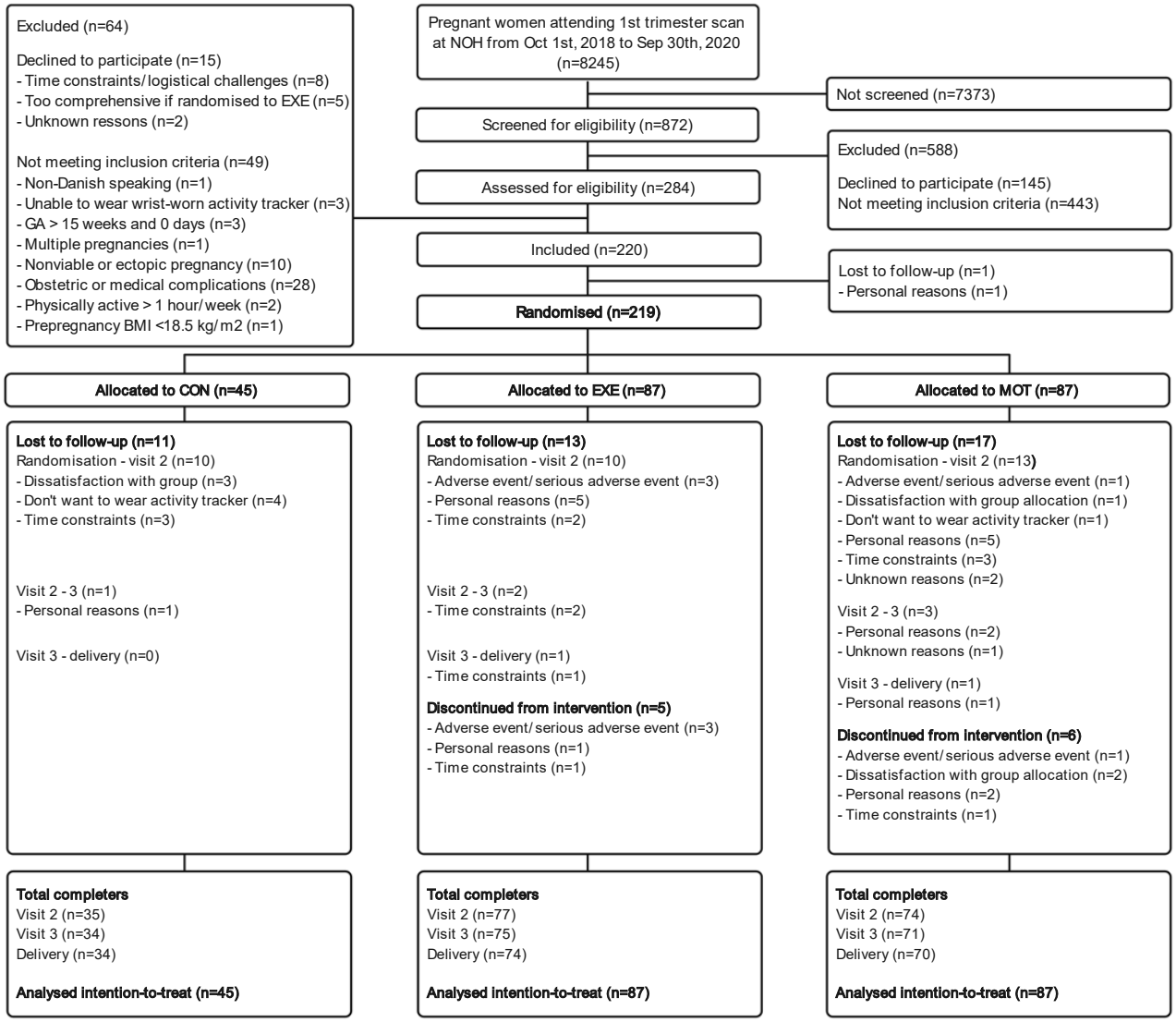

Figure 1. Inclusion, randomization, allocation and completion of the FitMum study reported in accordance with the CONSORT (Consolidated Standards of Reporting Trials) guidelines.[55] CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity. The figure was created with BioRender.com.

Table 1. Maternal baseline characteristics

Characteristic	ALL	CON	EXE	MOT
Number of participants	***n=219***	***n=45***	***n=87***	***n=87***
Age, years	31.5 ± 4.3	32.0 ± 4.6	31.1 ± 4.3	31.7 ± 4.1
Prepregnancy BMI, kg/m^2*	24.1 (21.8-28.7)	23.5 (21.3-26.8)	25.2 (21.6-29.8)	24.1 (22.4-28.9)
Gestational age, weeks (median)	12.9 (9.4-13.9)	12.9 (9.7-13.9)	12.6 (9.3-13.7)	12.9 (9.6-13.9)
Gestational age, weeks (mean)	11.7 ± 2.5	11.9 ± 2.6	11.7 ± 2.4	11.8 ± 2.4
Parity, nulliparous, n (%)	82 (37%)	16 (36%)	40 (46%)	26 (30%)
Education level, n (%)**				
School ≥12 years	191 (87%)	41 (91%)	74 (85%)	76 (87%)
Further education ≥3 years	175 (80%)	33 (73%)	73 (84%)	69 (79%)
Employed/studying, n (%)	199 (91%)	39 (87%)	83 (95%)	77 (89%)
Smoking, n (%)				
During pregnancy	2 (1%)	0 (0%)	1 (1%)	1 (1%)
Quit smoking before pregnancy	27 (12%)	6 (13%)	12 (14%)	9 (10%)

Baseline characteristics in the randomization groups. * Prepregnancy BMI; n=218 (CON; n=45, EXE; n=86, MOT; n=87). ** School ≥12 years corresponds to high school, and further education ≥3 years corresponds to university degree (bachelor or master level). Data are mean ± SD, median (IQR) and n (%). No statistical comparisons have been performed on baseline characteristics in accordance with CONSORT recommendations. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, BMI; Body mass index, IQR; interquartile range.

Gestational weight gain after each trimester

Estimated GWG did not differ between groups at GA 12+0 weeks (p=0.310) or GA 28+0 weeks (p=0.396) (Figure 2A-B). Total GWG (GWG at GA at 40+0 weeks) was 14.9 kg [13.6;16.1] in CON, 15.7 kg [14.7;16.7] in EXE and 15.0 kg [13.6;16.4] in MOT and did not differ between groups (p=0.538) (Figure 2C). Pairwise comparisons of total GWG showed no differences in total GWG between MOT and EXE (-0.7 kg [-2.6;1.3], p=0.710), MOT and CON (0.2 kg [-2.0;2.3], p=0.985), or EXE and CON (0.8 kg [-1.1;2.7], p=0.562). Figure 2D-F illustrates the estimated relationship between self-reported and measured body weight observations (dots) and predicted body weights by the mixed effects model (lines) for all individuals in the three groups.

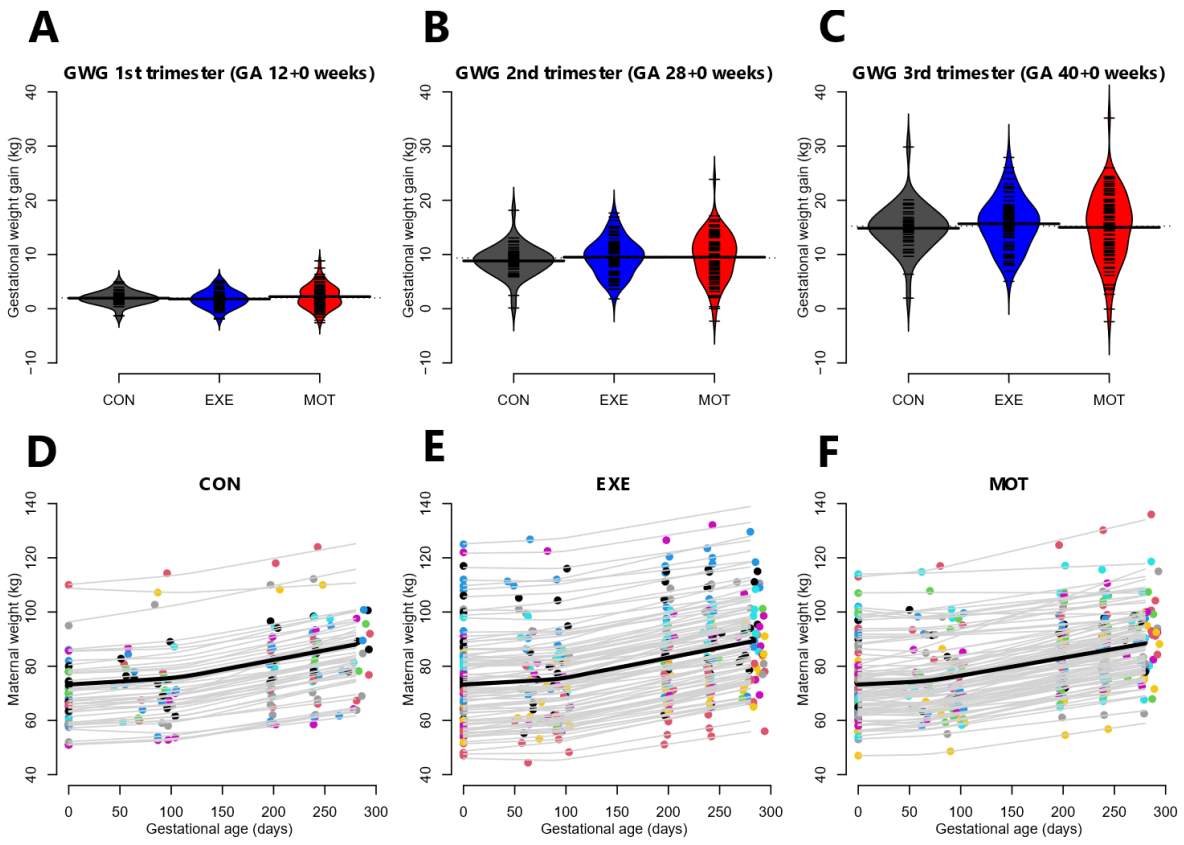

Figure 2. Gestational weight gain (GWG) for all participants (n=219) after first trimester/GA 12+0 weeks **(A)**, second trimester/GA 28+0 weeks **(B)**, and third trimester/GA 40+0 weeks (total GWG) **(C).** Self-reported and measured weights (dots) and predicted weights by mixed effects model (lines) for all individuals throughout pregnancy in the three groups **(D-F)**. ANOVA was used for **A-C** and showed no differences between groups at GA 12+0 weeks (p=0.310), GA 28+0 weeks (p=0.396) and GA 40+0 weeks (p=0.538). CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, GWG; Gestational weight gain, GA; Gestational age

A complete case analysis of GWG at delivery calculated as measured weight at delivery (available for n=131) minus self-reported prepregnancy weight showed no difference between groups (p=0.612) (Figure S.1). Sensitivity analyses showed higher total GWG (4.7 kg [1.6;7.8], p=0.003) in MOT during COVID-19 compared with before COVID-19, but no differences were found in EXE (1.6 kg [-0.8;4.0], p=0.184) and CON (-1.2 kg [-4.3;1.9], p=0.425). We found no between-group differences (p=0.537) in total GWG among participants, whose weight were measured at the hospital only, similarly to the ITT results.

Obstetric and neonatal outcomes

Overall, apart from the number of unassisted vaginal deliveries and GA at delivery, none of the obstetric and neonatal outcomes differed between groups (Table 2 and 3). Overall, unassisted vaginal deliveries differed between groups (CON: 88%, EXE: 81%, MOT: 69%, p=0.04987), however results of Holm-corrected post-hoc pairwise comparisons showed no differences between any of the groups (Table 2). GA at delivery differed between groups (p=0.048) and Holm-corrected post-hoc pairwise comparisons showed that EXE had higher GA at delivery compared to MOT (EXE: 40.6 weeks (39.9-41.3), MOT: 40.0 weeks (39.3-40.9), p=0.038) (Table 3).

