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194

Tuija Leskinen

Long-Term Leisure-Time Physical Activity vs. Inactivity, Physical Fitness, Body Composition and Metabolic Health Characteristics: a Co-Twin Control Study

JYVÄSKYLÄN YLIOPISTO

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Life is like exercise - the harder it is, the stronger you become.

ABSTRACT

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If an individual is physically active or inactive, then this may have a major impact on health and disease development. In this thesis, the associations between long-term leisure-time physical activity vs. inactivity and health outcomes were studied among 16 middle-aged and older twin pairs (7 were monozygotic pairs who share all of their genes at the sequence level) with a persistent discordance in their leisure-time physical activity habits. At the end of the follow-up, clinical exercise test with cycle ergometer, maximal isometric knee extensor force, and body composition were assessed. Visceral fat area, liver fat score and thigh composition were measured using MRI.

The results revealed that the physically active twins had 23% higher VO_{2peak} values than their inactive co-twins but also 20% higher knee extensor forces although no further significant intrapair differences in skeletal muscle properties were found. The follow-up weight gain was attenuated among the physically active twins but there was no significant intrapair difference in body weight at the end of the follow-up ($p=0.12$). However, the inactive twins had significantly more body fat, 1.5 times more visceral and midthigh intramuscular (extramyocellular) fat and a higher liver fat score compared to their active co-twins. The intrapair difference in the level of ectopic fat was also significant among monozygotic co-twins. To further understand the underlying mechanisms, biopsies were taken from *m. vastus lateralis* and abdominal subcutaneous adipose tissue from 10 volunteer pairs for the gene expression analysis. Gene Set Enrichment Analysis revealed up-regulated metabolic pathways (such as oxidative phosphorylation and valine, leucine and isoleucine degradation) among the active compared to the inactive twins. The expression centroids of these gene sets were associated with cardio-metabolic risk factors, including fitness.

In conclusion, long-term leisure-time physical activity is associated with a better physical fitness and in particular with a lowered rate of ectopic fat accumulation. Therefore regular long-term leisure-time physical activity may confer a major benefit in combatting the appearance of cardio-metabolic disease.

Keywords: physical activity, twins, body composition, ectopic fat, gene expression

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in text by their Roman numerals. Additionally, some unpublished data are included in the thesis.

I Leskinen T, Waller K, Mutikainen S, Aaltonen S, Ronkainen PHA, Alen M, Sipilä S, Kovanen V, Perhonen M, Pietiläinen KH, Cheng S, Suominen H, Kainulainen H, Kaprio J, Kujala UM. 2009. Effects of 32-year leisure time physical activity discordance in twin pairs on health (TWINACTIVE study): aims, design and results for physical fitness. Twin Research and Human Genetics 12(1), 108-117.

II Leskinen T, Sipilä S, Alen M, Cheng S, Pietiläinen KH, Usenius J-P, Suominen H, Kovanen V, Kainulainen H, Kaprio J, Kujala UM. 2009. Leisure-time physical activity and high-risk fat: a longitudinal population-based twin study. International Journal of Obesity 33, 1211-1218.

III Leskinen T, Rinnankoski-Tuikka R, Rintala M, Seppänen-Laakso T, Pöllänen E, Alen M, Sipilä S, Kaprio J, Kovanen V, Rahkila P, Oresic M, Kainulainen H, Kujala UM. 2010. Differences in muscle and adipose tissue gene expression and cardio-metabolic risk factors in the members of physical activity discordant twin pairs. PLoS ONE 5(9), e12609.

IV Leskinen T, Sipilä S, Kaprio J, Kainulainen H, Alen M, Kujala UM. 2013. Physically active vs. inactive lifestyle, muscle properties and glucose homeostasis in middle-aged and older twins. AGE, doi:10.1007/s11357-012-9486-7.

ABBREVIATIONS

BCAA	Branched-chain amino acids
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CI	Confidence interval
DNA	Deoxyribonucleic acid
DXA	Dual-energy X-ray absorptiometry
DZ	Dizygotic
EMCL	Extramyocellular lipids
FC	Fold change
FDR	False discovery rate
GSEA	Gene Set Enrichment analysis
^{1}H-MRS	^{1}H-magnetic resonance spectroscopy
Hb_{A1C}	Glycated hemoglobin
HDL	High-density lipoprotein
HOMA	The homeostatic model assessment
HR	Hazard ratio
HR_{max}	Maximum heart rate
IMCL	Intramyocellular lipids
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MET	Metabolic equivalent task
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MZ	Monozygotic
NAFLD	Nonalcoholic fatty liver disease
NEAT	Non-exercise activity thermogenesis
OXPHOS	Oxidative phosphorylation
PUFA	Polyunsaturated fatty acid biosynthesis
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RM	Repetition maximum
ROI	Region of interest
SAT	Subcutaneous adipose tissue
SD	Standard deviation
TE	Echo time
TG	Triglycerides
TR	Repetition time
VO_{2max}	Maximal oxygen uptake
VO_{2peak}	Peak oxygen uptake
VAT	Visceral adipose tissue
WHO	World Health Organization

CONTENTS

1 INTRODUCTION

Research in the field of physical activity epidemiology has been progressing for over 60 years. In 1950s, Professor J.N. Morris and his colleagues reported the results of their work on London´s double-decker bus drivers vs. conductors. They examined the tailored uniform sizes, and found that the trouser waist girths and the jacket´s breast girths were greater among the bus drivers than among the conductors. They considered that due to the *sedentary* nature of the work, the drivers might have become fatter and more liable to coronary heart disease than the more *active* conductors (Heady, Morris & Raffle 1956). Subsequently, many large observational studies have shown that physical activity and good fitness decrease the risk of coronary heart disease (Paffenbarger, Wing & Hyde 1978, Sesso, Paffenbarger & Lee 2000, Lakka et al. 1994), cardiovascular disease (Blair et al. 1996), type 2 diabetes (Hu et al. 1999, Tuomilehto et al. 2001) and mortality (Wei et al. 1999). Thus, it seems clear that an increase in leisure-time physical activity level (Byberg et al. 2009, Kujala et al. 1998, Schnohr, Scharling & Jensen 2003) and enhancement in cardiorespiratory fitness (Blair et al. 1995) are ways to reduce the risk of death. Regular physical activity during adult life has been shown to reduce the occurrence of chronic diseases, even in a dose-response manner (Physical Activity Guidelines Advisory Committee 2008). Therefore, physical activity can also be used as a therapy for multiple chronic diseases (Pedersen & Saltin 2006).

However, current estimations are that physical inactivity causes globally 6 to 10% of the major non-communicable diseases i.e. too many of us, in fact over one in three, are hazardously inactive (Lee et al. 2012, Hallal et al. 2012). According to the World Health Organization, physical inactivity is the fourth leading risk factor for global mortality, immediately after high blood pressure, tobacco use and high blood glucose levels. The fact is that the lack of physical effort in work and in routine daily activities calls compensatory activities like leisure-time exercise training (Hallal et al. 2012).

Personal (age, sex, health, motivation), genetic and environmental factors all can influence the level of physical activity (Stubbe et al. 2006, Bauman et al. 2012). The heritability (an estimate of the genetic influences on a trait at the

population level) of exercise participation is found to be at least moderate (e.g. Stubbe et al. 2006). In addition, cardiorespiratory fitness (Bouchard et al. 1998, Bouchard et al. 1999) and responsiveness to exercise (Bouchard et al. 1994, Bouchard & Rankinen 2001, Bouchard 2012) are heritable traits. If the genetic components determining physical activity and fitness are shared with those influencing chronic diseases, some inherited biological characteristics could both make it easier for some individuals to achieve high levels of physical activity or fitness and endow them with low morbidity or longevity (Heady, Morris & Raffle 1956, Booth, Chakravarthy & Spangenburg 2002, Kujala, Kaprio & Koskenvuo 2002, Kujala et al. 2003).

Evidence of the health effects of physical activity/fitness has been gathered from both observational studies and randomized controlled trials. Observational studies are usually done with large sample sizes and with population-based samples but these studies are affected by a (genetic) selection bias that may weaken the evaluation of the cause-and-effect. Randomized controlled trials (RCT) are more reliable for the evaluation of cause-and-effect and are often considered as a golden standard. However RCTs are frequently too short to document effects of long-term exercise training on health and do not reach the same sample sizes as those in observational studies (Kujala 2011). The Finnish Twin Cohort has been used to investigate whether genes or other familial factors can cause a selection bias in epidemiological studies on the associations between physical activity and future morbidity and mortality (Kujala et al. 1998, Kujala, Kaprio & Koskenvuo 2002, Kaprio et al. 1978, Kaprio & Koskenvuo 2002, Kaprio et al. 2000, Kujala, Kaprio & Koskenvuo 2000, Waller et al. 2010a, Waller et al. 2010b). The fundamental concept behind twin studies is that monozygotic (MZ) twins come from one fertilized egg and share all of their genes at the sequence level. Therefore, it can be hypothesized that any intrapair difference (that is *discordance*) between the co-twins of a MZ pair must be due to environmental factors. Dizygotic (DZ) twins come from two separately fertilized eggs and that makes them as genetically similar as any other pair of siblings, sharing 50% of same genes.

Physical activity interacts with a plethora of metabolic and health-related functions but many of those such as exercise capacity are partly affected by genetic factors. Therefore, some of the associations between physical activity and metabolic and cardiovascular health may be explained by shared genetic factors. The goal here was to study the associations between life-long physical activity vs. inactivity and health outcomes in a design in which the genetic background could be standardized. Therefore, 16 middle-aged and older twin pairs (of which seven MZ pairs) with an adult-life-long discordance in their physical activity habits were identified from the Finnish Twin Cohort. This thesis is a combination of a literature review and a critical summary of four original articles focusing on clarifying the associations of long-term physical activity vs. inactivity on physical fitness, body composition, and on metabolic health characteristics by comparing active vs. inactive members of physical activity discordant twin pairs.

2 LITERATURE REVIEW

2.1 Physically active vs. inactive

The World Health Organization (WHO) defines physical activity as any bodily movement produced by the contraction of skeletal muscle that increases energy expenditure above the basal level. The daily total energy expenditure consists of the resting metabolic rate (also referred to as basal metabolic rate) (60-75%), thermic effects of feeding (~10%) and posture, spontaneous or voluntary physical activity (15-30% of the total energy expenditure in non-athlete individuals). Energy expenditure is elevated by performing daily routines like sitting, standing, walking and talking (i.e. non-exercise activity thermogenesis (NEAT)) and by undertaking voluntary physical activity. The latter activity has a major effect on the inter-individual variability in energy expenditure (Neilson et al. 2008, Lagerros & Lagiou 2007, Vanhees et al. 2005) although both voluntary physical activity and NEAT contribute to the overall energy balance. In other words, an increase in NEAT also offers the possibility to increase the net energy expenditure and to gain the associated health benefits (Westerterp 2001, Hamilton, Hamilton & Zderic 2007, Levine et al. 2005). However, physical activity is the only component of energy expenditure that can be modified behaviorally in a significant manner.

Physical activity recommendations

In 1995, Centers for Disease Control and Prevention and American College of Sports Medicine recommended that individuals should accumulate 30 minutes or more of moderate-intensity physical activity on most, preferably all, days of the week (Pate 1995). In 2007, the recommendations were updated by the American College of Sports Medicine and American Heart Association such that the physical activity of 30 minutes on five days/week was set as a minimum. Moderate and vigorous activities were advised to be conducted separately or in combination. Also recommendations on the possibility to accumulate short bouts of physical activity with a minimum length of 10

minutes and how best to conduct muscular training were added. Moreover the updated recommendations emphasized that by exercising more than minimum recommendations then more health benefits can be gained (Haskell et al. 2007).

The first-ever Physical Activity Guidelines for Americans was released in 2008 recommending 150 minutes a week (2h and 30 min) of moderate-intensity or 75 minutes a week of vigorous-intensity aerobic physical activity or an equivalent combination of these two types in order to maintain health (Physical Activity Guidelines Advisory Committee 2008). Aerobic activity was recommended to be performed in episodes of at least 10 minutes. Muscular training (strength training) was advised to be performed at least twice a week for all major muscle groups. For children (aged 6 to 17 yrs) at least 60 minutes of daily moderate to vigorous physical activity was recommended (Physical Activity Guidelines Advisory Committee 2008). For older adults (aged ≥65 yrs), the recommendations were a minimum of 30 minutes of moderate five times per week or minimum of 20 minutes of vigorous physical activity at least three times per week or a combination of these two forms. Furthermore it was recommended that muscle-strengthening activities should be performed at least twice a week and exercises for improving balance should be done by older adults (Nelson et al. 2007, Physical Activity Guidelines Advisory Committee 2008).

As revealed in these physical activity recommendations the health benefits of physical activity can be achieved by dividing a weekly amount of 150 minutes into at least 10 minute episodes (Murphy, Blair & Murtagh 2009, Lee, Sesso & Paffenbarger 2000). There are also studies that have found that even less than the recommended amount of physical activity would be sufficient to i) increase fitness in previously sedentary and obese postmenopausal women (Church et al. 2007), ii) to lower the risk for coronary heart disease among older men (Lee et al. 2003) and iii) to achieve a reduced risk for type 2 diabetes (Waller et al. 2010a). In the study of Lee et al. (2004) conducted in healthy men, the mortality risk was shown to be lowered by centralizing large amounts of activity e.g. to weekend days while still adhering to the current physical activity recommendations (Lee et al. 2004). However these findings need more support before they are widely accepted as facts.

What can be concluded is that all physical activity is good for an individual´s health; more exercise results in increased benefits and some exercise is always better than nothing (Haskell et al. 2007, Lee 2007, Leitzmann et al. 2007). Age, health problems and possibly the intensity of physical activity may increase the risk level individually; such cases, the net health effects of exercise may be decreased (Kesäniemi et al. 2001).

Inactivity and sedentariness

In physical activity trials, the term sedentary or inactive is used to refer to individuals whose activity level ranges from <20 to <150 minutes/week and frequency from <1 to <3 days/week while the term active person usually meets the current physical activity recommendations (Bennett et al. 2006). The WHO

defines inactivity as a lack of moderate-to-vigorous activity. Inactivity is nowadays one of the main causes of cardiovascular disease and, thus, a global burden (Lee et al. 2012, Booth, Roberts & Laye 2012). Although inactivity is known to be a hazardous behavior the underlying mechanisms are poorly known. Much of the evidence has been gathered from (sedentary) control groups in clinical trials (Patel, Slentz & Kraus 2011) and from bed rest studies (Bergouignan et al. 2011). It was found that a six-month-long inactivity in the Studies of Targeted Risk Reduction Interventions through Defined Exercise (STRRIDE) resulted in metabolic abnormalities such as an increase in fat mass and visceral fat, and an increase in fasting insulin level, fasting glucose level and small low-density lipoprotein (LDL) particle number. Lack of exercise also worsened cardiorespiratory fitness (Patel, Slentz & Kraus 2011). According to bed rest studies, this kind of extreme inactivity could induce so-called metabolic inflexibility; i.e. insulin resistance followed by a lack of use of muscles may lead to an imbalance between the fatty acid oxidation and fatty acid uptake resulting in an increased accumulation of high-risk fat (also called lipotoxicity) (Bergouignan et al. 2011).

Sedentary behavior (excessive sitting) is not the same as inactivity (too little exercise) although these terms are often used as synonyms (Owen et al. 2010). In general, sedentary time reduces energy expenditure and might increase energy intake (snacking). Moreover sedentary behavior may have an independent and qualitative effect on human metabolism (studied in the field of inactivity physiology). The mechanisms are perhaps related to the suppression of skeletal muscle lipoprotein lipase (LPL) activity and in glucose uptake (Hamilton, Hamilton & Zderic 2007, Thorp et al. 2011). There is an emerging body of evidence that also sedentary behavior is a risk factor for health, including mortality (Katzmarzyk et al. 2009, Katzmarzyk 2010). However, it is still not known if an inactive person will increase his/her risk for cardio-metabolic diseases even more by simply sitting too much (Hamilton, Hamilton & Zderic 2007). On the other hand, it has been found that exercise training may not decrease the daily sedentary time (Finni et al. 2012), and that excessive times spent doing sedentary activities may even weaken the effects of a substantial amount of exercise (Owen et al. 2010). Breaking up the daily sitting time is found to have beneficial effects on health outcomes (Dunstan, Thorp & Healy 2011) and can therefore be recommended for all office workers.

2.2 Assessment of the dose of physical activity

It is important to measure the dose of physical activity accurately if one wished to study the association, causality or dose-response between physical activity and health-related outcomes (Kesäniemi et al. 2001). The main aim of the physical activity studies is to produce a detailed physical activity recommendation for the maintenance of good health and to answer the ultimate question: how much physical activity is enough?

Physical activity can be assessed with subjective (self-reported) and/or objective methods (Figure 1). It is important to remember that physical activity (the behavior) is not the same as energy expenditure (the energy cost) of the behavior (Lamonte & Ainsworth 2001). In view of the health effects of daily activities such as stair climbing and bouts of exercise under 10 minutes, more sophisticated methods are needed to measure physical activity if one wishes to fully capture the true amount of daily activity (e.g. Murphy, Blair & Murtagh 2009). Epidemiological studies frequently estimate the amount of physical activity by using questionnaires (Neilson et al. 2008, Shephard 2003). Unfortunately physical activity questionnaires are prone to measurement error and bias due to mis-/overreporting (e.g. Troiano et al. 2008). Questionnaires are also problematic as they cannot capture the overall free-living physical activity by self-report although they can be used to study long-term physical activity (Helmerhorts et al. 2012).

		SUBJECTIVE *(Self-reported)*	OBJECTIVE
INDIRECT	ACTIVITY	Questionnaires, Surveys	Physical activity recordings
	Energy cost	MET-min/day	Oxygen uptake, Heart rate, Body temperature, Ventilation
DIRECT	ACTIVITY	Recalls, Diaries	Accelerometer, Pedometer
	Energy cost	MET-min/day	Calorimetry, Doubly labelled water

FIGURE 1 Chart of the assessment of physical activity (collected from Lagerros & Lagiou 2007, Vanhees et al. 2005, Warren et al. 2010, Shephard 2003, Bonomi & Westerterp 2012, Lamonte & Ainsworth 2001).

Voluntary physical activity can be categorized as occupational, leisure-time, daily and commuting (transportation) activities. Activity itself has four dimensions: type, intensity, duration, and frequency (Warren et al. 2010). The intensity of aerobic/endurance exercise can be expressed as the rate of energy expenditure e.g. millilitres per kilograms per minute of oxygen being consumed or in METs (metabolic equivalent tasks). The intensity of activity can also be expressed as the speed of activity (e.g. walking 6 kilometres per hour, jogging 10 kilometres per hour) or via a physiological response such as heart rate. The relative intensity can be expressed in several ways e.g. as percentage of person´s

aerobic capacity (%VO_{2max}) or as the percentage of maximum heart rate (%HR_{max}) or through subjective indices of the perceived exertion (such as the Borg scale). According to the latest American College of Sports Medicine Position Stand moderate intensity exercise is 46 to 63% of one´s VO_{2max} whereas vigorous intensity exercise is referred to 64 to 90% of one´s VO_{2max} (Garber et al. 2011). In other words, the threshold between light and moderate physical activity is around 40% of the heart rate reserve of a healthy non-sedentary adult (Garber et al. 2011). The intensity of strength training is frequently expressed as percentage of one repetition maximum (1 RM), e.g. 50-69% of the 1 RM for moderate intensity activity (American College of Sports Medicine 2009, Garber et al. 2011, Westcott 2012). The duration of one session can be expressed in either minutes or hours with the frequency usually being reported as times per day/week/month.

MET

The MET (metabolic equivalent task) value differentiates the intensity of the physical effort exerted during different types of activities. For an average adult, 1 MET is equal to resting energy expenditure, i.e., 1 kcal/kilogram of body weight/hour or to an average resting metabolic rate of 3.5 ml/kg/min. The energy cost of sedentary behavior (e.g. sitting, watching TV, driving) is considered to be under 1.5 METs, low (light) intensity physical activity 2.0 to 2.9 METs, moderate physical activity (such as brisk walking) 3.0 to 5.9 METs, and vigorous intensity activity over 6.0 METs (corresponding to ≥21 ml/kg/min) (Ainsworth et al. 2011, Ainsworth et al. 2000, Garber et al. 2011). The dose of activity is expressed as a sum-score of intensity (MET) x duration (min or h) x frequency of activity, e.g. MET-min/day or MET-h/week. For example, jogging at 7 METs for 30 minutes on 3 days/week equals to 630 MET-min/week (500 to 1000 MET-min/week is enough to meet the current physical activity recommendations) (e.g. Physical Activity Guidelines Advisory Committee 2008). However there might be problems related to the self-reported activity as age, sex and fitness level affect to the perception of its intensity. Therefore, it is possible that recommendation for moderate intensity activity, especially if given in an absolute number (like MET), would represent more strenuous activity for an elderly person with poor fitness than for a young and fit person. According to the latest American College of Sports Medicine Position Stand the term moderate intensity activity refers to a range between 4.8 and 7.1 METs for a young person (20-39 yrs), between 4.0 and 5.9 METs for middle-aged (40-64 yrs) and between 3.2 and 4.7 METs for older (>65 yrs) people (Garber et al. 2011). Therefore, it could be more useful to express moderate intensity activity as a relative value of one´s maximal capacity.

2.3 Principles of exercise training

Exercise is physical activity that is planned, structured and repetitive and purposive in the sense that the improvement or maintenance of one or more components of physical fitness is the objective. Thus, exercise training is leisure-time physical activity that improves physical fitness and it can have different goals, e.g. to increase muscle strength or size, to improve sport performance, to improve aerobic capacity or to improve body composition. In order to set a realistic goal for the training (program), the principles of exercise training need to be considered (Hoffman 2002, Campbell, Neil & Winters-Stone 2012). These are summarized in Table 1.

Each of the exercise sessions should be aimed to unbalance the normal body homeostasis so that improvements (compared to the baseline level) can be seen (*overload principle*). It is clear that training adaptations are different after aerobic vs. strength training (*specificity principle*). For example, for a non-exerciser, brisk walking is more beneficial than strength training as a way to increase cardiorespiratory fitness and to lose weight. If exercise sessions are not performed frequently or stopped, the gains achieved in exercise training (e.g. fitness enhancement) start to revert toward baseline levels (*reversibility principle*) (Hoffman 2002). Also the fact is that people will respond differently to similar training program (*individuality principle*).

TABLE 1 Summary of the training principles.

PRINCIPLE	**The basis of the principle**	**Training requirements**
OVERLOAD	The dose of activity is greater than what the individual is already doing	Expected improvements compared to baseline level are seen
SPECIFICITY	Training adaptations are specific to the type of exercise training	Strength training to increase muscle strength, aerobic training to increase cardiorespiratory fitness
REVERSIBILITY	When training stimulus is removed or reduced, the gains are lost	Regularity of exercise training
INDIVIDUALITY	Individuals might respond differently to the same training stimulus. Influenced by initial training status, genetic background and gender.	Individualized training programs
PROGRESSION	When body adapts to exercise, the dose of activity must be increased	Progressive training programs
DIMINISHING RETURNS	The degree of an improvement decreases as the individual become fitter	Progression, increasing effort and periodization for continued improvements

The table is modified from Campbell, Neil & Winters-Stone (2012).

Exercise training should be progressive if continued improvements are desired (*progression principle*). This is especially the case in sports. However, the degree of an improvement e.g. in fitness is high at the beginning of the exercise program but the fitter the individual becomes, the smaller are the further changes (*principle of diminishing returns*) (Hoffman 2002). Therefore increasing effort, including periodization of heavy, moderate and light phases, is needed. However, a continued progression is not needed in exercise for health but planned changes in exercise intensity or type (strength/endurance/skills) are beneficial for the training responses and this might increase the individual´s motivation.