Table 2. Obstetric outcomes

Outcome	ALL	CON	EXE	MOT	p-value
Pregnancy complications	***n=219***	***n=45***	***n=87***	***n=87***	
GDM, n (%)	13 (6%)	2 (4%)	5 (6%)	6 (7%)	0.934[a]
Gestational hypertensive disorders, n (%)*	12 (6%)	2 (4%)	5 (6%)	5 (6%)	1.000[a]
Delivery related outcomes	***n=178***	***n=34***	***n=74***	***n=70***	
Induction of labor, n (%)	53 (30%)	11 (32%)	22 (30%)	20 (29%)	0.925[a]
Mode of delivery, n (%)					
Unassisted vaginal	138 (78%)	30 (88%)	60 (81%)	48 (69%)	0.050[a#]
Instrumental assisted vaginal	8 (5%)	1 (3%)	1 (1%)	6 (9%)	0.103[a]
Planned caesarean section	11 (6%)	1 (3%)	5 (7%)	5 (7%)	0.791[a]
Emergency caesarean section	21 (12%)	2 (6%)	8 (11%)	11 (16%)	0.333[a]
Epidural analgesia, n (%)	58 (33%)	9 (27%)	25 (34%)	24 (34%)	0.698[a]
Oxytocin augmentation, n (%)	46 (26%)	5 (15%)	24 (32%)	17 (24%)	0.138[a]
Rupture degree 3 + 4, n (%)**	8 (5%)	1 (3%)	3 (4%)	4 (6%)	0.805[a]
Postpartum hemorrhage, ml	350 (250-508)	300 (200-445)	350 (250-593)	400 (250-540)	0.212[b]
Postpartum hemorrhage > 1000 ml, n (%)	19 (11%)	2 (6%)	11 (15%)	6 (9%)	0.300[a]
Duration of labor nulliparous					

Total duration vaginal delivery, min**	443 (273-617)	523 (199-582)	481 (363-678)	298 (198-488)	0.163[b]
Duration of active second stage labor, min**	41 (22-65)	53 (16-70)	43 (23-67)	34 (21-44)	0.436[b]
Active second stage labor >60 min, n (%)**	15 (27%)	3 (27%)	9 (31%)	3 (20%)	0.797[a]
Duration of labor multiparous					
Total duration vaginal delivery, min**	150 (86-262)	126 (102-254)	152 (79-277)	160 (88-262)	0.994[b]
Duration of active second stage labor, min**	13 (7-19)	13 (6-20)	14 (7-19)	11 (6-19)	0.849[b]
Active second stage labor >30 min, n (%)**	11 (13%)	3 (16%)	2 (7%)	6 (16%)	0.592[a]

Obstetric outcomes in the randomization groups. Pregnancy complications are reported for all randomized participants (n=219) and delivery related outcomes are reported for participants still enrolled in the study at delivery (n=178). * Defined as preeclampsia, gestational hypertension, HELLP or eclampsia. ** For some variables the total number is lower due to missing values: For nulliparous women: Total duration of vaginal deliveries; n=49 (CON; n=10, EXE; n=24, MOT; n=15), duration of active second stage labor; n=55 (CON; n=11, EXE; n=29, MOT; n=15), active second stage labor >60 min; n=55 (CON; n=11, EXE; n=29, MOT; n=15), for multiparous women: total duration of vaginal deliveries; n=81 (CON; n=19, EXE; n=27, MOT; n=35), duration of active second stage labor; n=86 (CON; n=19, EXE; n=29, MOT; n=38), active second stage labor >30 min; n=86 (CON; n=19, EXE; n=29, MOT; n=38), rupture degree 3 + 4; n=173 (CON; n=31, EXE; n=72, MOT; n=70). Data are mean ± SD, median (IQR) and n (%). [a]Pearson's χ^2 test, [b]Kruskal-Wallis test. [#]p=0.04987 but results of pairwise comparisons by Pearson's χ^2 tests with Holm-corrected p-values were inconclusive. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, GDM; Gestational diabetes mellitus.

Table 3. Neonatal outcomes

Outcome	ALL	CON	EXE	MOT	p-value
Number of participants	***n=178***	***n=34***	***n=74***	***n=70***	
Gestational age delivery, weeks (median)	40.4 (39.4-41.1)	40.2 (38.8-41.3)	40.6 (39.9-41.3)	40.0 (39.3-40.9)	0.048[b#]
Gestational age delivery, weeks (mean)	40.1 ± 1.6	39.8 ± 1.9	40.4 ± 1.2	39.8 ± 1.7	
Premature delivery (GA<34), n (%)	3 (2%)	1 (3%)	0 (0%)	2 (3%)	0.309[a]
Premature delivery (GA 34+0-36+6), n (%)	3 (2%)	2 (6%)	1 (1%)	0 (0%)	0.093[a]
Birth weight, g	3715 (3289-3979)	3630 (3024-3899)	3768 (3410-4069)	3665 (3266-3880)	0.083[b]
Birth length, cm*	52 (51-53)	52 (51-54)	53 (51-54)	52 (51-53)	0.354[b]
Birth weight adjusted for GA at delivery and sex, z-score	0.10 ± 1.0	-0.02 ± 1.0	0.17 ± 1.0	0.09 ± 1.0	0.648[c]
SGA (<10th percentile), n (%)	15 (8%)	4 (12%)	3 (4%)	8 (11%)	0.208[a]
LGA (>90th percentile), n (%)	18 (10%)	2 (6%)	7 (10%)	9 (13%)	0.526[a]
5-min apgar score <7, n (%)	1 (1%)	0 (0%)	0 (0%)	1 (1%)	0.580[a]

Neonatal outcomes in the randomization groups. * Birth lenght; n=177 (CON; n=34, EXE; n=74, MOT; n=69). Data are mean ± SD, median (IQR) and n (%). [a]Pearson's χ^2 test, [b]Kruskal-Wallis test, [c]Analysis of variance (ANOVA). [#]EXE vs. MOT (p=0.038) (pairwise Wilcoxon rank sum test with Holm-corrected p-value). CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, GA; Gestational age, SGA; Small for gestational age infants, LGA; Large for gestational age infants, IQR; interquartile range.

Gestational weight gain in relation to prepregnancy BMI and IOM recommendations

Stratification of participants into subgroups based on their prepregnancy BMI resulted in 122 women with normal weight (CON: n=30; EXE: n=43; MOT: n=49), 52 women with overweight (CON: n=10; EXE: n=23; MOT: n=19) and 45 women with obesity (CON: n=5; EXE: n=21; MOT: n=19). Overall, women with obesity had lower total GWG compared to women with normal weight (-5.2 kg [-7.2;-3.2], p<0.001) and overweight (-5.1 kg [-7.4;-2.7], p<0.001) (Figure 3A). Participants with obesity in MOT had lower total GWG compared to both all three normal weight groups (CON: -5.9 kg [-10.4;-1.5], p=0.001; EXE: -8.2 kg [-12.4;-4.0], p<0.001; MOT: -7.3 kg [-11.4;-3.2], p<0.001) and to all three overweight groups (CON: -6.7 kg [-12.7;-0.8], p=0.013; EXE: -6.4 kg [-11.1;-1.6], p=0.001; MOT: -8.4 kg [-13.4;-3.5], p<0.001). Likewise, participants with obesity in EXE had lower total GWG compared to EXE participants with normal weight (-4.4 kg [-8.4;-0.3], p=0.023) (Figure 3A). We found no differences between CON, EXE, and MOT in total GWG within versus outside IOM recommendations for neither women with normal weight (p=0.230), overweight (p=0.275) nor obesity (p=0.730) (Figure S.2).

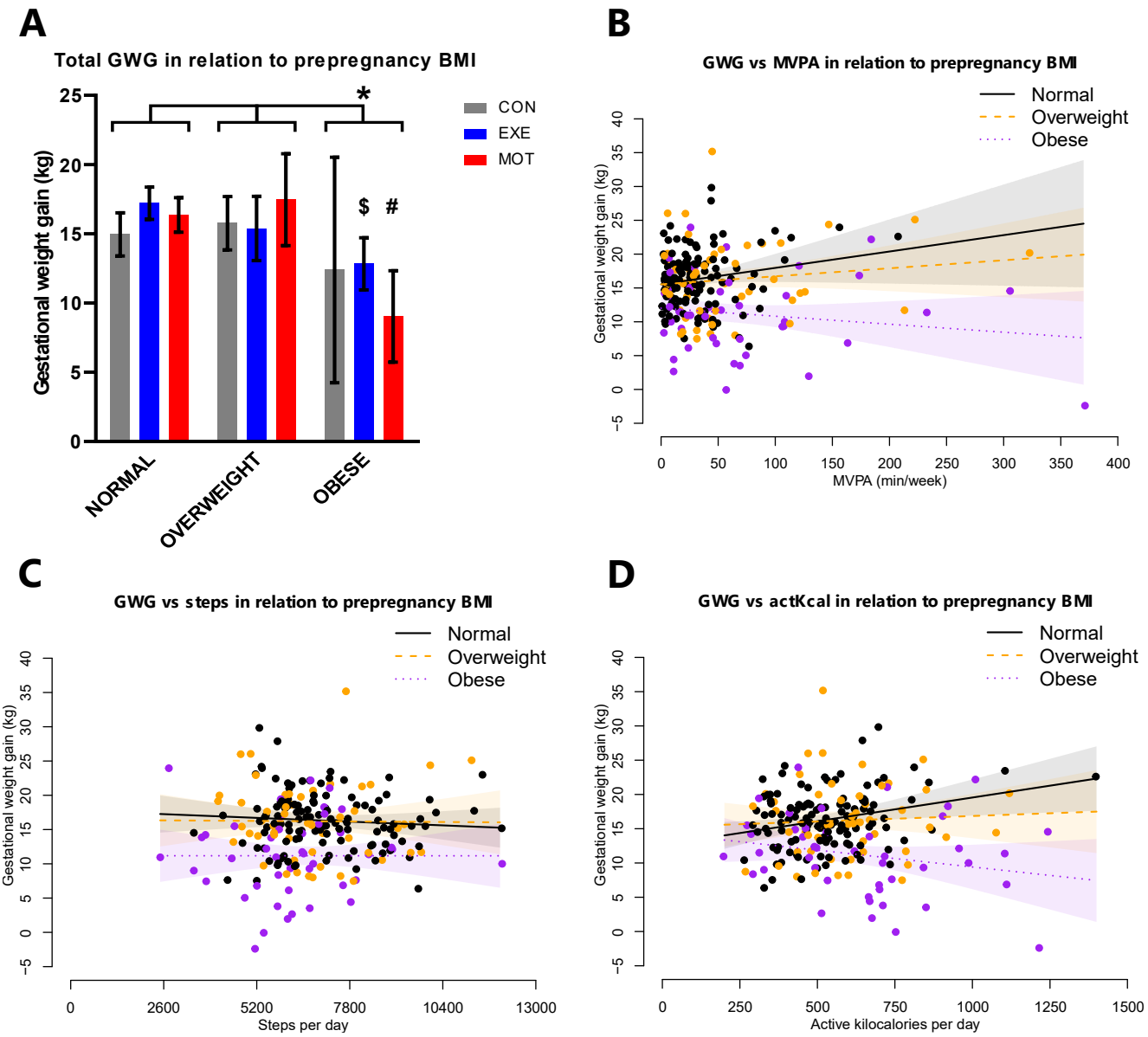

Figure 3. Total gestational weight gain (GWG) for participants with normal weight, overweight and obesity in CON, EXE and MOT **(A)** and associations between MVPA (min/week) and total GWG **(B),** steps per day and total GWG **(C)**, and active kilocalories per day and total GWG **(D)** for all participants (n=219). **A,** * represents differences compared with normal weight (p<0.001) and overweight (p<0.001). # represents differences compared with all normal weight and overweight groups (p<0.05). $ represents differences compared with normal weight EXE participants (p=0.023) (two-way ANOVA and Tukey's method). **B-D,** associations between MVPA (min/week) and total GWG, as well as active kilocalories per day and total GWG differed (MVPA: p=0.034; Active kilocalories: p=0.003) between women with obesity and normal weight, but associations between steps per day and total GWG did not (p=0.685) (linear regression). Data points are visualized based on average MVPA, steps and active kilocalories of 25 imputed data sets (B-D). CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, GWG; Gestational weight gain, BMI; Body mass index, MVPA; moderate to vigorous intensity physical activity.