2.4 Physical activity, fitness and health

Regular physical activity results with enhanced cardiorespiratory fitness in a dose-response manner (Wei et al. 1999, Church et al. 2007, Blair, Cheng & Holder 2001). There is a strong relationship between cardiorespiratory fitness and health. In the Swedish population, the odds ratio for having three or more CVD risk factors was 0.5 (0.25 to 0.99) for the persons with high physical activity but low cardiorespiratory fitness and 0.08 (0.04 to 0.16) for those individuals with high physical activity and high cardiorespiratory fitness when both were compared to individuals displaying the low physical activity and low cardiorespiratory fitness phenotype (Ekblom-Bak et al. 2010). A low cardiorespiratory fitness is found to be an independent risk factor for cardiovascular disease (Blair, Cheng & Holder 2001) and mortality (Blair et al. 1996, Kokkinos et al. 2008, Myers et al. 2002). Kokkinos et al. (2008) found that the mortality risk for exercise capacity of 7 to 10 METs (~24.5 to 35 ml/kg/min) was half of that of the exercise capacity under 5 METs (Kokkinos et al. 2008). The mortality risk has been shown to decrease by 12 to 20% for each MET-unit increase in fitness (Kokkinos & Myers 2010). In addition, a meta-analysis revealed that the minimal cardiorespiratory fitness level with which to achieve a significant prevention of all-cause mortality and coronary heart disease and cardiovascular disease risk was 7.9 METs (~27ml/kg/min) (Kodama et al. 2009). In the study of Sassen et al. (2009), physical activity was found to be inversely associated with clustering of metabolic abnormalities; the higher the intensity of physical activity, the stronger the effect of exercise on cardiovascular disease risk (Sassen et al. 2009).

VO_{2max}

Maximal oxygen uptake (VO_{2max}) is the maximal amount of oxygen which can be delivered to the peripheral organs, including the skeletal muscles, during peak muscular contraction. The VO_{2max} is a measure of cardiorespiratory fitness and can be at least theoretically regarded as an upper limit for the aerobic performance (Levine 2008). The age-related decrement in VO_{2max} is 1 to 2% per year (Tanaka & Seals 2008, Hakola et al. 2011). Also the peak exercise

performance starts to decline after 35 yrs of life and declines more dramatically after 50 to 60 years of age. The reduction in VO_{2max} is the primary mechanism causing the age-related reductions in endurance performance (Tanaka & Seals 2008). The Baltimore Longitudinal Study of Aging has shown that a decrease in VO_{2max} is inevitable with age among healthy adults, but the level of aerobic capacity remains higher among those who have exercised (Fleg et al. 2005).

The heritability (an estimate of the genetic influences on trait at the population level) of VO_{2max} has been found to be moderate (51% by Bouchard et al. 1998) as well as the heritability of the trainability of the VO_{2max} (47% by Bouchard et al. 1999). The responsiveness to exercise also seems to aggregate in families (Bouchard et al. 1994, Bouchard & Rankinen 2001, Bouchard 2012). In the HERITAGE Family Study of 742 healthy sedentary subjects, the average increase in VO_{2max} after a 20-week-long controlled endurance-training program was 384±202 ml, but the individual responses varied from "non-responders" (0 ml) to "high responders" (>1000 ml of oxygen per minute) (Bouchard et al. 1999, Bouchard & Rankinen 2001). Extensive plasticity in VO_{2max} was also found among healthy young untrained men after they participated in a 6-week-long aerobic (cycling) training program (Vollaard et al. 2009). Furthermore the changes in VO_{2max} did not correlate with the changes seen in aerobic capacity after the exercise intervention (Vollaard et al. 2009). On the other hand, a recent population based findings have indicated that even by training less than recommended but at a very high intensity may be beneficial for VO_{2peak} (Nes et al. 2012). Thus, the total amount of (leisure-time) physical activity does not always correlate to an individual´s VO_{2max} value (Talbot, Metter & Fleg 2000).

In summary, all physical activity is good for health even if cardiorespiratory fitness is not enhanced (as physical activity have also many other pathways to decrease disease risk, e.g. via lipid profile) but greater health benefits may be gained if cardiorespiratory fitness is increased by exercise training (including exercise at high intensity) (Sassen et al. 2009). For example, an inverse relationship between physical activity and metabolic syndrome has been found to be steeper among unfit persons (Franks et al. 2004).

2.5 Physical activity and metabolic health

Physical activity has both acute and chronic effects on cardio-metabolic risk factors (Thompson et al. 2001). Each (single) exercise session exerts a metabolic response but the health effects of the exercise are dependent on the frequency of the sessions (Haskell 1994). There is clear evidence that (aerobic) exercise acutely decreases blood pressure for up to 12-16h, reduces levels of triglycerides (TG) and increases those of high-density lipoprotein (HDL) for up to 72h, and enhances glucose control for up to 72h (Thompson et al. 2001). Thus, the acute effects of exercise are rather short-lived if they are not followed by another bout of exercise within 2 to 3 days. If the exercise sessions are repeated frequently, then the net health benefit of exercise is larger, including fitness enhancement.

Regular aerobic exercise has been shown to modestly (up to 20%) increase HDL levels but the chronic effects of exercise on TGs, low-density lipoprotein (LDL) levels or on total cholesterol are less clear (Kodama et al. 2007, Kelley, Kelley & Tran 2005, Tambalis et al. 2009). In the STRIDDE study, 11 to 16 km of regular weekly aerobic exercise was found to be sufficient to maintain a healthy lipid profile (Kraus & Slentz 2009). After a 6-month-long training period in STRIDDE, the acute improvements in HDL cholesterol levels as well as those in the HDL particle size and large HDL levels after training were sustained for at least two weeks after the intervention cessation among the subjects who had trained with high amounts and at high intensity (Slentz et al. 2007). The results from resistance training are more inconsistent although it has been found to exert beneficial effects on blood pressure and on the lipid profile (mainly LDL) (Tambalis et al. 2009, Strasser & Schobersberger 2011, Westcott 2012).

Regular physical activity has also been demonstrated to maintain glucose homeostasis (Boule et al. 2005, Hawley & Lessard 2008, Church et al. 2010). In the study of Mikus et al. (2012) a mere three days of dramatically lowered levels of activity were enough to increase postprandial glucose levels in young, active and healthy individuals (Mikus et al. 2012). Resistance training is also known to induce beneficial effects on skeletal muscle insulin sensitivity (Flack et al. 2010, Westcott 2012). Thus, results from the literature highlight the importance of regularity in exercise training in order to reduce the risk for chronic diseases via these biological mechanisms (Warburton, Nicol & Bredin 2006).

Heterogeneity of responses

There is considerable variability in the individual responses to a standard dose of activity (Bouchard & Rankinen 2001, Vollaard et al. 2009, Boule et al. 2005, King et al. 2009, Bouchard et al. 2012, Hautala et al. 2012, Hautala, Kiviniemi & Tulppo 2009, Karavirta et al. 2011). Genetics, eating habits (energy balance), the amount of non-exercise activities and the use of medications all influence an individual´s training response (Patel, Slentz & Kraus 2011). At present the most extensively individual training responses were studied in the HERITAGE Family Study among 742 healthy sedentary subjects and after a 20-week-long controlled endurance-training program (Bouchard et al. 1999). Inter-individual heterogeneity in responses to regular physical activity was found in blood pressure levels, HDL levels, fasting insulin levels, plasma triglycerides levels and in cardiorespiratory fitness levels (Bouchard & Rankinen 2001). Recently Bouchard et al. (2012) found that 7% of over 1500 adults experienced adverse responses to two or more risk factors after an exercise intervention (Bouchard et al. 2012). In summary, individual responsiveness reflects the normal biological diversity and while this may represent a goal it is also a challenge for personalized training programs (Bouchard 2012).

2.6 Physical activity vs. inactivity, body composition and muscle properties

2.6.1 Weight, fat mass and fat free mass

Observational cohort studies (Tammelin, Laitinen & Näyhä 2004, Parsons, Manor & Power 2006, Pietiläinen et al. 2008a) and longitudinal studies (e.g. Hankinson et al. 2010) have shown that if an individual is physically inactive or active in adolescence then this has a role in his/her obesity level at adulthood. People who are obese (body mass index greater than 30 kg/m^2) are clearly at an increased risk for suffering cardio-metabolic diseases (Poirier et al. 2006). According to the Nurses' health study population, inactive and obese women had 2.42 times higher, obese and active 1.91 times higher and lean but inactive 1.55 times higher risk of death compared to the lean and active women (Hu et al. 2004).

If one wishes to lose weight, energy expenditure must exceed energy intake. It is noteworthy that achievement of a positive energy balance is unfortunately all-too-easy in modern society (Hill et al. 2003). According to the WHO if no actions are taken, then soon 1.5 billion adults are going to weigh more than they should. Exercise training is a very important component in the maintenance of energy balance and thus in the prevention of (further) weight gain (Hill, Wyatt & Peters 2012). Moreover it seems that if one has a good cardiorespiratory fitness, then the health risks are lower at any degree of fatness (Ross & Janiszewski 2008).

Fat mass (and thus weight) tends to increase in middle-age (Hughes et al. 2001, Jackson et al. 2012). However, longitudinal studies have shown that physically active individuals are less likely to gain weight in middle-age as compared to the inactive subjects (Waller, Kaprio & Kujala 2008, Littman, Kristal & White 2005, Gordon-Larsen et al. 2009) but there are also conflicting results (Fogelholm & Kukkonen-Harjula 2000, Wareham, van Sluijs & Ekelund 2005). The current physical activity recommendation of weekly 150 minutes of moderate-intensity activity should be sufficient to prevent a significant gain of weight although the recommendation has not been made with the intention of weight loss (Donnelly et al. 2009). Thus, more activity is needed if the goal is to lose weight or to maintain weight loss (Donnelly et al. 2009, Hill & Wyatt 2005, Saris et al. 2003). The RCT study conducted by Slentz et al. (2004) suggested that an energy expenditure corresponding to 9.6 to 11.2 km of walking or jogging per week is needed in order to prevent further weight gain in sedentary overweight individuals (Slentz et al. 2004). In a prospective cohort study of middle-aged healthy women, 60 minutes per day of moderate-intensity activity was enough to either maintain weight or to result in a weight gain less than 2.3 kilograms after 13-year-long follow-up (Lee et al. 2010).

Exercise vs. diet

Although the role of exercise in the issue of weight loss is frequently questioned, it seems that any weight loss achieved by exercise (with or without caloric restriction) results in an improved metabolic status and better fitness (King et al. 2009, Larson-Meyer et al. 2010, Slentz, Houmard & Kraus 2009, Thompson et al. 2012). However, diet is a powerful means to lose weight/fat mass especially among highly obese persons who initially are not able to increase their exercise levels substantially (Wadden et al. 2012).

In the RCT study of Ross and co-workers (2000) slightly abdominally obese men were randomly assigned to control group, diet-induced weight loss group (-700 kcal/d), exercise-induced weight loss group (~700 kcal/d) and exercise without weight loss group (caloric compensation in order to maintain weight). Firstly, the weight loss achieved by either diet or exercise was similar in both groups (~8% of initial body weight). Secondly, both of the exercise groups improved their fitness by 16% with no fitness enhancements in the control or diet group (Ross et al. 2000). In a RCT study of the physiological effects of weight loss achieved by diet with or without exercise, 35 overweight men were randomly assigned to the control group, diet only group, diet and aerobic exercise group and diet with aerobic and strength training group (Kraemer et al. 1999). At the 12-week assessment, the weight loss among the three intervention groups was rather similar (~9kg from the initial weight) but the composition of the weight loss was different between the groups (Figure 2). In the diet alone group, the loss of fat free mass was significant. By combining exercise training with diet restriction, the loss of fat free mass was considerably lower than that of dieting alone. By combining both endurance and strength training with diet restriction more fat was lost and more fat free mass was preserved than in any other group. In addition, both endurance groups increased their fitness levels (Kraemer et al. 1999).