Observational associations with prenatal physical activity per se

We investigated if PA was associated with total GWG independent of group allocation. We found no associations between any of the PA measures and total GWG (MVPA: slope -0.01 [-0.02;0.01], p=0.363; Steps: slope $0.14 \cdot 10^{-3}$ [$-0.03 \cdot 10^{-2}$;$0.06 \cdot 10^{-2}$], p=0.537; Active kilocalories: slope $-0.09 \cdot 10^{-2}$ [$-0.05 \cdot 10^{-1}$;$0.03 \cdot 10^{-1}$], p=0.637) (Figure S.3A-C). However, when the participants were stratified according to their prepregnancy BMI we found that associations between both MVPA and total GWG (Figure 3B) as well as active kilocalories and total GWG (Figure 3D) differed between women with obesity and normal weight (MVPA: difference between slopes -0.04 [-0.07;$-0.03 \cdot 10^{-1}$], p=0.034; Active kilocalories: difference between slopes -0.01 [-0.02;$-0.04 \cdot 10^{-1}$], p=0.003), but associations between steps and total GWG did not (difference between slopes $0.02 \cdot 10^{-2}$ [$-0.80 \cdot 10^{-3}$;$0.12 \cdot 10^{-2}$], p=0.685) (Figure 3C). Associations between neither MVPA, steps nor active kilocalories and total GWG differed (MVPA: difference between slopes -0.01 [-0.05;0.02], p=0.492; Steps: difference between slopes $0.02 \cdot 10^{-2}$ [$-0.84 \cdot 10^{-3}$;$0.12 \cdot 10^{-2}$], p=0.729; Active kilocalories: difference between slopes -0.01, [-0.01;$0.04 \cdot 10^{-1}$], p=0.252) between women with overweight and normal weight. Higher MVPA and active kilocalories were associated with higher total GWG within women with normal weight (MVPA: slope 0.02 [$0.61 \cdot 10^{-3}$;0.05], p=0.044; Active kilocalories: slope 0.01 [$0.02 \cdot 10^{-1}$;0.01], p=0.003), whereas these associations were not present within women with obesity (MVPA: slope -0.01 [-0.03;0.01], p=0.288; Active kilocalories: slope $-0.05 \cdot 10^{-1}$ [-0.01;$0.02 \cdot 10^{-1}$], p=0.145), but had negative slopes, meaning that increasing PA decreases total GWG.

Comment

We found no overall effect of neither structured supervised EXErcise training nor MOTivational counselling on GWG or obstetric and neonatal outcomes in healthy pregnant women compared to standard care. Hence, our predefined hypotheses that GWG would be lower in EXE compared to MOT, and in MOT compared to CON were rejected. Importantly, the interventions did not seem to influence mother or offspring negatively, which is in line with the literature.[34] Women with obesity in EXE and MOT had lower total GWG compared to EXE and MOT women with normal weight. Further, associations between PA and total GWG differed between women with obesity and normal weight, with slopes being in negative and positive directions, respectively. Interestingly, this could indicate a differential influence of BMI on the association between PA and total GWG and that women with obesity may be more susceptible to beneficial effects of exercise interventions than women with normal weight, in line with previous studies.[1,21,24,35] Establishing effective interventions to reduce obesity, excessive GWG and associated adverse maternal[36–38] and infant[36,38–40] outcomes among pregnant women with overweight and obesity is crucial, since excessive weight gain is more prevalent among women with obesity[36,41].

The low number of participants with obesity in CON possibly explains why an intervention effect of MOT on total GWG compared to CON in women with obesity could not be confirmed. The lower total GWG among women with obesity compared to women with normal weight could be due to recommendations of GWG[26] being dependent on prepregnancy BMI and that women with obesity tend to gain less during pregnancy. However, we observed no difference in total GWG between women with obesity and normal weight in CON, which however might be due to the low number of CON participants with obesity.

It is noteworthy that our interventions were not effective on reducing GWG compared to CON in our entire study population that were normal weight on average, where more than 50% of the

participants with normal and overweight in EXE and MOT had excessive GWG[26]. This is in contrast with several studies showing reduced GWG compared to standard care after prenatal exercise in healthy[3] and normal weight women.[42] However, some studies also found no effect of exercise on GWG in women with normal weight.[17,43] We found no effects of our interventions compared to CON on obstetric and neonatal outcomes. This is in contrast to most studies reporting a protective effect of prenatal exercise on GDM and hypertensive disorders[3] and reduced preterm delivery, SGA and LGA[8] in women with normal weight, overweight and obesity. However, similar to our study, other studies found no effects of prenatal exercise on GDM, preeclampsia, preterm delivery and birth weight.[43–45] The literature is inconsistent regarding effects of prenatal exercise on mode of delivery, induction of labor and epidural analgesia.[4,46,47] Some studies mention rather low adherence to their exercise interventions as a possible explanation for the lack of exercise effect. Likewise, we only had moderate adherence to interventions, which is a weakness for investigation of health outcomes in our study, given the importance of achieving a certain amount of PA to obtain beneficial effects.[48,49] However, none of the PA measures were per se associated with total GWG. Further investigation is needed on whether maternal body composition might be improved in EXE or MOT compared to CON, since exercise-induced improvement of body composition has been shown in both pregnant[50] and non-pregnant populations.[51,52]

Ideally, GWG calculation is based on last measured available weight in pregnancy.[26] Most studies calculate GWG based on a weight measured at the last pregnancy visit,[2,3,35] and only few studies have reported GWG all the way to delivery.[53,54] A strength of the current study is that the last weight measurement is obtained at delivery enabling us to estimate GWG for the entire pregnancy period by a new method accounting for individual differences in GA at delivery. We found a very good fit between weight observations and predicted weights by the statistical model, supporting use of our method to estimate GWG. The method can advance state-of-the-art in the obstetric research field.

Conclusion

Overall, neither structured supervised EXErcise training nor MOTivational counselling on PA during pregnancy affected GWG as estimated by a novel method or obstetric and neonatal outcomes in healthy pregnant women compared to standard care. However, women with obesity in both intervention groups gained less weight compared to women with normal weight within the same intervention groups. Further, associations between PA and total GWG differed between women with obesity and normal weight. This indicates that pregnant women with obesity may be susceptible to beneficial effects of exercise.

Acknowledgements

The authors would like to acknowledge and thank all the participants for signing up for the project and delivering important data. We also thank the students, research assistants, and staff at Department of Gynecology and Obstetrics, Nordsjaellands Hospital, Hillerod, who contributed to conduct the intervention activities and data collection. Additionally, we would like to thank the technical staff, especially Susanne Månsson and Charlotte Pietraszek, from the Clinical Research Unit, Department of Clinical Research, Nordsjaellands Hospital, Hillerod, for their contribution to planning practicalities and collecting data.

Authors' contribution

B.S. initiated and directed the FitMum study. C.B.R., S.dP.K., J.B., T.D.C., S.M., S.A.A., E.L and B.S. developed the study protocol. C.B.R., S.dP.K., A.D.A., N.B. and I.K.B.J. conducted intervention activities and collected data assisted by S.A.A., research assistants and master students. E.L. is the clinical trial manager and supervised the clinical part of the study in collaboration with J.B., T.D.C., S.M., and B.S. T.D.C and E.L. supervised analysis of data and writing of the manuscript, and A.K.J. performed and supervised statistical analyses. C.B.R.

analyzed data and drafted the manuscript. All authors read, contributed to, and approved the final version of the manuscript.

Supplementary material

Supplementary material includes supplementary figures S.1, S.2, and S.3A-C.

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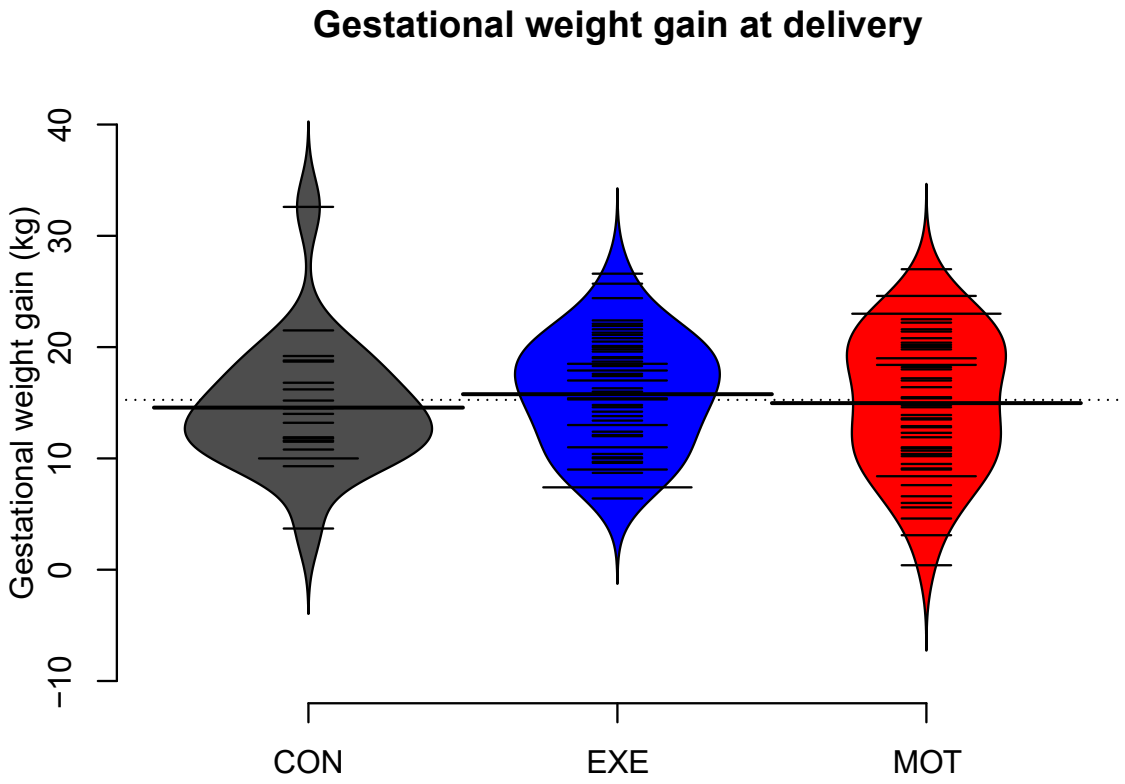

Figure S.1: Complete case analysis of gestational weight gain at delivery including participants with available weight measurements from delivery only (n=131). ANOVA showed no difference between groups (p= 0.612). CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity.

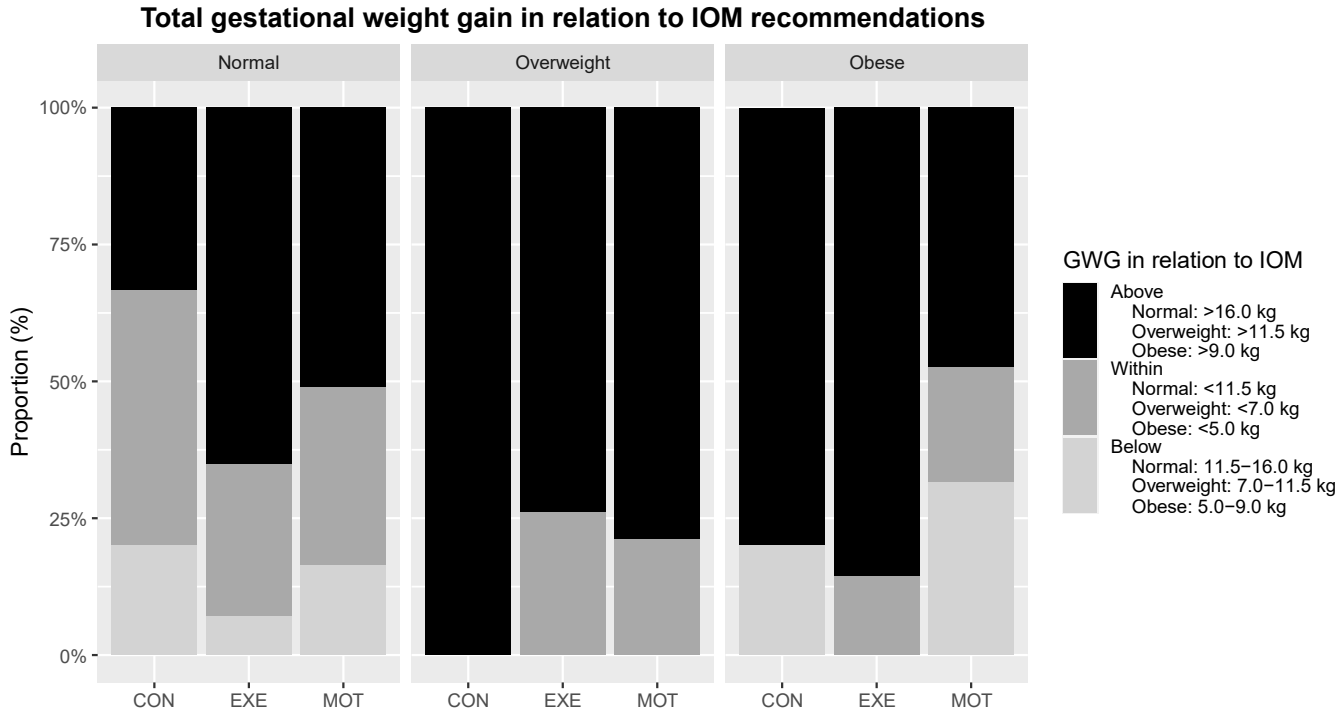

Figure S.2: Total gestational weight gain above, within and below IOM recommendations for participants with normal weight, overweight and obesity in CON, EXE, and MOT. No differences between groups in total gestational weight gain within versus outside (below and above combined) IOM recommendations for neither women with normal weight (p=0.230), overweight (p= 0.275) nor obesity (p= 0.730) (Fisher's Exact tests). IOM; Institute of Medicine, GWG; Gestational weight gain, CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity.

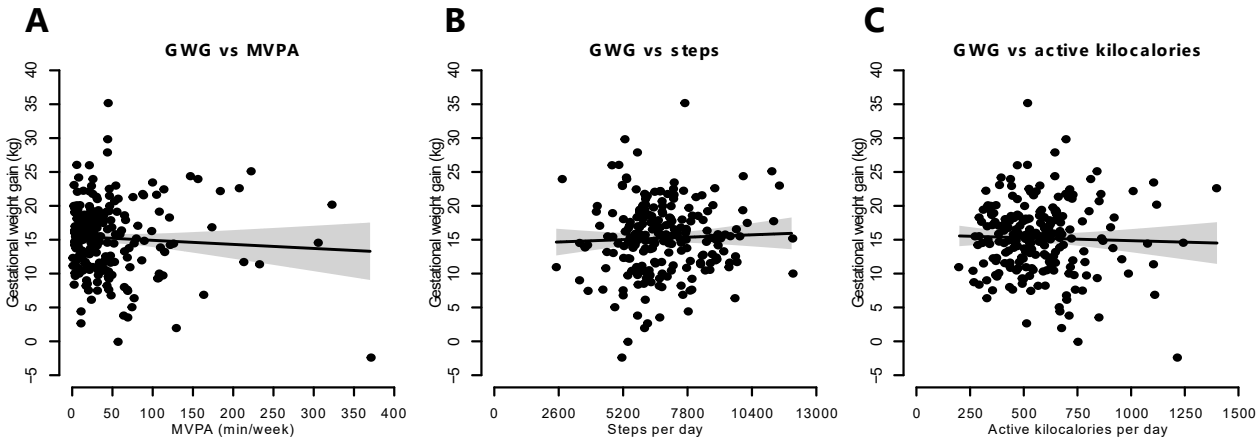

Figure S.3: Associations between MVPA (min/week) and total gestational weight gain (GWG) **(A),** steps per day and total GWG **(B),** and active kilocalories per day and total GWG **(C)** for all participants (n=219). Data points are visualized based on average MVPA, steps and active kilocalories of 25 imputed data sets. A linear regression analysis showed no associations between MVPA (p=0.363), steps (p=0.537), active kilocalories (p=0.637) and total GWG, respectively. GWG; Gestational weight gain, MVPA; moderate to vigorous intensity physical activity.

Paper 3

The effects of prenatal exercise interventions on breast milk composition in puerperal mothers

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Abstract

Maternal exercise during pregnancy is associated with beneficial effects on offspring health, and exercise-induced adaptations in breast milk have recently been proposed as an underlying mechanism. Metabolomic and lipidomic profiling was performed on human breast milk samples of 99 mothers, 7-14 days after birth to investigate the effects of various exercise interventions during pregnancy or a control group. Overall, some metabolites and lipids in human breast milk changed after both structured supervised exercise training and motivational counselling on physical activity during pregnancy interventions compared to control. Moreover, some of the metabolites and lipids correlated with physical activity level. However, prenatal exercise interventions did not elicit major metabolite or lipid changes compared to control. The data indicate that maternal exercise during pregnancy may induce changes to the human breast milk metabolome and lipidome, which in part could explain improved offspring metabolic health.

Introduction

Maternal exercise during pregnancy is an effective tool to improve metabolic health of offspring in animal models (1–3), but it is unclear whether the same improvement applies to humans. Prenatal exercise has been shown to normalize offspring birth weight in humans (4,5), but conflicting evidence exists regarding the long-lasting effects of prenatal exercise on offspring obesity-related outcomes (6,7). A recent systematic review and meta-analysis indicated no overall association between prenatal lifestyle interventions, including physical activity (PA), and weight and body mass index (BMI) in offspring aged one month to seven years (6), whereas maternal prenatal exercise has been associated with lower risk of being overweight/obese among 5,125 eight-year-old offspring compared to offspring of sedentary mothers (7). Thus, maternal exercise might optimize offspring health and decrease the risk for development of cardiovascular and metabolic diseases in humans, but the underlying mechanisms remain incompletely understood.

Recent research has proposed exercise-induced adaptations to breast milk as a possible mechanism underlying the beneficial effects of prenatal exercise on offspring health (8,9). Harris et al. identified an exercise-induced increase in the oligosaccharide 3′-sialyllactose (3′-SL) in breast milk in mice and showed that the beneficial effects of maternal exercise on offspring health were mediated by 3′-SL in breast milk. Further, they showed a positive correlation between PA and 3′-SL content in breast milk in humans (8). Additionally, Wolfs et al. reported that the lipokine (lipid compounds that are predominantly secreted from adipose tissue and can act as signaling molecules and influence systemic metabolism (10,11)) 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) was increased in human breast milk after an acute bout of moderate intensity exercise, and that breast milk abundance of 12,13-diHOME was inversely associated with several measures of infant adiposity (9). These intriguing findings call for further investigation of whether exercise training during pregnancy induces changes in human breast milk composition.

To expand our understanding on how various prenatal exercise interventions affect the human breast milk metabolome and lipidome, we investigated the effects of structured supervised exercise training (EXE) and motivational counselling on PA (MOT) compared to standard care (CON) during pregnancy on the human breast milk metabolome and lipidome in the early postpartum period.

Methods

Participants and study procedures

Participants (n=99) were pregnant women enrolled in FitMum, a single-site randomized controlled trial (ClinicalTrials.gov #NCT03679130) conducted in 2018-2021 at the Department of Gynecology and Obstetrics at Nordsjaellands Hospital, Hillerod, Denmark (12). In brief, the eligibility criteria included being healthy, gestational age (GA) ≤ 15+0 weeks, inactive during early pregnancy (structured exercise at moderate to vigorous intensity < one hour/week) and without pre-existing or ongoing obstetric or medical complications, or alcohol/drug abuse. Demographic information was obtained at inclusion and obstetric (e.g. gestational diabetes mellitus and gestational hypertensive disorders) and neonatal outcomes (birth weight and birth length) were collected from medical records. Prepregnancy BMI (kg/m^2) was calculated based on self-reported prepregnancy weight and height. Gestational weight gain at GA 40+0 weeks was estimated based on predicted body weights from a mixed effects model as described previously *(reference to another FitMum paper in review in AJOG will be inserted when published, before submission of this paper)* and excessive gestational weight gain was defined according to the Institute of Medicine's recommendations (13). Postpartum maternal weight was measured to the nearest 0.1 kg on a calibrated electronic scale (SECA 799) 7-14 days after delivery (the day of breast milk collection). Small for gestational age (<10th percentile) and large for gestational age (>90th percentile) (14) were defined according to a Danish standard population and calculated from the Marsal formula (15), which includes fetal sex, birth weight and GA at delivery. All participants wore an activity tracker (Garmin Vivosport) that measured PA, including moderate to vigorous intensity PA (MVPA, min per week), steps (per day), and active min (everything beyond sedentary time per day), continuously from inclusion to delivery. Randomization to either CON, EXE, or MOT followed a one-week baseline period (latest GA 16+0). Participants in the EXE intervention were offered supervised exercise training at

moderate intensity three times a week. Participants in the MOT intervention were offered three group and four individual motivational counselling sessions on PA during pregnancy and one weekly SMS-reminder to increase PA level. During the Covid19-pandemic (from March 11th, 2020 and throughout the intervention period) exercise training sessions and motivational counselling sessions were conducted online using Zoom Cloud Meetings or by telephone. In a period, test visits were also conducted online and no breast milk samples were collected (12). The study was approved by the Danish National Committee on Health Research Ethics (#H-18011067) and the Danish Data Protection Agency (#P-2019-512). Written informed consent was obtained from all participants.

Collection of breast milk

Participants collected a milk sample from a single feed at the first feeding after 6:00 AM at home 7-14 days after delivery. The breast was cleaned with a wet washcloth before pumping. One breast was expressed fully into a clean bottle using a manual breast pump (Medela Harmony breast pump). The bottle was shaken for 30 sec to account for any variation between fore- and hindmilk, and 2.5 mL was collected in a plastic vial and immediately stored in the participant's own freezer at around -18 °C for approximately eight hours. Milk samples were kept frozen and transported in insulated bags to the hospital for storage at -80 °C. Samples were shipped to the Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Denmark, for metabolomics and lipidomics analysis. Eight breast milk samples were not obtained exactly as described above, since they were stored at around -18 °C for longer time (three weeks), obtained on day five and 15 after birth (not within the 7-14 days postpartum interval), or from women who delivered prematurely, or experienced challenges obtaining the sample. However, these samples did not appear as outliers in

our metabolomics and lipidomics analyses and hence, data from all participants were included in the analysis.

Metabolomics and lipidomics analysis

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade water, acetonitrile, 2-propanol, and methanol (MeOH) were purchased from Honeywell (Charlotte, NC, USA). Labeled standards were acquired from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA), Sigma-Aldrich (St. Louis, MO), and Cayman Chemical (MICH, USA). SPLASH Lipidomix stable isotope labeled internal standards were purchased from Avanti (Alabaster, Alabama, USA). ESI-L Low Concentration Tune Mix was purchased from Agilent Technologies (Santa Clara, CA, USA). Formic acid 99.5+%, Optima™ LC/MS grade was purchased from Fisher Chemical (Pittsburgh, PA, USA). Sodium hydroxide solution (1 N) was purchased from Merck (Darmstadt, Germany).

Sample preparation for metabolite and lipid extraction

For metabolomics analysis, sample preparation has been described elsewhere (16). Briefly, 200 µL of each breast milk sample was aliquoted in 1mL Eppendorf tubes and stored in a −80 °C freezer until use. The samples were treated with 595 µL of MeOH. For quality control and normalization, a 5 µL of internal labeled standards mix (10 µg/mL for all) containing octanoyl-L-carnitine-d_3, butyryl L-carnitine-d_3, palmitoyl L-carnitine-d_3, glycoursodeoxycholic acid-d_4, glycochenodeoxycholic acid-d_4, taurolithocholic acid-d_4, decanoic acid-d_{19}, prostaglandin E_2-d_4, dihomo-γ-linolenic acid-d_6, 12S-HETE-d_8, and oleic Acid-d_{17} (1 mg/L) in MeOH was added to the extraction solvent. Samples were vortexed and precipitated on ice for 20 min. After precipitation, extracts were centrifuged at 14,000 rpm at 4 °C for 15 min for protein precipitation and metabolite

extraction. The supernatant (700 µL) containing the polar metabolites was collected in new 2mL Eppendorf tubes. A volume of 700 µL of dichloromethane and 50 µL of water was then added to the supernatant. Samples were vortexed and left at room temperature for 10 min. A volume of 620 µL of the aqueous layer was then collected into a new glass HPLC vial containing a 300 µL glass insert. Samples were evaporated with a gentle stream of nitrogen to dryness using Zipvap 48 position evaporator heater (Terre Haute, IN, USA). The dried samples were redissolved in 50 µL of water.

For lipidomics analysis, 10 µL of each breast milk sample was aliquoted in 1 mL Eppendorf tubes and stored in a −80 °C freezer until use. Samples were treated with 10 µL 0.9% w/v NaCl, and 120 µL chloroform/methanol (2:1) containing 14 internal standards (SPLASH Lipidomix stable isotope labeled internal standards; 2.5 µg/mL for all). The mixture was vortexed and precipitated on ice for 30 min followed by centrifugation at 1000g for 3 min at 4 °C. Sixty µL supernatant of the lipid-containing chloroform phase (lower level) was extracted to a glass HPLC vial containing 60 µL chloroform: methanol (1:1).