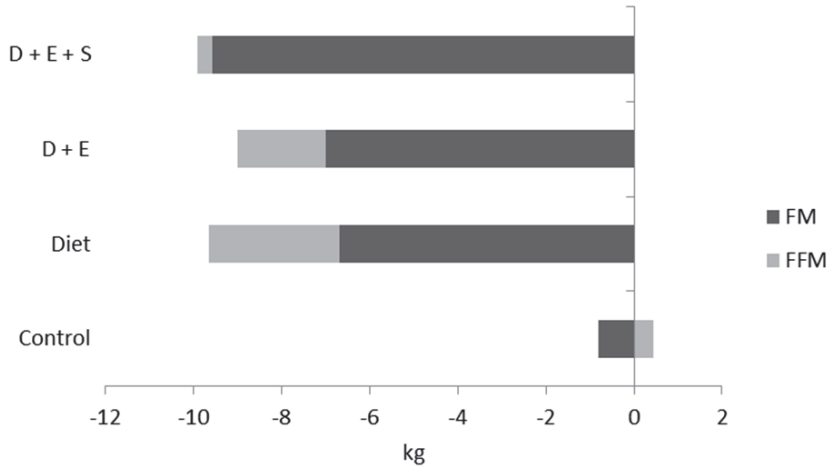

FIGURE 2 The effect of exercise training added to dietary regimen to the changes in fat mass (FM) and fat free mass (FFM). D, Diet; E, endurance training; S, strength training. Modified from Kraemer et al. (1999).

Results from other RCT studies have also shown that the maintained (or increased) lean mass by exercise could interfere with the expected weight changes (Slentz et al. 2004, Velthuis et al. 2009, Nicklas et al. 2009). Velthuis et al. (2009) reported the results of their RCT study that the 12-month exercise program combining endurance and strength training did not affect body weight but decreased fat and waist circumference and increased lean mass among initially sedentary postmenopausal women (Velthuis et al. 2009). In the RCT study of Slentz et al. (2004) three exercise groups (low amount/moderate intensity groups, low amount/vigorous intensity, and high amount/vigorous intensity) and a control group were studied after a 6-month-long aerobic exercise intervention (Slentz et al. 2004). A clear dose-response relationship between the amount of aerobic exercise and weight loss and fat mass loss but not with that of lean mass was found. In fact the vigorous exercise groups increased their lean body mass nearly twice as much as the controls (Slentz et al. 2004).

Overall it seems that the total amount of physical activity in exercise interventions could frequently be too small to induce changes in weight, which also might be due to the low adherence of the participants (Catenacci & Wyatt 2007). However, when diet and exercise induced energy deficits were matched to produce similar negative energy balances in a controlled study design (~500-700 kcal/day), the weight losses were similar (Ross et al. 2004, Ross et al. 2000, respectively). Thus, as recently also reviewed by Thompson et al. (2012), exercise can reduce masses of adipose tissue depots similar to that of caloric restriction if the exercise-induced energy deficit is the same as the one with energy restriction. Individual variations in exercise-induced weight/fat mass reductions are also believed to be due to the inter-individual differences in the amounts of non-exercise physical activity and/or concomitant changes in eating habits (caloric compensation) (Manthou et al. 2010, Thompson et al. 2012, Thomas et al. 2012).

The exercise-induced weight loss is not necessary in order to gain health benefits although the health benefits are greater when the body weight is lost (Ross & Janiszewski 2008, Janiszewski & Ross 2007). In an intervention study conducted by King et al. (2009) a 12-week-long supervised aerobic exercise in sedentary and obese participants achieved a mean weight loss of only one kilogram but it significantly increased aerobic capacity and lowered blood pressure, the length of waist circumference and resting heart rates (King et al. 2009). There is also evidence that the cardiorespiratory fitness could be an even more important cardio-protective factor than low adiposity (Janiszewski & Ross 2007). In other words, being fat and fit maybe better than being lean but unfit (Blair et al. 1995, Lee, Jackson & Blair 1998). However, although the risk for all-cause and cardiovascular mortality is found to be lower in fat but fit compared to lean and unfit persons, the risk for the incidence of type 2 diabetes and the prevalence of cardiovascular and type 2 diabetes risk factors is found to be high in overweight individuals irrespective of their fitness levels (Fogelholm 2010).

2.6.2 Visceral fat, liver fat and intramuscular fat

Characteristics of adipose tissue

The body weight starts to rise when the energy intake exceeds the energy expenditure for a period of time, yet, some individuals may also be genetically predisposed to have a high body weight (e.g. Bouchard & Tremblay 1997). The expansion of adipose tissue defines obesity as adipose tissue lipid content (fat mass) reflects energy balance, i.e., the balance between fat deposition and fat mobilization. Fat is an important substrate during prolonged, moderate exercise. The exercise-induced weight loss is mostly related to a reduction in adipocyte sizes (Thompson et al. 2012) as the number of adipocytes is determined at an early age (Spalding et al. 2008).

However, adipose tissue is not only a homogenous fat store. Evidence is accumulating that different compartments of adipose tissue have characteristics of their own and the link between obesity–related metabolic consequences are more related to the distribution of the body fat and to the activity of the excessive fat stores than to the amount of fat *per se* (Karelis 2008, Pajunen et al. 2011). Adipose tissue can be divided into two major depots: subcutaneous and internal adipose tissue (Shen et al. 2003). Internal adipose tissue can be further subdivided into visceral and non-visceral adipose tissue. The visceral adipose tissue is fat located within the chest, abdomen and pelvis. Nonvisceral adipose tissue is mostly found within a muscle (intramuscular) or perimuscular e.g. between the muscles (intermuscular) but also between muscle and bone (Shen et al. 2003). Subcutaneous adipose tissue can also be divided into superficial and deep layers; the superficial part acts as the "storage layer" and the deep layer is more metabolically active one (Wong, Janssen & Ross 2003).

Surprisingly adipose tissue might be the largest endocrine organ of obese individual that have a prominent role in the regulation of energy metabolism (Trayhurn 2007). The increased white adipose tissue mass secretes adipokines (such as leptin, adiponectin) which are linked to overall metabolic regulation and proinflammatory cytokines, like tumor necrosis factor (TNF-α), which are related to the metabolically triggered inflammation and insulin resistance (Trayhurn 2007, Hotamisligil 2006). In particular, the expanded visceral (also called intra-abdominal) adipose tissue is well recognized as a major contributor for the metabolic perturbations and increased cardio-metabolic risk (Despres & Lemieux 2006, Despres 2006). The visceral adipose tissue releases the free fatty acids and pro-inflammatory adipokines into the portal vein, referred to as "portal hypothesis" (Björntorp 1990). This might then induce the hepatic insulin resistance, hepatic steatosis and further hyperglycemia (Rector & Thyfault 2011).

The fact that visceral adipose tissue represents a rather small part of the total adipose but nonetheless may have a major contribution to the cardio-metabolic disease risk underlines the importance to avoid the expansion of this "high-risk" fat store. Therefore screening for people predisposed to metabolic syndrome, that is, they are abdominally obese and having several other cardio-metabolic risk factors, is an ongoing health mission (e.g. Cornier et al. 2011). One of the theories for the visceral fat accumulation is that the subcutaneous

"energy sink" overflows in the state of prolonged positive energy balance resulting also with the accumulation of ectopic fat in the liver, muscle, pancreas, heart tissues which are non-adipose tissues (Despres & Lemieux 2006). A good marker of the partitioning of fat to visceral (VAT) instead of subcutaneous depot (SAT) is the ratio between these two fat depots (VAT/SAT). Recently the VAT/SAT ratio was found to be an independent cardio-metabolic risk factor (Kaess et al. 2012). However the hazardousness of visceral fat has still not been differentiated from the risk related to total adiposity.

Findings from RCT studies

The evidence of the effects of different exercise amounts (vs. controls) on fat mass and fat distribution from RCT studies has been available for as long as 15 years (Ross & Janssen 1999, Ross & Janssen 2001). Due to the lack of use of imaging techniques, it was initially virtually impossible to study the dose-response relationship between physical activity and abdominal obesity (Ross & Janssen 1999, Snijder et al. 2006). One of the first RCT studies using imaging techniques to measure exercise-induced changes in intra-abdominal adipose tissue was conducted by Irwin et al. (2003). It was found that if previously sedentary and overweight postmenopausal women took approximately 200 min/week of moderate intensity exercise (such as brisk walking <3 METs for 12 months) this resulted in a minimal loss of weight (1.3 kg) but the loss of intra-abdominal fat was 8.5 g/cm^2 at the level of L4-L5 vertebrae disc. A dose-response relationship for greater body fat loss with increasing duration of exercise (when divided into tertiles according to the exercise adherence) was also found. The loss of intra-abdominal fat was 6.9% for the highest tertile (>195 min/wk) (Irwin et al. 2003). Ross et al. (2004) conducted a 14-week-long controlled trial by randomizing overweight premenopausal women into four groups: control, diet weight loss, exercise weight loss and exercise without weight loss. The average energy deficit in the weight loss groups (~500kcal/day) resulted in similar weight losses in both groups (~6% of initial body weight). The exercise weight loss group reduced total fat mass and abdominal (abdominal visceral and subcutaneous) fat more than the diet weight loss group (Figure 3). One noteworthy finding was that also the exercise without weight loss group reduced substantially their amounts of total and abdominal fat (Ross et al. 2004).

A 6-month-long RCT study conducted by Slentz et al. (2005) revealed a dose-response relationship between the amount exercise and visceral fat among sedentary overweight men and women (Slentz et al. 2005). The highest amount of activity in their RCT study groups was comparable to 32 km/week of vigorous activity and it resulted in a 6.9% reduction in visceral fat area. In the same study, a low amount of moderate (corresponding to 19.2 km/week 40-55% VO_{2max}) and a low amount of vigorous (corresponding to 19.2 km/week 65-80% VO_{2max}) activities were enough to prevent the visceral fat gain (Slentz et al. 2005).

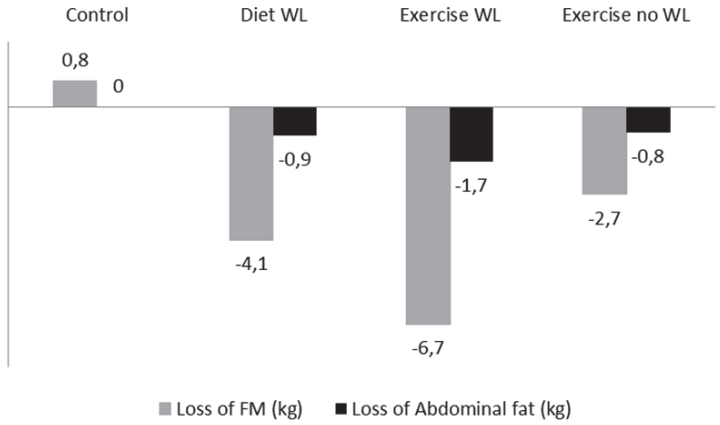

FIGURE 3 The effects of diet (-500 kcal/d) with weight loss and exercise (energy expenditure of 500 kcal/d) with or without weight loss on total and abdominal (visceral + subcutaneous) fat. FM, fat mass; WL weight loss. Adapted from Ross et al. (2004).

In their review Kay and Fiatarone Singh (2006) concluded that when imaging methods were used to measure abdominal fat in RCT studies, short-term exercise training (≥8 weeks) could reduce abdominal fat in overweight and obese subjects even in the absence of changes in body weight (Kay & Fiatarone Singh 2006). Janiszewski and Ross (2007) also concluded in their review that the weight loss was not necessary in order to gain health benefits as the abdominal and ectopic fat accumulation could be decreased by exercise even without weight loss (Janiszewski & Ross 2007). Further, Ohkawara and co-workers (2007) stated that at least 10 MET h/week of aerobic exercise (like brisk walking) was needed in order to significantly reduce visceral fat. They also found, that in healthy obese subjects there was a dose-response relationship between aerobic exercise and visceral fat reduction (Ohkawara et al. 2007). The visceral adipose tissue depot has been shown to be sensitive to even modest weight loss in overweight and obese persons (Ross et al. 2000, Chaston & Dixon 2008). Finally in a cohort study of 288 498 men and women aged 25 to 79 yrs physical activity predicted a lower waist circumference and this was independent of baseline or concomitant weight changes in normal-to-overweight individuals (Ekelund et al. 2011).