Quality control

A pooled sample (quality control sample, QC) was prepared by mixing equal volumes of each breast milk sample for both the metabolomics and lipidomics extracts. To condition the column, the QC sample was injected at least three times before initiating the run. Then the sample was reinjected every 10 injections, and at the end of the run to assess instrument stability and analyte reproducibility. An equal volume of a blank sample consisting of the internal labeled standard mix (blank IS) in MeOH and a performance check (PEC) sample were also analyzed. For metabolomics analysis, the PEC consisted of reserpine, Leu-Enk, and Val-Tyr-Val in MeOH and for the lipidomics analysis, the PEC consisted of the LIGHTSPLASH primary standard mix (Avanti;

Alabaster, Alabama, USA). The blank IS and PEC were randomly inserted among the real sample queue for monitoring the performance of the analytical platform. The analytical performance was evaluated by calculating the technical precision within each blank IS & PEC sample for retention time (RT), mass accuracy, and MS intensity. In this way, the precision of data was based on within run precision data of all standards present in the blank IS and PEC solution, while for the QC plasma pool endogenous metabolites and lipids were assessed both in positive and negative modes. The analytical reproducibility in terms of intensities of the detected *m/z* features was evaluated by calculating the coefficient of variation (% CV) of detected peaks in QC samples.

Analysis by Ultrahigh Performance Liquid Chromatography mass spectrometry (UHPLC-MS)

Metabolomics and lipidomics profiling were performed using an Ultrahigh Performance Liquid Chromatography (UHPLC) system (Agilent 1290 Infinity II) connected to a Bruker timsTOF Pro™ instrument equipped with trapped ion mobility spectrometer (TIMS) coupled to a hybrid quadrupole, time-of-flight mass spectrometer (TOF-MS; Bruker, Bremen, Germany). Ions were generated in both positive and negative *electrospray ionization mode*. The ESI source used 10 L/min of drying gas at a temp of 220 °C. The ESI was set at 4500 V and 3600 V capillary voltage for pos and neg mode, respectively, and a 2.2 bar nebulizer pressure. Detection of the mass/charge ratio (*m/z)* of ions was set from 50 to 1000 over 17 min. To facilitate the compound identifications, QC samples were analyzed by auto MS/MS. The absolute threshold was set to 1000 counts. MS and MS/MS spectra acquisition rates were set to 2 and 4 Hz, respectively, with a total cycle time of 1 sec for precursor ions collection. The collision energy in MS/MS varied between 10 eV to 60 eV. For metabolomics analysis, the samples were randomized and analyzed using reversed-phase ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 mm X 100 mm (Waters, Milford, MA). The column and auto-sampler temperatures were maintained at 40 °C and 6 °C, respectively. Solvent A

consisting of 0.1% HCOOH v/v in H_2O and solvent B consisting of 0.1% HCOOH v/v in acetonitrile and isopropanol (IPA; 3:1, v/v) were used as mobile phases. The injection volume and flow rate were 3 µL and 0.4 mL/min, respectively. The mobile phase gradient was programmed as follows: a linear gradient from 3% B to 97% B over 9 min, 97% B for 5 min followed by 3% B in 0.5 min, and equilibration at 3% B for 2.5 min (17).

For lipidomics analysis, the samples were randomized and analyzed using reversed-phase ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm (Waters, Milford, MA). The column and auto-sampler temperatures were maintained at 50 °C and 6 °C, respectively. Solvent A consisting of H_2O + 1% NH_4Ac (1M) + 0.1% v/v HCOOH and solvent B consisting of ACN: IPA (1:1, v/v) + 1% NH_4Ac (1M) + 0.1% HCOOH (3:1, v/v) were used as mobile phases. The injection volume and flow rate were 1 µL and 0.4 mL/min, respectively. The mobile phase gradient was programmed as follows: The gradient was: 0-2 min 35-80% B; 2-7 min 80–100% B; and 7-14 min 100% B, followed by a 4 min re-equilibration period under the initial conditions (35% B) (18).

MS-data processing for metabolomics and lipidomics analysis

Data acquisition was controlled by the otofControl software version 6.0 and Bruker Compass HyStar version 5.0 (Bruker Daltonics, Bremen, Germany). Data processing was performed with Bruker Compass Data Analysis 5.2 software and MetaboScape version 5.0 (Bruker Daltonics, Bremen, Germany). Molecular features selection, bucketing, filtering, and scaling were performed by MetaboScape to generate the peak lists from MS and MS/MS spectra. An internal calibrant of Na format injected at the beginning of each analysis was used to calibrate the acquired MS and MS/MS data in MetaboScape.

Detected molecular features were annotated in two steps. First masses and retention times were compared with an in-house library based on authentic standards. Secondly, the recorded MS/MS spectra in MetaboScape were annotated using SmartFormula and by comparing with the in-house MS/MS spectral library, LipidBlast, Bruker HMDB Metabolite Library, Bruker MetaboBASE Personal Library 3.0, MoNA, and MSDIAL-TandemMassSpectralAtlas using a confidence limit of 5 mDa for parent mass tolerance.

Statistical analysis

Data are presented as mean ± SD/± SEM/95% confidence intervals [95% CI], median and interquartile range (IQR), or frequencies and proportions. Statistical analyses were performed using R (19), GraphPad Prism (version 9, GraphPad Software), Metaboanalyst (www.metaboanalyst.ca), and Microsoft Excel. Metabolite and lipid relative abundances were log transformed and autoscaled to each individual metabolite; further analyses were performed on normalized data. Statistical significance was defined as p-value <0.05 and determined by Student's t-tests or Kruskal-Wallis tests with wilcoxon rank sum test with holm-corrected p-values for pairwise comparisons. Tendencies were defined as p-values of 0.05-0.1 and marked with parentheses in figures. P-values of 0.05 corresponded to -Log10 p-values of 1.3. Linear regression analyses were used for analyses of correlations between specific metabolites/lipids in breast milk, prenatal PA measures (MVPA, active min, and steps), and postpartum maternal weight. PA data were the average of 25 imputed data sets for missing data from randomization to delivery.

Results

The FitMum study included 220 pregnant women. We obtained breast milk samples from 99 women 7-14 days after birth (CON: n=18, EXE: n=38, MOT: n=43) and performed untargeted metabolomics and lipidomics to investigate the effects of prenatal exercise interventions on early postpartum breast milk metabolome and lipidome (Figure 1). Descriptive characteristics for the breast milk population are presented in Table 1. Overall, most participants were healthy, without pregnancy or delivery related complications. More than 50% had an excessive gestational weight gain according to the Institute of Medine's recommendations. Overall, most offspring were delivered full term and had normal anthropometric measures. Neonatal characteristics seemed to be similar between the three groups, except from fewer offspring in the EXE group that seemed to be born small or large for gestational age. The descriptive characteristics and the distribution of participants in the three study groups did not differ compared to the entire study population *(reference to another FitMum paper in review in AJOG will be inserted when published, before submission of this paper)*. Adherence to EXE and MOT among participants in the breast milk population was on average 1.6 [1.3;1.8] sessions/week and 6.5 [6.3;6.8] counselling sessions during pregnancy, respectively. The UHPLC–MS untargeted metabolomics and lipidomics analysis detected 219 annotated metabolites and 172 annotated lipids, respectively, and 21,434 and 14,278 non-annotated metabolite and lipid mass features, respectively. Statistical analyses were performed on the annotated metabolites and lipids.

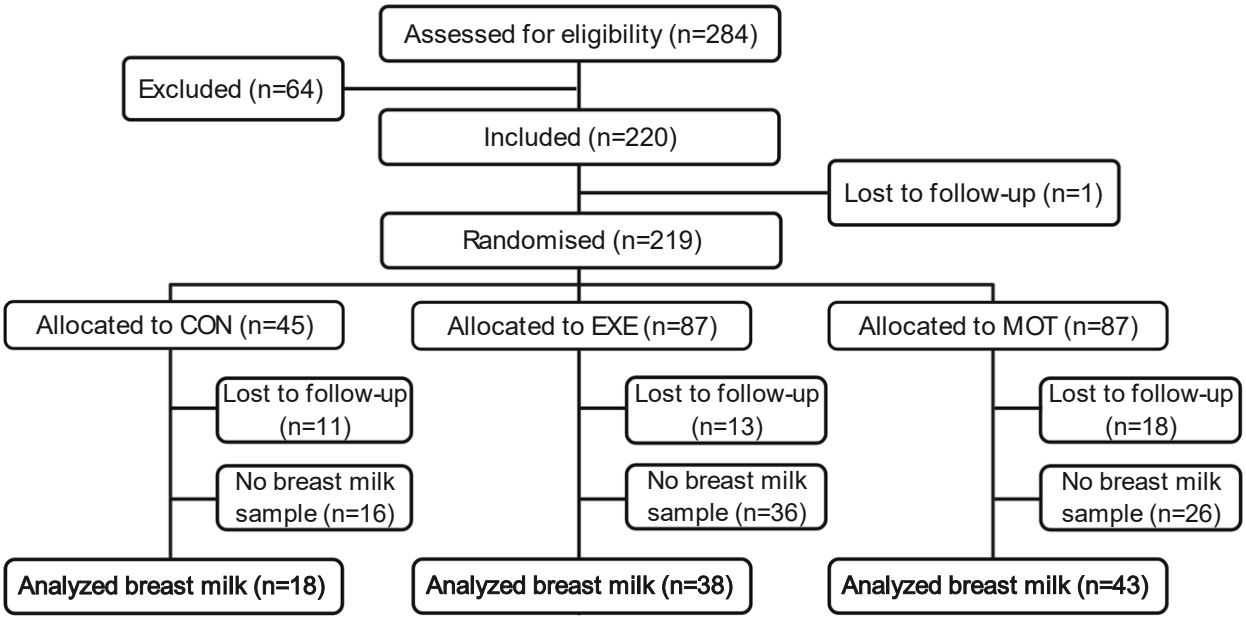

Figure 1. Inclusion, randomization, allocation, and analyzed breast milk samples in the FitMum study. Breast milk samples were obtained from n=18 in CON, n=38 in EXE and n=43 in MOT 7-14 days after delivery. A detailed flow diagram of the FitMum study has been published previously *(reference to another FitMum paper in review in AJOG will be inserted when published, before submission of this paper)*. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity. The figure was created with BioRender.com.

Table 1. Descriptive characteristics

	ALL (n=99)	CON (n=18)	EXE (n=38)	MOT (n=43)
Age, years	31.1 ± 4.1	31.3 ± 4.0	30.2 ± 3.8	31.9 ± 4.2
Prepregnancy BMI, kg/m^2***	23.7 (21.6-28.6)	22.6 (20.8-24.7)	23.9 (21.6-28.7)	24.3 (22.3-28.9)
GA at inclusion, weeks	12.4 (9.3-14.0)	12.4 (9.3-13.1)	11.9 (9.2-13.9)	13.0 (10.2-14.1)
Parity, nulliparous, n (%)	39 (39%)	7 (39%)	20 (53%)	12 (28%)
Education level, n (%)*				
School ≥12 years	89 (90%)	17 (94%)	33 (87%)	39 (91%)
Further education ≥3 years	84 (85%)	14 (78%)	32 (84%)	38 (88%)
Employed/studying, n (%)	92 (93%)	18 (100%)	37 (97%)	37 (86%)
Smoking, n (%)				
During pregnancy	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Quit smoking before pregnancy	6 (6%)	2 (11%)	3 (8%)	1 (2%)
Obstetric characteristics				
GDM, n (%)	7 (7%)	1 (6%)	2 (5%)	4 (9%)
Gestational hypertensive disorders, n (%)**	6 (6%)	0 (0%)	2 (5%)	4 (9%)
GWG until GA40+0, kg	14.5 ± 6.1	15.4 ± 5.4	15.1 ± 5.2	13.5 ± 7.1
Excessive GWG according to IOM recommendations, n (%)	52 (53%)	10 (56%)	24 (63%)	18 (42%)
GA at delivery, weeks	40.4 (39.7-41.3)	40.3 (39.7-41.4)	40.6 (39.8-41.3)	40.1 (39.7-41.1)
Premature delivery (GA<37), n (%)	3 (3%)	1 (6%)	1 (3%)	1 (2%)

Epidural analgesia, n (%)	29 (29%)	5 (28%)	12 (32%)	12 (28%)
Oxytocin augmentation, n (%)	23 (23%)	3 (17%)	11 (29%)	9 (21%)
Vaginal delivery, n (%)	80 (81%)	16 (89%)	29 (76%)	35 (81%)
Caesarean section, n (%)	19 (19%)	2 (11%)	9 (24%)	8 (19%)
Maternal weight 7-14 days postpartum, kg***	77.8 ± 15.0	74.1 ± 13.9	78.3 ± 16.4	79.0 ± 14.1
Neonatal characteristics				
Birth weight, g	3670 (3270-3910)	3640 (3220-3900)	3690 (3460-3960)	3660 (3210-3900)
Birth length, cm	52 (51-53)	52 (51-54)	52 (51-53)	52 (51-53)
SGA, n (%)	11 (11%)	3 (17%)	2 (5%)	6 (14%)
LGA, n (%)	6 (6%)	1 (6%)	0 (0%)	5 (12%)

Descriptive characteristics in the randomization groups. * School ≥12 years corresponds to high school and further education ≥3 years corresponds to university degree (bachelor or master level). ** Defined as preeclampsia, gestational hypertension, HELLP or eclampsia. *** For some variables the total number is lower due to missing values: Prepregnancy BMI; n=98 (CON; n=18, EXE; n=37, MOT; n=43), maternal weight 7-14 days postpartum; n=98 (CON; n=18, EXE; n=38, MOT; n=42). Data are mean ± SD, median (IQR) and n (%). No statistical comparisons have been performed on descriptive characteristics in accordance with CONSORT recommendations. CON; Control group, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, GA; Gestational age, GDM; Gestational diabetes mellitus, GWG; Gestational weight gain, IOM: Institute of Medicine, SGA; Small for gestational age, LGA; Large for gestational age, IQR; interquartile range.