Liver fat

Rector and Thyfault (2011) have recently claimed that habitual physical inactivity, low fitness and overnutrition seems to be the actual causes for the development and progression of non-alcoholic fatty liver disease (NAFLD) (Rector & Thyfault 2011). NAFLD is characterized by increased hepatic triglyceride accumulation without heavy use of alcohol possibly due to inactivity-induced insulin resistance and, further, ectopic fat accumulation via increased hepatic free fatty acids uptake, activation of lipogenesis and

suppressed triglyceride export (Rector & Thyfault 2011). Hannukainen et al. (2007) have shown that hepatic free fatty acid uptake is lower among physically active, fit and lean monozygotic twins as compared to their less active and intra-abdominally obese co-twins (Hannukainen et al. 2007). Recently they also observed that the hepatic fat content was lower in physically active and fit twins compared to their inactive and unfit co-twins (Hannukainen et al. 2011). Perseghin and co-workers (2007) have shown that a lower hepatic fat content is associated with a higher level of habitual physical activity (Perseghin et al. 2007). Physical activity seems to affect to the liver fat content similarly as it does to visceral fat, perhaps even independently of visceral and weight reductions (Magkos 2010).

Intramuscular fat

Intramyocellular lipids (IMCL) can be distinguished from extramyocellular lipids (EMCL) by using an ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) technique which is a non-invasive way to measure the skeletal muscle fat content. IMCL is stored as intramuscular triglyceride in lipid droplets and used as energy during exercise. Magnetic resonance imaging and computed tomography are also non-invasive ways to quantify intramuscular fat; these techniques have a larger perspective than MRS but they cannot separate IMCL from EMCL (Schrauwen-Hinderling et al. 2006).

Prolonged disuse of muscles usually decreases metabolic flexibility (insulin action) and induces lipotoxicity, that is, muscles start to become filled with fat (Eckardt, Taube & Eckel 2011). Elevated IMCL stores have been associated with reduced skeletal muscle insulin sensitivity in sedentary, obese and insulin resistant subjects, thus, possibly reflecting an imbalance between the storage and the oxidation of the fatty acids (van Loon & Goodpaster 2006). There are many theories to explain the possible connection between the intramyocellular lipids and insulin resistance (such as lipid droplet size, level of physical activity, training status, oxidative capacity, muscle fiber type distribution, lipid-derived metabolites (Taube, Eckardt & Eckel 2009), mitochondrial (dys)function (Petersen et al. 2003), incomplete β-oxidation), but the underlying mechanisms are still not yet fully understood (Eckardt, Taube & Eckel 2011, van Loon & Goodpaster 2006, Goodpaster et al. 2001a, Goodpaster & Brown 2005, Dube et al. 2008). Moreover, there are findings that endurance trained muscle, which are known to be highly insulin sensitive, can also be characterized by their elevated IMCL stores (Goodpaster et al. 2001a) and that obese and previously sedentary older adults can increase their IMCL content by exercising (Dube et al. 2008). Therefore it can be concluded that it is not the amount of IMCL *per se* that interferes with the insulin-signaling pathway, but instead, it is the deranged lipid metabolites (producing compounds such as diacylglycerol and ceramide) which might be the interfering factors (Taube, Eckardt & Eckel 2009, Goodpaster & Brown 2005).

There are few studies examining extramyocellular lipids and glucose homeostasis (Taube, Eckardt & Eckel 2009). Goodpaster and coworkers found

that it is possible to separate different thigh adipose tissue depots by computed tomography and that the intermuscular adipose tissue was increased in obesity and that this was related to insulin resistance (Goodpaster, Thaete & Kelley 2000). Four weeks of reduced physical activity by unilateral limb suspension led to a significant increase in intramuscular fat measured by magnetic resonance imaging (Manini et al. 2007). In a 1-year trial, middle aged men and women were randomly assigned to exercise, diet, or control group (Murphy et al. 2012). The results indicated that the exercise group lost twice as much visceral and intramuscular fat (adjusted for whole body fat mass reduction) than the diet group although the energy deficit was similar in both intervention groups. However, Murphy and coworkers could not find any relationship between changes in intramuscular fat and glucoregulation (Murphy et al. 2012).

2.6.3 Muscle mass, strength and quality

In humans, the amount of muscle tissue starts to decline around the fifth decade of life (Hughes et al. 2001, Jackson et al. 2012). The age-related loss of muscle mass (sarcopenia) could be due to the loss of muscle fiber number and size and motor units, thus, leading to decreased muscle strength, power, quality (force/contracting unit), and endurance capacity (Deschenes 2004, Faulkner et al. 2007). The age-related loss of muscle mass is around 0.2 kg/year after 30 years of age and 0.4 kg/year after 50 years of age (Westcott 2012). Decline in muscle strength exceeds the decline in muscle mass (Visser et al. 2005, Goodpaster et al. 2006, Hughes et al. 2001).

Regular physical activity can not only preserve but also increase skeletal muscle mass and strength at all ages (Physical Activity Guidelines Advisory Committee 2008). According to a recent meta-analysis, resistance training (20.5 weeks) can achieve an 1 kilogram increase in lean body mass among adults aged 50 yrs or more (Peterson, Sen & Gordon 2011). The maintenance of muscle properties has notable benefit for the prevention of age-related disability (Rantanen et al. 1999, Janssen 2006). In prospective cohort studies, good muscle strength is also found to associate with lower mortality (Newman et al. 2006, Ruiz et al. 2008). Moreover, exercise-induced positive changes in skeletal muscle structure, function and metabolism have been linked to a delay in the progression of multiple diseases (Wolfe 2006, Kujala 2009, Garber et al. 2011). Importantly, the loss of skeletal muscle, the primary site for glucose and triglyceride disposal, may impair the glucose metabolism with health consequences (Westcott 2012).

The results from the cohort study of Health, Aging and Body Composition (ABC) conducted in older sedentary adults aged 70 to 79 yrs have shown that although the correlation between muscle mass loss and strength decline is not very high nonetheless the loss of muscle strength and quality with age does seem to be the major contributor for the risk of disability and mortality (Hughes et al. 2001, Newman et al. 2006, Goodpaster et al. 2006, Visser et al. 2005, Visser et al. 2002). Furthermore, muscle fat infiltration (measured by muscle attenuation) has been found to contribute towards the poor muscle quality,

strength and function among older sedentary adults (Goodpaster et al. 2001b) but also among young adult after a single leg immobilization (Manini et al. 2007). Longitudinal studies have also shown that muscle fat infiltration increases with age and is associated with muscle strength loss and mobility limitations (Visser et al. 2005, Delmonico et al. 2009).

According to cross-sectional (Tarpenning et al. 2004, Wroblewski et al. 2011) and RCT studies (Sipilä & Suominen 1995, Goodpaster et al. 2008) physical activity seems to be an efficient way to slow down the loss of muscle strength and muscle atrophy from middle to old age. Good fitness at old age has also been shown associate with higher lean mass and strength (Koster et al. 2010). In a longitudinal study, Frontera et al. (2008) revealed that the force-generating capacity of a single muscle fiber could be maintained into very old age despite the loss of muscle size or strength (Frontera et al. 2008). The elite athletes are the very best evidence of the power of an active lifestyle in the maintenance of the "machinery" (Tanaka & Seals 2008, Tarpenning et al. 2004, Wroblewski et al. 2011, Louis et al. 2009). For example the cross-sectional study done with 40 high-level endurance elite athletes showed that muscle mass can be maintained and muscle fat infiltration can be protected by long-term exercise despite the increase in body fat mass (Wroblewski et al. 2011).

2.7 Exercise-induced changes in gene expression

Exercise training is intended to unbalance body homeostasis regularly so that different molecular responses are initiated that lead to training adaptations and phenotypic changes (Coffey & Hawley 2007). Gene expression levels are widely used to describe exercise-induced changes at the molecular level (Mahoney et al. 2005).

Training adaptations can be viewed as merely an accumulation of specific proteins. A single bout of exercise leads to increased mRNA levels of metabolically related proteins but the altered gene expression that triggers these changes in protein concentration is of major importance for any subsequent training adaptation (Coffey & Hawley 2007, Pilegaard et al. 2000). Thus changes in the gene expression are required before one observes phenotypic changes.

The signaling-transcription network mediates exercise-induced skeletal muscle adaptations. Muscle contractile activity or endurance training activates a wide variety of protein phosphatase and kinases, which in turn regulate transcriptional factors, coactivators and repressors in the control of i) contractile protein genes in fiber type transformation, ii) mitochondrial genes in mitochondrial biogenesis and iii) angiogenic growth factor genes in angiogenesis (Yan et al. 2011). Numerous experimental findings have shown that e.g. coactivator PGC-1α mediates and coordinates gene regulation during endurance training-induced skeletal muscle adaptation including mitochondrial metabolic proteins (mitochondrial biogenesis) (Olesen, Kiilerich & Pilegaard 2010) fitting with the concept that endurance type of training is

known to result in an enhanced oxidative capacity of the muscles (e.g. Holloszy & Coyle 1984).

Similarly, adipose tissue is known to respond to acute exercise in several ways (increased adipose tissue blood flow, lipolysis i.e. fat mobilization and secretion of IL-6). If exercise training is sufficiently regular, these acute changes persist for longer after each bout of exercise and form part of the training response (Thompson et al. 2012). It is important to clarify the mechanisms involved in regulating these processes (e.g. lipolysis) since they represent also potential drug targets.

3 AIMS OF THE STUDY

A phenotype characterized by high physical activity and/or aerobic fitness predicts low cardio-metabolic morbidity and mortality more strongly than any other known biological risk factor (Myers et al. 2002, Kujala et al. 2003, Kujala 2009, Kodama et al. 2009, Blair et al. 1995). The associations between physical activity and health seem to be explained mechanistically by the interactions between a complex network of pathways, including systemic level changes in fitness, body composition, fat distribution and cardio-metabolic risk factor levels (Kujala 2009, Warburton, Nicol & Bredin 2006).

To study the associations between long-term leisure-time physical activity vs. inactivity and health outcomes in a design in which the genetic background could also be standardized, 16 middle-aged and older twin pairs (of which seven were MZ pairs) with an adult-life-long discordance in their leisure-time physical activity habits were identified from the Finnish Twin Cohort. Discordance was initially defined in 1975 and the same co-twin remained significantly more active during the 32-year-long follow-up.

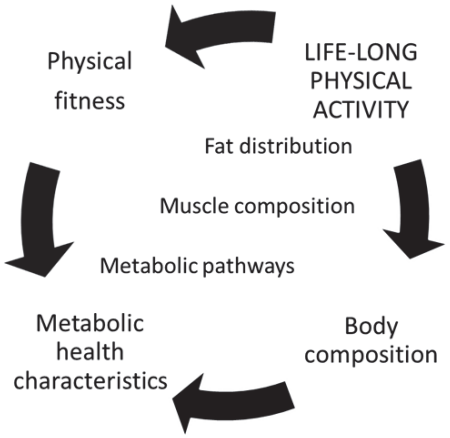

FIGURE 4 The main outcomes of the study.

The main aim was to study the associations of habitual physical activity vs. inactivity on physical fitness, body composition, fat distribution and metabolic health characteristics among the active vs. inactive co-twins of twin pairs (Figure 4). The hypothesis was that long-term physical activity could be associated with better health characteristics when compared to long-term inactivity. By studying discordant monozygotic twin pairs, it was possible to control for the genetic background and the childhood environment.