Metabolites

Metabolic profiling can provide rapid indications of metabolic perturbations in response to exercise. In the present study, two-way hierarchical clustering of normalized metabolites showed differences in normalized metabolite abundances in both EXE and MOT compared to CON, but no major metabolite shifts were identified in response to EXE or MOT. Therefore, we performed a cluster analysis, identifying clusters based on the hierarchical clustering, to stratify metabolites into six main groups (Figure 2A). To probe possible exercise-induced up- and downregulations of individual metabolites in milk, we performed volcano plot analysis compared to CON (Log2 fold scale with $p<0.05$). Compared to CON, EXE increased 1,7-dimethyluric acid ($p=0.026$) and decreased p-cresol sulfate and xanthurenate ($p<0.05$) (Figure 2B). MOT increased nine metabolites including caffeine and 1,7-dimethyluric acid ($p<0.05$) compared to CON (Figure 2C). Neither 3′-SL nor 12,13-diHOME were changed in EXE or MOT compared to CON (Figure 2B-C). However, both EXE and MOT significantly increased the abundance of 1,7-Dimethyluric acid, compared to CON (Figure 2D). Thus, while no major differences were observed between groups, our analysis identified the caffeine byproduct 1,7-dimethyluric acid to be increased in both EXE and MOT.

For all six clusters that were identified during the cluster analysis, z-scores were calculated and plotted to visualize interesting differentiations between groups. We proceeded with analyses of cluster 1-5 (Figure S.1), since interesting differentiations of EXE and MOT compared to CON and of each other were found in these clusters. In cluster 1, EXE and MOT differed by being in a positive direction compared to CON (Figure 2E). In cluster 2 (Figure S.1E) and 3 (Figure S.1H), MOT differed by being in a positive and negative direction, respectively, compared to the other two groups. In cluster 4 (Figure 2I), and 5 (Figure S.1P), EXE was in a positive and negative direction, respectively, compared to the other two groups. Enrichment analyses in cluster 1 (Figure 2F) showed that caffeine metabolism pathway, which might be involved in lipolysis during

exercise, was enriched in EXE and MOT combined compared to CON (-Log10 p=2.010). Identifying significantly enriched metabolites in the caffeine metabolism pathway revealed that relative abundance of the metabolite caffeine was higher in MOT compared to CON ($p<0.01$) (Figure 2G). A positive correlation was found between caffeine and active min per day ($p=0.029$) (Figure 2H). Enrichment analysis in cluster 4 (Figure 2J) showed that EXE increased 23 different metabolic pathways, among them citric acid cycle (TCA cycle), compared to CON and MOT combined (-Log10 $p>1.3$). The metabolite oxoglutarate was involved in the regulation of all 23 pathways with a higher relative abundance in EXE compared to MOT ($p<0.05$) (Figure 2K). Oxoglutarate is involved in the TCA cycle, indicating an overall change in TCA cycle activity in EXE. Positive correlations were found between oxoglutarate and MVPA ($p=0.041$) (Figure 2L) as well as between oxoglutarate and maternal body weight at day 7-14 after delivery ($p=0.042$) (Figure 2M). Overall, we observed higher relative abundances of caffeine and oxoglutarate after prenatal exercise interventions and positive correlations between these metabolites and PA level.

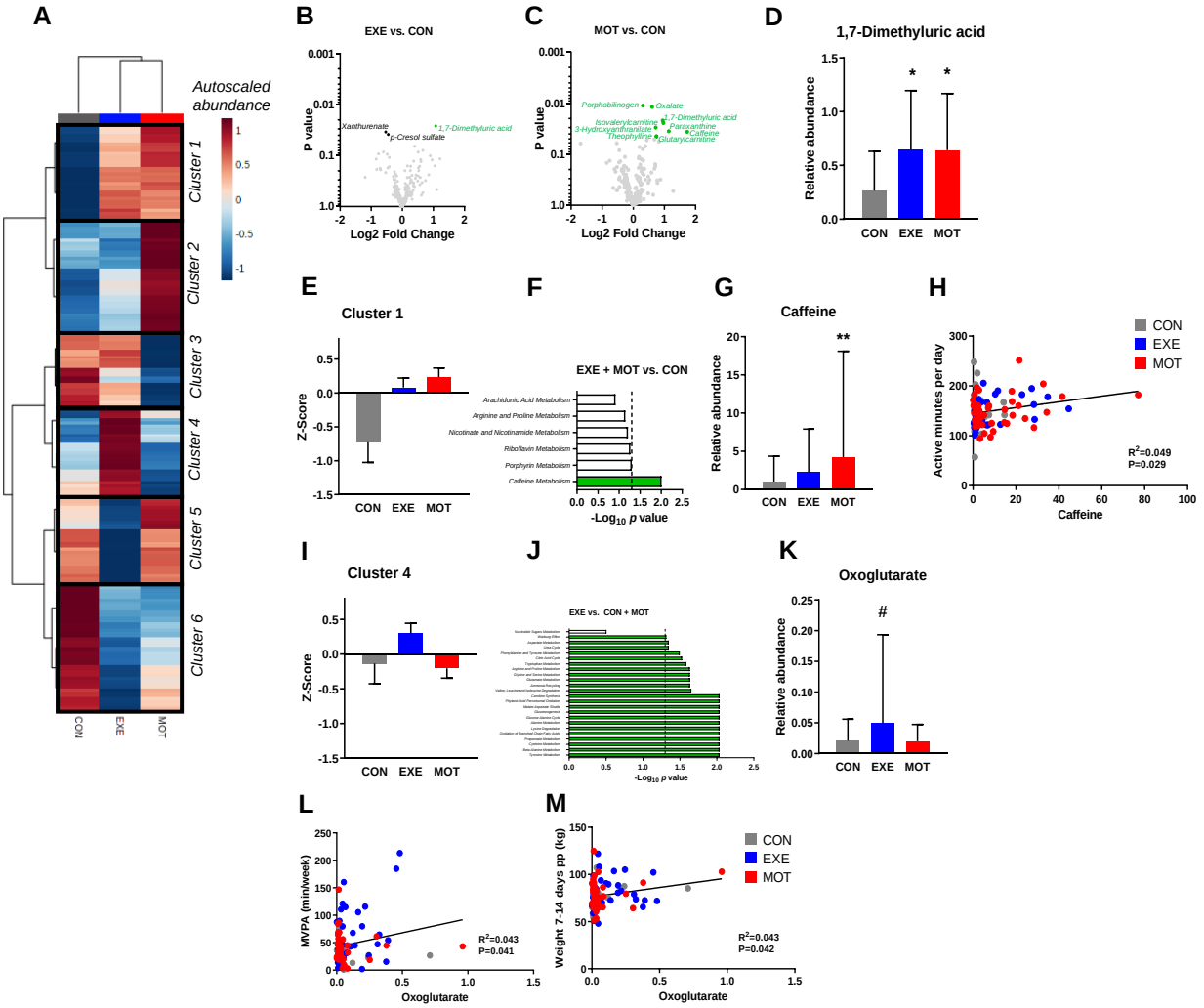

Figure 2. Structured supervised exercise training or motivational counselling on physical activity influence 1,7-dimethyluric acid, caffeine, and oxoglutarate.

Heatmap **(A)** and volcano plots **(B-C)** representing metabolites comparing the fold induction of control with the P value. Relative abundance of **(D)** 1,7-dimethyluric acid. Z-scores for CON, EXE, and MOT in cluster 1 **(E)** and 4 **(I)**. Pathway regulations in cluster 1 **(F)** and 4 **(J)** with significantly enriched pathways (-Log10 p value>1.3) in green. Relative abundances of metabolites involved in pathway regulations; **(G)** caffeine, and **(K)** oxoglutarate (involved in all 23 significantly changed pathways). Data are median (IQR) **(D, G, K)** or mean ± SEM **(E, I)** (CON: n=18; EXE: n=38; MOT: n=43). * represents difference versus CON (* p<0.05, **p<0.01), # represents differences versus MOT (p<0.05). Significant correlations between caffeine and active min per day **(H)**, oxoglutarate and MVPA **(L),** and oxoglutarate and maternal weight 7-14 days pp **(M)** (n=97). Student's t-tests were used for **B-C** and Kruskal-Wallis tests with wilcoxon rank sum tests for **D, G,** and **K**. Linear regression analyses were used for **H**, **L** and **M**. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, MVPA; Moderate to vigorous physical activity, PP; Postpartum, IQR; interquartile range.

Lipids

Lipidomic profiling can expand our understanding of lipid alterations in response to exercise. Two-way hierarchical clustering of normalized lipids showed differences in relative abundances in both EXE and MOT compared to CON, with overall lower normalized lipid abundances in EXE and MOT compared to CON (Figure 3A). To explore possible exercise-induced changes in individual lipids in milk, we performed volcano plot analysis compared to CON (Log2 fold scale with $p<0.05$). Compared to CON, EXE decreased seven lipids, including PC(34:0), PC(36:1), and PC(36:2) ($p<0.05$) (Figure 3B), and MOT decreased 14 lipids, also including PC(34:0), PC(36:1), and PC(36:2) ($p<0.05$) (Figure 3C). To investigate changes in lipids between the three groups, we performed principal component analysis, which showed no clear separation of the three datasets, suggestive of no distinct influence of the two interventions on metabolic perturbations of lipids (Figure 3D).

We divided the lipids into six classes including oxidized phosphatidylethanolamines (O-PE) & phosphatidylethanolamines (PE), fatty acid hydroxy fatty acids (FAHFA), lysophosphatidylcholines (LPC) & phosphatidylcholines (PC) (Figure 3E-G), triglyceride (TG) & fatty acids (FA), phosphatidylglycerol (PG), and sphingolipids (SM) (Figure S.2A-D). In the O-PE and PE class, relative abundances of the three lipids O-PE(36:6), O-PE(38:7), and PE(38:6) were lower in MOT compared to CON ($p<0.05$). PE(38:6) ($p=0.033$) and PE(34:2) ($p=0.014$) were also lower in MOT compared to EXE (Figure 3E). Compared to CON, MOT decreased relative abundance of FAHFA(36:3) ($p=0.045$) (Figure 3F). In the LPC and PC class, EXE decreased LPC(18:0) compared to CON ($p=0.042$) (Figure 3G). Thus, our data suggest that prenatal exercise interventions decrease several lipids. Positive correlations were found among LPC(18:2) and steps per day ($p=0.034$) (Figure 3H), PC(36:1) and active min per day ($p=0.009$) (Figure 3J), as well as between PC(36:2) and active min per day ($p=0.046$) (Figure 3K), indicating that these lipids are

directly correlated to the amount of steps as well as duration of PA. In addition, maternal weight at day 7-14 after delivery was negatively correlated with both LPC(18:2) (Figure 3I) and PC(36:2) (Figure 3L), meaning that increases in these lipids correlated with lower maternal weight. Overall, we observed lower relative abundances of some phospholipids and FAHFA(36:3) with prenatal exercise interventions but positive correlations between several other phospholipids and PA level.