The specific research questions are:

1. Is long-term leisure-time physical activity associated with good fitness? (Article I)
2. Is long-term leisure-time physical activity associated with healthy body composition and fat distribution? (Article II)
3. Is long-term leisure-time physical activity associated with maintained muscle strength and composition? (Article IV)
4. What is the association of lifelong physical activity vs. inactivity with metabolic pathway regulation and cardio-metabolic risk? (Article III)

4 PARTICIPANTS AND METHODS

4.1 Twin pairs

The selection of physical activity discordant twin pairs fit the TWINACTIVE study was carried out in two waves. Both waves included three assessments; baseline identification based on the questionnaire data of leisure-time physical activity in 1975, 1981; retrospective follow-up physical activity interview (conducted in 2005, 2000, 1995, 1990, 1985 and 1980); and physical activity questionnaires and interviews at the laboratory visit carried out in 2007. The second wave of this study was conducted in order to increase the number of physical activity discordant MZ twin pairs. Therefore the early identification criteria needed to be somewhat different for this subgroup of MZ pairs as they had not been included in the first wave selection. The same physical activity questionnaires where used in both waves. A detailed description of the selection of twin pairs is given below. A summary of the selection process is illustrated in Figure 5.

4.1.1 Early identification of physical activity discordant twin pairs

The Finnish Twin Cohort includes all same-sex twin pairs born in Finland before 1958, and with both co-twins alive in 1967 (Kaprio & Koskenvuo 2002). In 1981, the cohort comprised of 5663 healthy same-sex twin pairs (1772 MZ and 3551 DZ, age limits 24 to 60 yrs) (Kujala, Kaprio & Koskenvuo 2002). Within these 5663 twin pairs, 146 pairs were found to be discordant for leisure-time physical activity both in terms of volume of the activity and in the participation in vigorous activity according to the physical activity questionnaires done in 1975 and 1981. The leisure-time physical activity volume was quantified as a metabolic equivalent task (MET index). The MET index was calculated as intensity (MET) x duration (h) x frequency (per day) of an average leisure-time activity and expressed as the sum-score of MET hours/day. The assessment of vigorous physical activity was based on the following question: Is

your physical activity during leisure-time about as strenuous on average as: 1) walking, 2) alternately walking and jogging, 3) jogging, 4) running. Those who chose 2, 3 or 4 were classified as engaging in vigorous activity (Kujala et al. 1998). Only those twin pairs in which the other twin did vigorous leisure-time activity equal or higher to a volume of 2 MET hours/day and his or her co-twin engaged in activity of less than 2 MET hours/day were selected (total 146 pairs) (Kujala, Kaprio & Koskenvuo 2002, Waller, Kaprio & Kujala 2008).

4.1.2 Follow-up interviews on leisure-time physical activity

A retrospective assessment

In 2005, a telephone interview for the group of 146 physical activity discordant twin pairs was carried out. The interview was conducted in 111 twin pairs (222 subjects) as only those pairs were included in which both twins were still alive, both were known to be living in Finland, and both spoke Finnish as their mother tongue. The telephone interview included questions on current and past leisure-time physical activity. The physical activity level was assessed by two sets of questions. The first was a shorter retrospective assessment of physical activity volume and participation in vigorous physical activity and it was conducted by using the same physical activity questions as in questionnaires from 1975 and 1981 (Kujala, Kaprio & Koskenvuo 2002, Waller, Kaprio & Kujala 2008). The past physical activity was assessed at five year intervals covering the years 1980, 1985, 1990, 1995, 2000 and 2005. To increase their recall, twins were asked their marital and work status for each year before the physical activity questions. The shorter questionnaire is presented in Table 2. The leisure-time activity was calculated by: ((frequency x duration x intensity)/60min)/30 days) and commuting activity as: ((frequency as five times per week x duration x intensity of 4 METs)/60min)/7days). The overall leisure-time MET index was a sum-score of leisure-time activity and commuting activity.

A previous 12-month assessment

The second set of questions asked in the retrospective follow-up interview was a detailed assessment of the volume of leisure-time, daily (non-exercise activities such as gardening, berry picking and repairing) and commuting activity over the previous 12 months using a modified version of the Kuopio Ischemic Heart Disease Risk Factor Study Questionnaire (Lakka & Salonen 1997). The modification refers here to an update of the list of activities included in the questionnaire. This questionnaire presented a 20-item list of different physical activity types including leisure-time (e.g. running, skiing, and swimming), daily (e.g. gardening, wood works), commuting activity (walking or cycling) and "other" physical activities specified by the responder. The twins reported monthly frequency and duration of each physical activity session covering the previous 12 months. They also reported the average intensity of activity sessions on a scale from 1 to 4: 1=recreational, outdoor activities that do not make you breathless or sweating, 2=conditioning exercise that makes you breathless but not sweating, 3=brisk conditioning exercise that makes you

breathless and sometimes sweating, 4=competitive, strenuous exercise that makes you breathless and sweating extensively. Each of the self-rated physical activity intensities were transferred into MET (metabolic equivalent task) values (Ainsworth et al. 2000, Lakka & Salonen 1997). The average duration per exercise session in each activity was also reported in order to calculate the overall dose of activity (MET x average duration x frequency, MET h/day). The overall dose of physical activity during the past 12-month was calculated by summing the leisure-time, daily and commuting activities.

TABLE 2 A short retrospective assessment of leisure-time and commuting activities.

How long does the physical activity last at one session on average?
a) less than 15 minutes (coded as 7 min)
b) 15 min to less than 30 min (22 min)
c) 30 min to less than 1 hour (45 min)
d) 1 hour to less than 2 hours (90 min)
e) over two hours (150 min)
Presently how many times per month do you engage in physical activity during your leisure time?
a) less than once a month (coded as 0.5 times per month)
b) 1 to 2 times per month (1.5)
c) 3 to 5 times per month (4)
d) 6 to 10 times per month (8)
e) 11 to 19 times per month (15)
f) more than 20 times per month (25)
Which of the alternatives would describe the best of the intensity of your average leisure time activity?
a) walking (coded as 4 MET/h)
b) alternatively walking and jogging (6 MET/h)
c) jogging (10 MET/h)
d) running (13 MET/h)
How much of your daily journey to work is spent in walking, cycling, running and/or cross-country skiing?
a) less than 15 min (coded as 7 min)
b) 15 min to less than an half an hour (22 min)
c) half an hour to less than an hour (45 min)
d) hour or more (75 min)
e) I am presently not at work (0 min)

Invited twin pairs

A total of 89 twin pairs (17 MZ, 72 DZ pairs) completed all the physical activity questions in the follow-up interviews. Of these 89 pairs, 42 pairs (5 MZ, 37 DZ pairs) had been consistently discordant for physical activity at every time point across the 30-year period (Waller, Kaprio & Kujala 2008). From the 37 consistently discordant DZ twin pairs, 16 DZ pairs, whose mean MET difference between the inactive and active co-twin of twin pair during the 30-

year period was as high as possible (9.84 MET h/day on average, 4.59 MET h/day as a minimum) were suitable for the TWINACTIVE study. Of these 16 DZ pairs, 14 DZ pairs were invited to the study measurements since two pairs had to be excluded due to Alzheimer disease and disability among one of the co-twins of the twin pairs. Four out of the five consistently discordant MZ pairs were invited as one pair had to be excluded due to rheumatoid arthritis and prednisolon medication use. Among the 47 pairs, who were not consistently discordant for thirty years, 3 MZ twin pairs, who were discordant for physical activity at least in four out of the six time points (mean MET difference 4.37 MET h/day on average), and with whom the physical activity discordance was seen in the detailed assessment of leisure-time physical activity volume over the previous 12 months were selected. Thus, overall 7 MZ and 14 DZ twin pairs discordant for physical activity habits were invited to the TWINACTIVE study measurements out of the 146 baseline discordant twin pairs.

4.1.3 Follow-up interviews for a sub-group of MZ pairs

To further increase the number of monozygotic twin pairs, an extra 151 MZ twin pairs who were discordant for the volume of leisure-time physical activity by 2 MET h/day or more both in 1975 and 1981 were selected from the original Finnish Twin Cohort. Among these 151 MZ pairs, 19 pairs were discordant for the volume of leisure-time physical activity such that the MET index difference between co-twins was at least 3 MET h/day in both 1975 and 1981, and the participation in vigorous activity was the same in both co-twins or greater in the active vs. inactive co-twin. They were interviewed by telephone and asked similar questions about their current and past physical activity as done in 2005. Two pairs had to be excluded from the interviews as one of the co-twins had died, and one pair had been excluded from the Finnish Twin Cohort follow-up. Of the interviewed 16 MZ pairs, 3 MZ pairs were consistently discordant for physical activity at all time points across the follow-up period (mean MET difference being 18.39 MET h/day on average). However, only two of these MZ pairs were invited as one pair had to be excluded due to missing information. In addition, 6 MZ pairs were discordant for physical activity at least in four of the six time points (mean MET difference 7.58 MET h/day on average), and the discordance was also present over the previous 12 months. However, only three pairs were invited as one elderly pair had to be excluded because in one case both co-twins were taking several pharmaceuticals and two pairs were excluded because one of the co-twins had cancer. Thus five extra MZ pairs from this later sub-group selection were invited to participate in the TWINACTIVE study measurements.

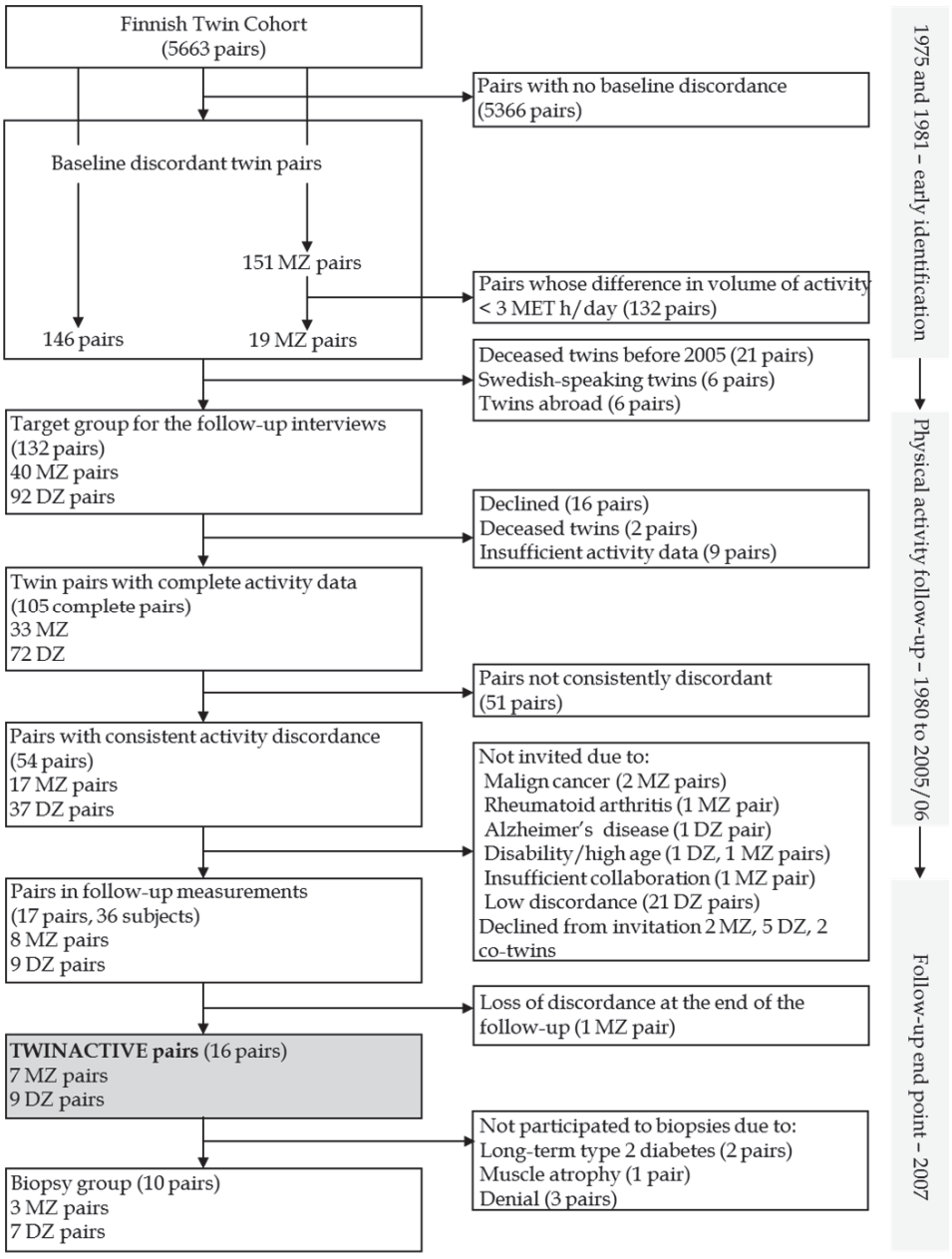

FIGURE 5 Summary of the selection of the twin pairs discordant for physical activity and the timeline of the recruitment (on the right).

4.1.4 TWINACTIVE pairs

After the comprehensive selection of the consistently discordant twin pairs, a total of 12 MZ and 14 DZ pairs having at least 30-year-long discordance in leisure-time physical activity habits received an invitation to the TWINACTIVE study measurements. Of these invited 26 pairs, 8 MZ and 9 DZ twin pairs (overall 34 co-twins and two twins without a co-twin) went through our detailed examinations related to health. Two MZ pairs, 5 DZ pairs and two co-twins from different pairs declined to participate in study measurements. The data from two twins (without their co-twins) was not usable for the study purposes. After careful intrapair examination of the leisure-time MET indices at the end of the follow-up, it was noted that in one MZ pair, the MET indices were higher among the previously inactive twin and lower among his previously active co-twin and thus this pair was excluded from the data analysis.

A total of 7 MZ (5 male and 2 female pairs) and 9 DZ pairs (6 male and 3 female pairs) fulfilled the discordance criteria, that is, baseline discordance with cut-off point of 2 MET h/day (3 MET difference in activity habits among the subgroup of MZ pairs); discordance in physical activity habits at all time points of the follow-up (discordance in at least 4 out of the six time points in the subgroup of MZ pairs) and having the highest possible intrapair difference in physical activity habits during the follow-up; finally, discordance in physical activity habits in the laboratory measurements and during the previous 12 months.

4.2 Measurements

4.2.1 The study timetable

Those pairs that agreed to participate in the study were advised not to change eating or physical activity habits before the measurements. Structured instructions were mailed two weeks before laboratory visit(s) arranged in Jyväskylä, Finland. The subjects were also advised not to exercise vigorously during the two days before their laboratory visits. The first laboratory visit lasted for two days. The timetable of the measurements is presented in Table 3.

At the end of the first visit, the subjects were informed of the possibility to participate in a second laboratory visit (muscle and fat biopsies). Ten pairs (3 MZ and 7 DZ) out of the 16 pairs further agreed to participate in the biopsies (see also Figure 5). Muscle and fat biopsies were not taken from the remaining pairs because of the following reasons: type 2 diabetes longer than 15 years (2 pairs), muscle atrophy in MRI (1 pair), and refusal (3 pairs). The second visit to the laboratory took place one month after the first visit.

TABLE 3 Timetable of the TWINACTIVE study measurements.

VISIT 1	
Day 1	
11 am	Blood sample
12 pm	Standardized interviews (smoking, use of alcohol, diet and motivation) (I)
1 pm	Echocardiography
1.45 pm	Standardized clinical examination (medication and health status)
2.15 pm	Resting electrocardiography
2.30 pm	Symptom-limited clinical exercise test (I)
3.15 pm	Maximal isometric left knee extensor strength, left hand grip strength (IV)
4 pm	Peripheral quantitative computed tomography (bone properties)
10 pm	Fasting begins
Day 2	
7.30 am	Anthropometric measurements, body composition by BIA (II)
8 am	Fasting blood and DNA samples (III, IV)
8.15 am	Oral glucose tolerance test
9 am	Standardized physical activity interviews (I)
12 pm	MRI (abdomen and thigh region) (II), angiography of macroscopic arteries
VISIT 2	
Overnight fast	
8-10 am	Muscle and subcutaneous adipose tissue biopsies (III)

4.2.2 Physical activity assessment

The volume of physical activity (MET index) was assessed with the same questions as used at the baseline and in follow-up assessments (see Table 2). The 12-month MET index was assessed by the questionnaire of leisure-time, daily and commuting activity during the previous 12 months as used in the telephone interviews (described in chapter 4.1.2). The two questionnaires on the volume of leisure-time physical activity (the retrospective vs. previous 12-month assessment) showed a good correlation in the present study population ($r=0.73$, $p<0.001$, $n=36$). Previously in the study of Waller et al. (2008), the intra-class correlation between the shorter MET index and the detailed 12-month physical activity MET index was 0.68 ($p<0.001$) for leisure-time physical activity and 0.93 ($p<0.001$) for commuting to and from work (Waller, Kaprio & Kujala 2008).

4.2.3 Fitness assessment

Symptom-limited maximal clinical exercise test with cycle ergometer was performed for the assessment of cardiorespiratory fitness using a slightly modified WHO protocol (Lange-Andersen et al. 1971). The testing protocol comprised of 2-minute stages beginning by learning stage at 20 W and warm-up stage at 25 W. Thereafter the increase in the workload was 25 W/stage. Recovery stage was performed at 25 W lasting for a period at least five minutes. At the end of each stage, heart rate was recorded from the electrocardiogram,

blood pressure was measured and subjective rating of perceived exertion (Borg's scale 6-20) was enquired. During the recovery stage, these measurements were performed at the end of 1, 3 and 5 minutes.

All subjects attended to the exercise test. The maximal oxygen uptake was not measured during the symptom-limited clinical exercise tests. The peak oxygen uptake, VO_{2peak} (ml/kg/min), was therefore evaluated from the time weighted peak load using formula (11.016 x peak load/ body weight) + 7 (American College of Sports Medicine, 2000). The correlation between follow-up mean MET index and estimated VO_{2peak} was 0.63 ($p<0.001$).

4.2.4 Strength measurements

Maximal isometric left knee extensor force was measured in a sitting position using an adjustable dynamometer chair (Good Strength, Metitur, Palokka, Finland) (Sipilä et al. 1996). The left knee was set at an angle of 60° from full extension. The ankle was attached by a belt above the malleolus to a strain-gauge system. After familiarization, the subject was advised to produce the maximal force as rapidly as possible. Overall four (4) maximal efforts separated with a 30 second pause were conducted. The best performance was accepted as a result. The knee extensor force was measured from 27 twins (13 complete pairs). Twins having multiple diseases (n=2), long-standing diabetes (n=2) and polio (n=1) were excluded.

The left maximal handgrip force was measured with an elbow flexed at 90° using the same adjustable dynamometer chair and the same protocol as described above. The left hand grip force was measured from 31 twins (15 complete pairs) excluding the twin with polio which affected the left hand.

4.2.5 Anthropometrics and body composition measurement

Height was measured to the nearest 0.5 cm. Body mass was measured with a digital scale with the participants wearing underwear and recorded to the nearest 100 g. Body mass index (kilograms per square meter) was calculated. Waist circumference was measured between lower rib and iliac crest, and it was recorded to the nearest half centimeter. Hip circumference was measured at the widest part of the pelvis and recorded to the nearest half centimeter. Three measurements for waist and hip were performed and the average value was used as the result. The waist-to-hip ratio and waist-to-height ratio were calculated.

Body composition was determined using InBody (720) (Biospace Co., Seoul, Korea) multifrequency eight-polar impedance plethysmograph body composition analyzer with the subjects wearing only undergarments and having had a ten-hour fast. The precision of repeated measurements in our laboratory is on average 0.3% (coefficient of variation) for FM% (Völgyi et al. 2008). Lookin'Body software (Biospace) was used for the output measures.

4.2.6 Abdominal MRI

T_1-weighted magnetic resonance imaging (MRI) axial scans were acquired from the abdomen with 1.5 T GE-Signa Exite HD CVi with a torso phase-array coil. Acquisition was made with matrix of 256 x 192, field of view of 40 x 30 cm, and gradient echo sequence with a repetition time/echo time (TR/TE) of 150/2.16 ms for out-phase and 150/4.97 for in-phase (FSPGR PulsSeg.) flip angle being 90 degrees in both occasions. The protocol involved acquisition of 32 axial images of 10 mm thickness and at 12 mm intervals from the abdomen region with the subject in a supine position. The subject held his/her breath for 15 seconds to reduce the effects of respiratory motion on image quality. The acquisition was done in all subjects.

Single slice analysis
A single T_1-weighted out-phase slice from the level of five centimeters above the L4-L5 level was used for the detailed measures of visceral and subcutaneous adipose tissue areas (Shen et al. 2004, Shen et al. 2007). Manual delineation was used to draw boundaries around the subcutaneous and visceral adipose tissue regions using OsiriX software (available at http://www.osirix-viewer.com/). In this study, the term visceral adipose tissue (VAT or visceral fat) is used to refer to the area that covers both intraperitoneal and extraperitoneal regions. The subcutaneous adipose tissue (SAT or abdominal subcutaneous fat) is the fat depot between skin and muscle fascia (Figure 6).

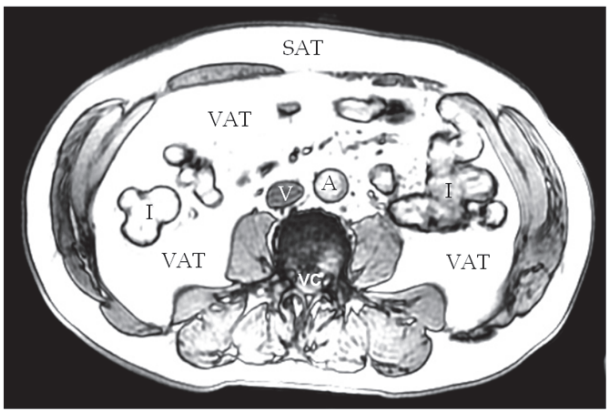

FIGURE 6 Differentiation of visceral adipose tissue area and subcutaneous adipose tissue area from the single slice image. SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; I, intestinal tract; A, arteria; V, vena.

Liver fat score
The liver fat score was calculated from a single slice image as a difference in mean signal intensity between in-phase (water +, fat +) and out-phase (water +, fat -) images (Fishbein et al. 1997). The single slice image was selected from the same or near to the same level in each subject. Overall six regions of interest

(ROIs), at size of 1cm^2, was drawn and placed in the liver parenchyma to the following segments: lobus caudatus (segment I), superior subsegment of lateral segment of left lobe (II), left medial segment of left lobe (IV), superior subsegment of anterior segment of right lobe (VIII), and superior subsegment of posterior segment of right lobe (VII) (Gazelle & Haaga 1992) using OsiriX software. The reference ROI was placed to spleen. The locations of the ROIs were the same in- and out-phase images. The same ROIs, as shown in figure 7, were used in all subjects. The place of an ROI was minimally changed only if there were vessels passing through the ROI. The fat fraction derived from the difference between the average pixel signal intensity of all the ROIs in in- and out-phase images were referred to the liver fat score. Magnetic resonance imaging has been shown to be a reliable method to assess liver fat content when compared to magnetic resonance spectroscopy ($r=0.851$, $p<0.001$) (Chan et al. 2006).

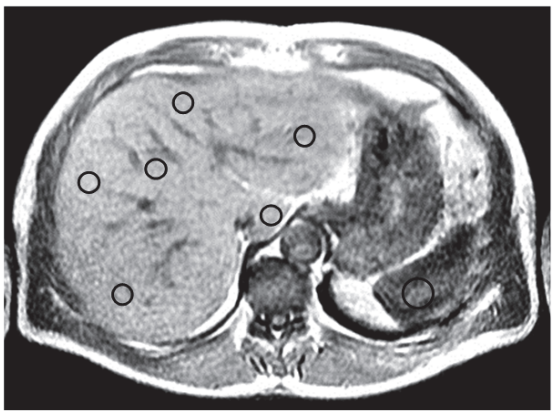

FIGURE 7 Localization of the liver fat ROIs in the MR image.