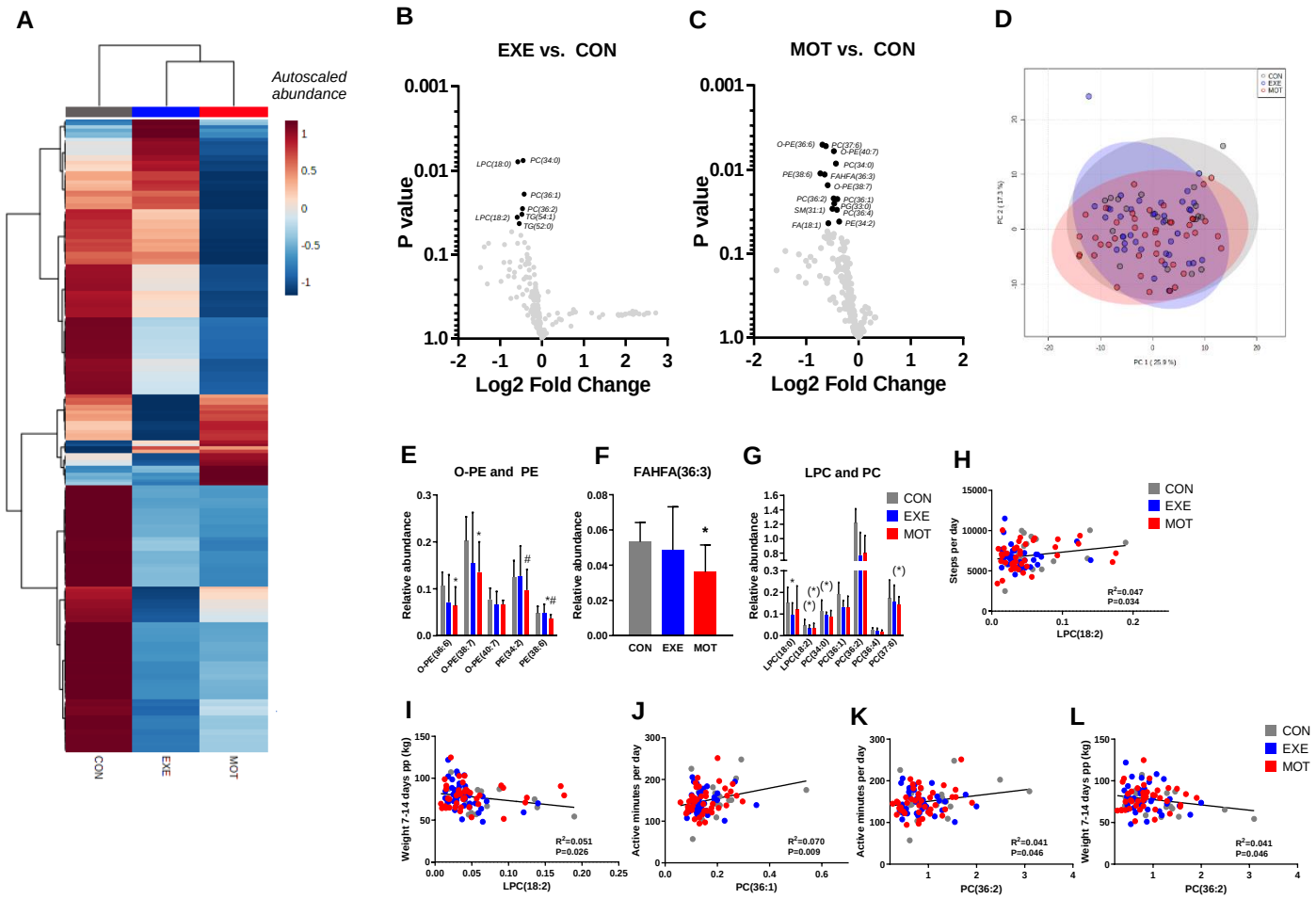

Figure 3. Structured supervised exercise training or motivational counselling on physical activity changes several lipids.

Heatmap **(A)** and volcano plots **(B-C)** representing lipids comparing the fold induction of control with the P value. Principal component analysis showing the first two components **(D).** Relative abundances of lipids; **(E)** O-PE and PE (5 lipids), **(F)** FAHFA(36:3), and **(G)** LPC and PC (7 lipids). Data are median (IQR) (CON: n=18; EXE: n=38; MOT: n=43). * represents difference versus CON ($p<0.05$), # represents differences versus EXE ($p<0.05$), and tendencies are marked with parentheses ($p=0.05$-0.1). Significant correlations of LPC(18:2) with steps **(H)** and maternal weight 7-14 days pp **(I)**, PC(36:1) and active min per day **(J),** and PC(36:2) with active min per day **(K)** and maternal weight 7-14 days postpartum **(L)** (n=97). Student's t-tests were used for **B-C** and Kruskal-Wallis tests with wilcoxon rank sum tests for **E-G**. Linear regression analyses were used for **H- L**. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, O-PE; Oxidized phosphatidylethanolamines, PE; Phosphatidylethanolamines, FAHFA; Fatty acid hydroxy fatty acids, LPC; Lysophosphatidylcholines, PC; Phosphatidylcholines, PP; Postpartum, IQR; interquartile range.

Discussion

Maternal prenatal exercise improves offspring metabolic health and decreases the risk of developing obesity and cardiovascular disease later in life, at least in animal models (2), but the underlying mechanisms remain unclear. Major technological advancements over the past decade have enabled the expansion of the application of metabolomics and lipidomics techniques to map metabolic responses to exercise or other stimuli (20). In the present randomized controlled trial, we used UHPLC–MS untargeted metabolomics and lipidomics to investigate the effects of two different prenatal exercise interventions (EXE and MOT) on the human breast milk metabolome and lipidome 7-14 days after delivery compared to control (CON). Overall, we found positive correlations of some metabolites and several phospholipids with PA measures, and changes in relative abundances of some metabolites and lipids with EXE and MOT compared to CON. However, EXE and MOT did not elicit major metabolite or lipid changes compared to CON. This could be due to only moderate adherence rate, at least among participants in EXE. Our data showed that three metabolites and seven lipids were changed in EXE compared to CON, whereas MOT increased nine metabolites and decreased 14 lipids, compared to CON.

While our study uncovered some changes in metabolites and lipids in response to exercise training during pregnancy, applied via EXE and MOT, the numbers of regulated metabolites and lipids in EXE and MOT compared to CON were markedly lower than findings of metabolic changes in previous studies after exercise. However, these studies investigated the effects of an acute bout of exercise (within 24 hours after endurance or resistance exercise) on metabolic profile measured by metabolomics in human blood, urine or sweat (21–23). The exercise-induced increase in breast milk concentration of the lipokine 12,13-diHOME as indicated by Wolfs et al. was also measured after acute exercise (9). The aim of our study was to investigate if exercise training performed on a regular basis throughout pregnancy affected mature breast milk

composition. Since mature breast milk cannot be pumped before around one week after delivery, an intermediate period between termination of prenatal interventions (at latest at delivery) and breast milk sampling 7-14 days postpartum could not be avoided. Nevertheless, prenatal exercise training may induce long-lasting adaptations in the breast milk metabolome and lipidome, but it is uncertain whether the metabolite and lipid changes found in EXE and MOT in our study were caused by lasting effects of the interventions or might be confounded by other factors. Moreover, confounding factors influencing breast milk composition might blur potential effects of prenatal exercise training on the metabolome and lipidome. Other factors that might influence breast milk composition could be medication use in relation to delivery (24), or maternal obesity, which has been associated with changes in the human milk metabolome (25,26). In our data we found correlations between maternal weight 7-14 days after delivery and some metabolites or lipids, indicating that other factors such as maternal weight at the sampling time point may have influenced the results.

Based on enrichment analysis in Metaboanalyst we reported that EXE changed metabolites related to several metabolic pathways, among them TCA cycle, gluconeogenesis, amino acid metabolism pathways, and urea cycle. Oxoglutarate was involved in the regulation of all the 23 changed metabolic pathways in EXE and was higher in EXE compared to MOT. Further, linear regression analysis showed a positive correlation between oxoglutarate and MVPA. Other studies have also found increased oxoglutarate in plasma and urine after both acute endurance and resistance exercise (21,22), indicating for example increased TCA cycle activity and ATP turnover with exercise.

Further, we found caffeine metabolism to be enriched in EXE and MOT combined compared to CON. Caffeine was the most relevant metabolite driving the enrichment of caffeine metabolism with prenatal exercise, and we showed that relative abundance of caffeine was higher in MOT compared to CON, but not in EXE compared to CON. We also reported a positive correlation

between caffeine and active min per day measured by the activity tracker. Caffeine might mobilize fatty acids to provide energy by stimulating lipolysis during exercise, as indicated by studies of caffeine supplementation prior to exercise (27). Caffeine metabolism has been shown to be increased in serum in young males after marathon running (28), but neither the marathon running study nor our breast milk study controlled for intake of dietary caffeine. Thus, it may be plausible that the increased caffeine metabolism could result from exogenous caffeine intake. The caffeine byproduct 1,7-dimethyluric acid was increased in both EXE and MOT, supporting the increased caffeine metabolism in human breast milk after prenatal exercise.

We found no changes in 3'-SL or 12,13-diHOME with EXE or MOT compared to CON. This is in contrast to previous studies on human breast milk that have showed that average prenatal PA measured with accelerometry three times during pregnancy correlated with 3'-SL concentration (8), and that 12,13-diHOME concentration was increased after postpartum acute maternal exercise (9). This might be explained by the moderate adherence rate to exercise as well.

With regards to changes in lipids, our data indicated lower relative abundances of several lipids from the O-PE & PE, FAHFA, and LPC & PC with prenatal exercise interventions compared to CON. In contrast, increased levels of phospholipids, including O-PE, PE, LPC, PC and LPE, after exercise have been reported in previous lipidomic studies in humans (23) and animals (29,30). Contrepois et al. showed a rapid and transient increase in 23 PC's after acute exercise in blood from human males and females aged 40-75 years (23). Likewise, Nolazco Sassot et al. demonstrated that several phospholipids including PC(34:1), PC(36:2), PC(36:4), and LPC(18:0) were increased in blood from horses after an acute bout of supramaximal exercise at 115% of maximal oxygen consumption (29). Moreover, Hoene et al. showed increased levels of several LPCs and LPEs in mice liver after acute exercise (30). The findings from these studies are in line with our data showing positive correlations of PA measures with LPC(18:2), PC(36:1), and

PC(36:2). Phospholipids are major structural components of cell membranes and increased phospholipid levels after exercise might be explained by increased turnover of cell membranes during exercise due to exercise-induced lipolysis and cell membrane damage. This may enable active mobilization of phospholipids as energy substrates or for cell membrane repair (20).

In contrast with the literature, our data indicated lower relative abundance of FAHFA(36:3) in MOT compared to CON, suggesting that prenatal exercise decreased FAHFA levels in breast milk. FAHFA, which is a lipid class that acts as lipokines (10), has been indicated to confer anti-diabetic and anti-inflammatory effects (31), and to be increased with exercise (32), and decreased in human breast milk from obese mothers collected 72 hours after delivery (33).

Strengths and limitations

In this study, we standardized breast milk sampling by providing the participants with thorough verbal and written instructions on how and when to sample at home 7-14 days after delivery. However, we did not include any restrictions on exercise or fasting prior to sampling, as seen in other studies applying metabolomics and lipidomics analyses to investigate metabolic response to acute exercise (9,21,23,34). The human metabolome and lipidome are dynamically changing and metabolomics/lipidomics analyses do only provide a molecular snapshot of phenotypic traits (20). Therefore, it might be important to control for confounding factors such as delivery mode as well as exercise and diet prior to sampling, when investigating exercise-induced changes in metabolites and lipids. However, a strength of our study is that it offered the possibility to investigate the effects of prenatal exercise on the human milk metabolome and lipidome in a real-life setting. Our breast milk study included 99 women of normal weight on average, non-smoking during pregnancy and overall likely healthier and more motivated to increase PA during pregnancy given their interest in participation in the study, compared to pregnant women in other western countries. This may have

resulted in a lower potential for exercise to induce changes in the breast milk metabolome and lipidome in our pregnant population compared to pregnant women in countries with a more pronounced overweight and obesity burden in pregnant women, for example the United States (35). In the study by Wolfs et al., exercise-induced changes in 12,13-diHOME breast milk concentration were found in both women with normal weight and obesity but the changes were more pronounced in women with obesity (9). This may indicate that the potential for exercise-induced improvements of breast milk composition might be more evident among populations with overweight or obesity. More than 50% of our study participants had an excessive gestational weight gain according to the Institute of Medine's recommendations, and similar prevalence of more than 50% of pregnant women having excessive gestational weight gain has been reported in both the United States and Europe (36).

Conclusion

This study provides comprehensive metabolomic and lipidomic profiling comparing the effects of two different prenatal exercise interventions on metabolite and lipid changes in human breast milk 7-14 days after delivery. Overall, we found changes in some metabolites and lipids in human breast milk after both EXE and MOT compared to CON during pregnancy, as well as positive correlations of some metabolites and lipids with PA measures. However, prenatal exercise interventions did not elicit major metabolite or lipid changes compared to control. Future research is needed on pre- and postnatal exercise-induced adaptations to breast milk and the long-lasting effects of maternal prenatal exercise on offspring metabolic health. Moreover, it needs to be investigated whether offspring metabolic and physiological adaptations are related to the reported changes in metabolites and lipids in breast milk. Ultimately, exercise-induced alterations in human breast milk composition

can be a potential therapeutic approach to prevent development of obesity, type 2 diabetes, and cardiovascular disease.

Abbreviations

3′-SL; 3′-sialyllactose, 12,13-diHOME; 12,13-dihydroxy-9Z-octadecenoic acid, BMI; body mass index, CON; standard care, CV; coefficient of variation, EXE; structured supervised exercise training, FA; fatty acids, FAHFA; fatty acid hydroxy fatty acids, GA; gestational age, HPLC; high-performance liquid chromatography, IQR; interquartile range, IS; internal labeled standard mix, LPC; lysophosphatidylcholines, MeOH; methanol, Min; Minute, MOT; motivational counselling on physical activity, MVPA; moderate to vigorous intensity physical activity, O-PE; oxidized phosphatidylethanolamines, PA; physical activity, PC; phosphatidylcholines, PE; phosphatidylethanolamines, PEC; performance check, PG; phosphatidylglycerol, QC; quality control, RT; retention time, SM; sphingolipids, TCA cycle; citric acid cycle, TG; triglyceride, TIMS; trapped ion mobility spectrometer, TOF-MS; time-of-flight mass spectrometer, UHPLC–MS; ultrahigh performance liquid chromatography mass spectrometry.

Conflicts of interest

The authors declare no conflicts of interest.

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Data availability

Individual participant data will not be available because the research data is confidential. A study protocol paper has been published (http://dx.doi.org/10.1136/bmjopen-2020-043671).

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Authors' contribution

B.S. initiated and directed the FitMum study. C.B.R., S.dP.K., J.B., T.D.C., S.M., S.A.A., E.L and B.S. developed the FitMum study protocol, and N.B. contributed to development of the breast milk sampling protocol. C.B.R., S.dP.K., A.D.A. and N.B. conducted intervention activities and collected data assisted by S.A.A., research assistants and master students. E.L. (clinical trial manager) supervised the clinical part of the study together with J.B., T.D.C., S.M. and B.S. A.K., K.T. and T.M. performed metabolomics and lipidomics analysis. D.H.S., K.I.S. and J.B. supervised analysis of data and assisted in writing the manuscript. C.B.R. analyzed data and drafted the manuscript. All authors read, contributed to, and approved the final version of the manuscript.

Supplementary material

Supplementary material includes supplementary figures S.1A-R and S.2A-D.

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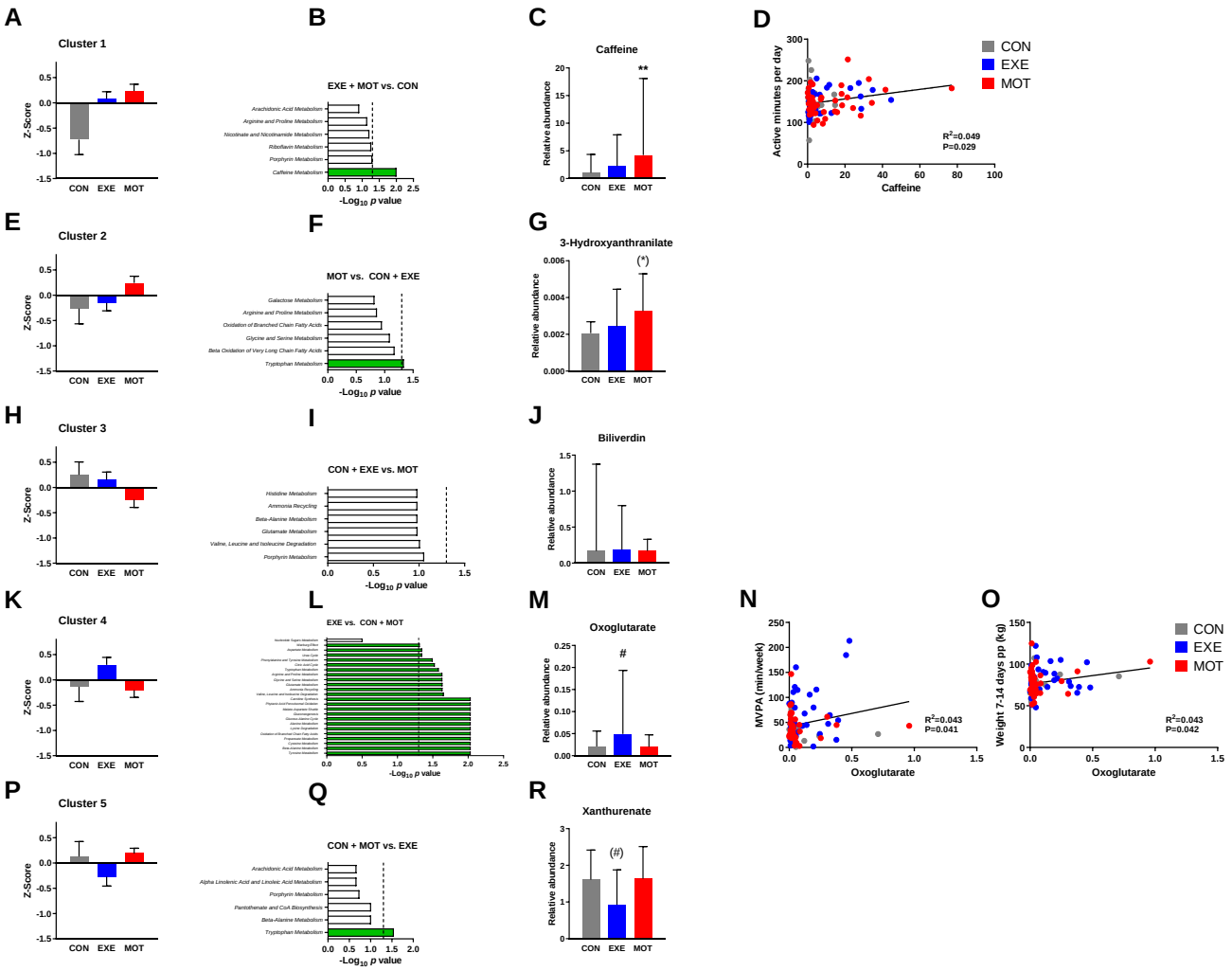

Figure S.1. Metabolites in cluster 1-5.
Z-scores for CON, EXE, and MOT in cluster 1-5 **(A, E, H, K, P)**. Pathway regulations in cluster 1-5 **(B, F, I, L, Q)** with significantly enriched pathways (-Log10 p value>1.3) in green. Relative abundances of metabolites involved in pathway regulations; **(C)** caffeine, **(G)** 3-hydroxyanthranilate, **(J)** biliverdin (involved in porphyrin metabolism that tend to be significantly changed), **(M)** oxoglutarate (involved in all 23 significantly changed pathways), and **(R)** xanthurenate. Data are mean ± SEM **(A, E, H, K, P)** or median (IQR) **(C, G, J, M, R)** (CON: n=18; EXE: n=38; MOT: n=43). * represents difference versus CON (* p<0.05, **p<0.01), # represents differences versus MOT (p<0.05), and tendencies are marked with parentheses (p=0.05-0.1). Significant correlations between caffeine and active min per day **(D)**, oxoglutarate and MVPA per week **(N),** and oxoglutarate and maternal weight 7-14 days pp **(O)** (n=97)**.**
Kruskal-Wallis tests with wilcoxon rank sum tests were used for **C, G, J, M** and **R**. Linear regression analyses were used for **D**, **N** and **O**. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, MVPA; Moderate to vigorous physical activity, PP; Postpartum, IQR; interquartile range.

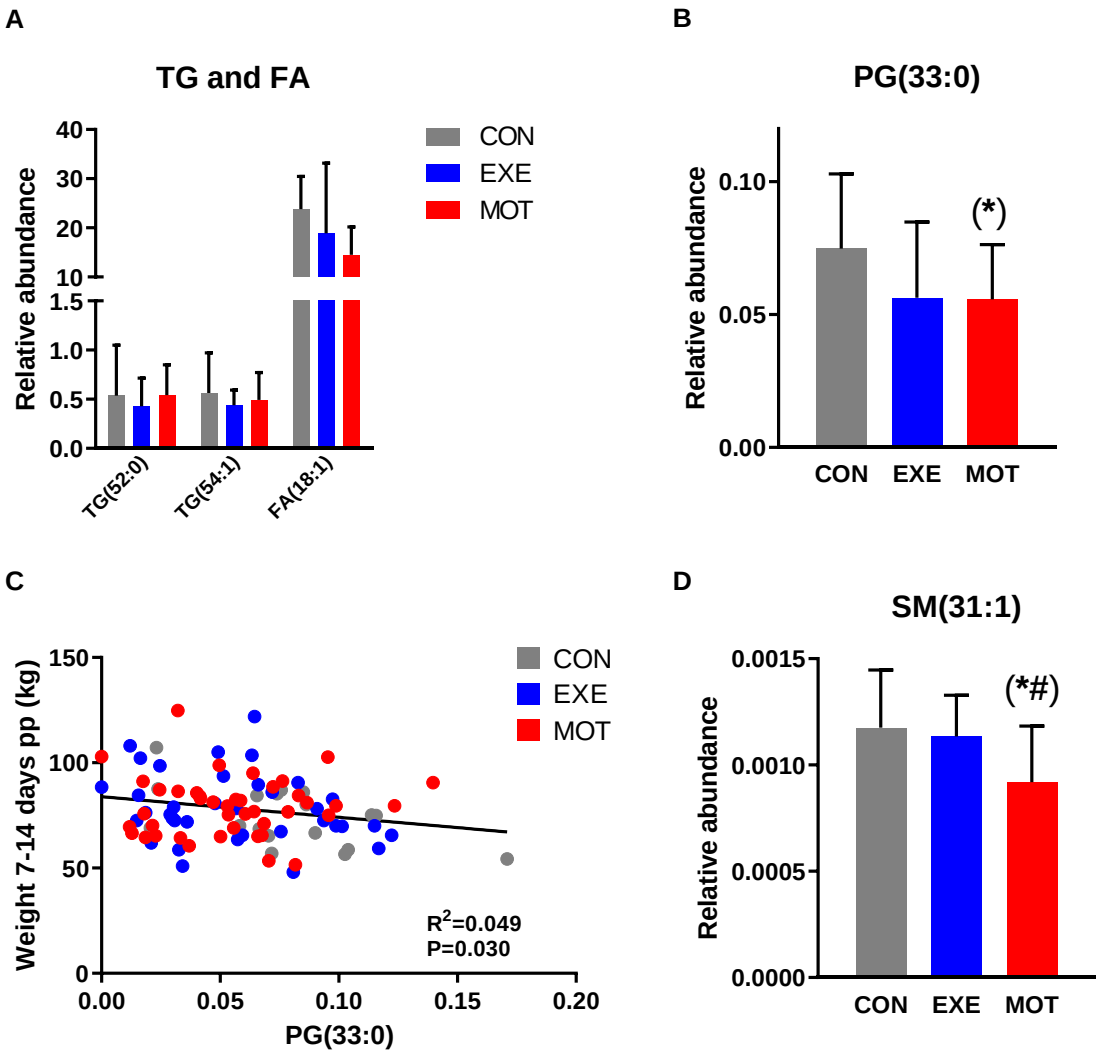

Figure S.2. TG, FA, PG, and SM in the three study groups
Relative abundances of lipids; **(A)** TG and FA (3 lipids), **(B)** PG(33:0), and **(D)** SM(31:1). Data are median (IQR) (CON: n=18; EXE: n=38; MOT: n=43). * represents difference versus CON ($p<0.05$), # represents differences versus EXE ($p<0.05$), and tendencies are marked with parentheses ($p=0.05$-0.1). Significant correlation of PG(33:0) and maternal weight 7-14 days pp **(C).** Kruskal-Wallis tests with wilcoxon rank sum tests were used for **A, B, and D**. Linear regression analysis was used for **C**. CON; Control, EXE; Structured supervised exercise training, MOT; Motivational counselling on physical activity, TG; Triglyceride, FA; Fatty acids, PG; Phosphatidylglycerol, SM; Sphingolipids, PP; Postpartum, IQR; interquartile range.