4.2.7 Midthigh MRI

Nine axial T_1-weighted MR-images of 10 mm thickness and with a 20-mm slice interval were acquired from the left midthigh using 1.5 T GE-Signa Exite HD CVi with a matrix of 384 x 256, field of view 40 x 28 cm, and a gradient echo sequence with TR/TE 550/15.2 ms (FSE-XL PulsSeg.). The midslice (5th image) was positioned at the midpoint lengthwise of the left femur. The midpoint of the femur was skin marked using the greater trochanter and lateral joint line of the knee as anatomical landmarks.

Single slice analysis
The midslice was used for the detailed analyses of the total midthigh cross-sectional area, muscle cross-sectional area, the intramuscular (extramyocellular) and subcutaneous fat areas (Figure 8). Segmentation was made manually using OsiriX software. Muscle tissue was detected by setting a density threshold and using automatic segmentation parameters. The midthigh volume was

calculated from the part of the thigh covered by all nine slices (lengthwise 16 cm) using automatic volume calculation. Fifteen complete pairs were included in the MRI analysis because one co-twin was not imaged. Intramuscular adipose tissue area from midthigh has been shown to display high correlations (r=0.60-0.72) with total intramuscular adipose tissue volume (Ruan et al. 2007).

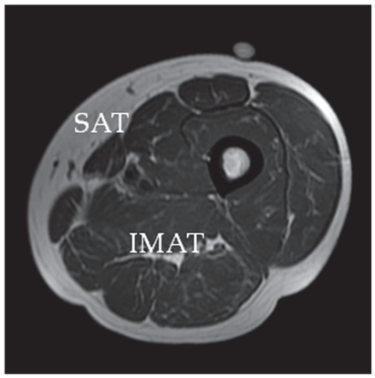

FIGURE 8 Marked left midthigh image. SAT, subcutaneous adipose tissue; IMAT, intramuscular (extramyocellular) adipose tissue (white stripes).

4.2.8 Muscle and fat tissue biopsies

Muscle and fat tissue samples were taken after an overnight fast between 8 am and 10 am. The subjects were advised not to exercise intensively during the preceding days and they were advised not to exercise except for slow walking to the laboratory in the morning before the samples were taken.

Needle muscle biopsies were taken after cooling and disinfection of the skin under local anaesthesia (2 ml lidocaine 10 mg/ml with adrenaline 10µg/ml, Orion, Finland). The muscle biopsy was taken from the mid-part of *m. vastus lateralis*, defined as the midpoint between the greater trochanter and the lateral joint line of the knee, using Bergström's needle (ø 5 mm) biopsy technique with suction. The muscle sample was cleaned of any visible connective and adipose tissue. Fat biopsy samples were taken with suction from abdominal subcutaneous fat tissue with a 12 G needle (ø 2 mm). Part of the biopsy samples was frozen in liquid nitrogen immediately after being removed from the needle and then stored at -80 °C until used for mRNA analysis.

4.2.9 Gene expression array

The RNA preparation, cRNA generation and microarray hybridization procedures in use were as previously described (Pöllänen et al. 2007). In brief, Trizol-reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from muscle biopsy samples of *m. vastus lateralis* homogenized on FastPrep FP120 apparatus (MP Biomedicals, Illkirch, France). From adipose tissue, total RNA was isolated following needle suspension with Ambion's RNAqueous -Micro

Kit (AM 1931, Applied Biosystems) according to the manufacturer's instructions. Experion (Bio-Rad Laboratories, Hercules, CA) was used to inspect the RNA concentration and quality. Only pure, good-quality RNA was used in the subsequent analyses (260/280 ratio >1.8). An Illumina RNA amplification kit (Ambion, Austin, TX) was used according to the manufacturer´s instructions to obtain biotinlabeled cRNA from 500 ng of total RNA. Experion was used to perform the quality control after amplification. Hybridizations (one array per tissue) to Illumina HumanWG-6 v3.0 Expression BeadChips (Illumina Inc., San Diego, CA, USA) containing probes for 48803 transcripts, were performed by the Finnish DNA Microarray Center at Turku Center for Biotechnology according to the Illumina BeadStation 500x manual (Revision C). Six samples were hybridized on the same chip with the twin and his/her co-twin always on the same chip. Hybridized probes were detected with Cyanin-3-streptavidin (1 µg/ml, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) using Illumina BeadArray Reader (Illumina Inc.) and BeadStudio v3 software (Illumina Inc.).

Initial data analyses were performed with R software environment for statistical computing (http://www.R-project.org), including Bioconductor development software (http://www.bioconductor.org). The raw data of each chip were quantile-normalized with affy package of Bioconductor (Gautier et al. 2004). Data quality was assessed by calculating Pearson correlations and clustering. In the pairwise analysis, normalized data was exported to Excel and SPSS statistical package. The fold change (FC) between twin pairs was calculated by dividing the normalized expression value (of each gene) of the active twin with the respective value of the inactive co-twin. Statistical analysis of this data was done using one-sample t-test (FC vs. 1). In both analyses, lists of genes at different significance levels ($p<0.05$, $p<0.01$ and $p<0.001$) were created. The gene expression data and the raw data sets are available in the GEO database, accession number GSE20319 for skeletal muscle and GSE20536 for adipose tissue data. MIAME guidelines were followed during array data generation, preprocessing, and analysis.

4.2.10 Gene Set Enrichment Analysis

The clustering of differentially expressed genes into functional groups and the significance of their distributions between groups were estimated with Gene Set Enrichment Analysis (version 2.0; GSEA, http://www.broad.mit.edu/gsea/) (Subramanian et al. 2005). GSEA is *"a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states (e.g. phenotypes)"*. A list of all transcripts on the chip ranked according to the intrapair expression ratio was utilized in the GSEA analysis with 1000 "gene set" permutations.

The most significant gene clusters obtained from the GSEA analysis were further correlated to anthropometric, physiological and biochemical data. For this purpose, leading-edge subsets (i.e. genes producing the GSEA enrichment score) were determined. The leading-edge genes contribute to the enrichment

score in each pathway. The centroid for each pathway was computed by normalizing the sum of expression levels of leading-edge genes to a mean of zero (Mootha et al. 2003).

4.2.11 Blood studies

Ten-hour fasting plasma samples were collected by venipuncture to Venosafe Gel tubes (Terumo Medical Co., Leuven, Belgium) after ten minutes of supine rest. Plasma glucose was determined using Biosen C-line (EKF-diagnostic, Magdeburg, Germany) and serum insulin by IMMULITE® 1000 Analyzer (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The HOMA index was calculated using the formula: (Fasting plasma glucose x Fasting plasma insulin)/22.5 (Muniyappa et al. 2008). Glycated hemoglobin (Hb_{A1C}) was analyzed using HPLC Variant II (Bio-Rad Laboratories, Munich, Germany). Serum lipids were analyzed by VITROS DT60 (Chemistry System Ortho-Clinical Diagnostics, Inc., Rochester, NY, USA). The low density lipoprotein level was calculated using Friedewald equation: (LDL (mmol/l) = Total Chol. – HDL – TG/2.2). Leptin levels were measured using commercial ELISA kit.

4.2.12 Assessment of confounders

Use of medications, smoking habits and use of alcohol were collected using questionnaire and interview methods as in this cohort earlier (Kujala et al. 1998, Waller, Kaprio & Kujala 2008, Hernelahti et al. 2004). Food and nutrient intake was studied via a 5-day diet diary and questionnaire of eating habits (Rintala et al. 2011).

4.2.13 Zygosity

The zygosity of the co-twins was verified at the Paternity Testing Laboratory (National Public Health Institute, Helsinki, Finland) using DNA extracted from a venous blood sample with a battery of ten highly polymorphic gene markers.

4.2.14 Ethics

The TWINACTIVE study was conducted according to the guidelines for good clinical and scientific practice laid down by the Declaration of Helsinki. The study was approved by the Ethics Committee of the Central Finland Health Care District on August 15, 2006. All participants gave their written informed consent before the measurements.

4.3 Statistical methods

Pairwise analyses were used to study the intrapair differences between the inactive vs. active co-twins of twin pairs. First the results were analysed for all twin pairs and then for the MZ and DZ twin pairs separately to find out whether the trends were similar for the MZ and DZ pairs. The normality of the mean variables was assessed by Shapiro-Wilk test. Student's paired t-test was used for the normally distributed variables and Wilcoxon matched-pair signed-rank test for the non-normally distributed variables. By studying same-sex twin pairs, all the pairwise analyses were adjusted for age and sex. Ninety-five percent confidence intervals (95% CI) were calculated for the absolute mean differences between the inactive vs. active co-twins. The symmetry test (Stata) was used to study the intrapair difference in categorical variables. Independent-samples t-test was used to compare the result between MZ and DZ twins. The Pearson correlation coefficient was used for the intrapair difference correlations. When calculating individual-based associations, the within-pair dependency of twin individuals was taken into account using the cluster option of Stata (svy:regress). The level of significance was set at $p<0.05$. Data were analyzed using IBM SPSS Statistics 19 and Stata 8 software.

For the high-dimensional gene expression array dataset, the p-values (see chapter 4.2.9) were corrected for multiple comparisons by calculating the False Discovery Rate (FDR) *q*-values (Storey 2002). R software version 2.4.1 was used for these analyses. The Pearson correlation coefficient was used for the intrapair difference (absolute differences between pairs) correlations and for individual-based correlations between gene set centroids and cardiovascular risk factors when the number of observations of continuous variables was ≥ 10 and the examination of distributions were suggestive of a normal distribution (all skewness values for cardio-vascular risk factors < 1 and for gene set centroids < 2.2).

5 RESULTS

5.1 Baseline characteristics

There were no baseline differences in anthropometrics or in work-related physical activity between the 16 inactive and active co-twins (Table 4).

TABLE 4 Baseline characteristics in 16 twin pairs discordant for physical activity (mean±SD or n). Significance bolded. (Adapted from article I).

Baseline characteristics (self-reported)	Inactive	Active	p-value
Sex (female:male)	5:11		
Age (yrs)	28 (min-max 18-42)		
Body height (cm) (n=15)	173.7 ± 9.8	172.9 ± 10.1	0.96†
Body weight (kg)	69.3 ± 16.4	66.0 ± 9.4	0.57†
Body mass index (kg/m^2) (n=15)	23.0 ± 4.2	22.3 ± 2.0	0.88†
Leisure time MET index (MET h/day)	**0.2 ± 0.3**	**3.3 ± 2.4**	**<0.001†**
Alcohol (g/day)	7.0 ± 8.3	9.6 ± 12.8	0.65†
Ever smoked regularly by 1975 (n)	6	9	0.25
Marital status (n)			0.37
Single	5	7	
Married	11	8	
Divorced	0	1	
Work-related physical activity (n)			0.23
Sedentary	3	7	
Standing or walking at work	3	4	
Light manual work	10	5	
Heavy manual work	0	0	
Occupational group (n)			0.43
Upper white-collar	1	3	
Clerical work	5	5	
Skilled workers	4	6	
Unskilled workers	1	0	
Farmers	3	0	
Other	2	2	

†Wilcoxon matched-pair signed-rank test used

5.2 Long-term physical activity discordance

5.2.1 Baseline leisure-time physical activity

The baseline was set to the year 1975 when the first physical activity data was available for the leisure-time MET index calculation. The baseline physical activity difference was 3.1 MET h/day ($p<0.001$) (see Table 4). The 1981 physical activity data was only used for the early identification of the consistently discordant twin pairs (see chapter 4.1.1 for baseline selection). The data from 1975 and 1981 were self-reported.

5.2.2 Follow-up leisure-time physical activity

During the whole follow-up period from 1980 to 2007 (Figure 9), thus, covering also the retrospective interviews by phone and the physical activity interview conducted at the TWINACTIVE laboratory measurements, the inactive twins were on average 8.8 MET h/day less active in their leisure-time (corresponding to about 2-h walk or 1-h jogging daily) than their active co-twins (2.2±2.3 vs. 11.0±4.1 MET h/day, $p<0.001$). The mean follow-up difference in leisure-time activity was similar for the MZ (3.4 vs. 12.2 MET h/day, $p=0.018$) and DZ pairs (1.3 vs. 10.1 MET h/day, $p=0.008$).

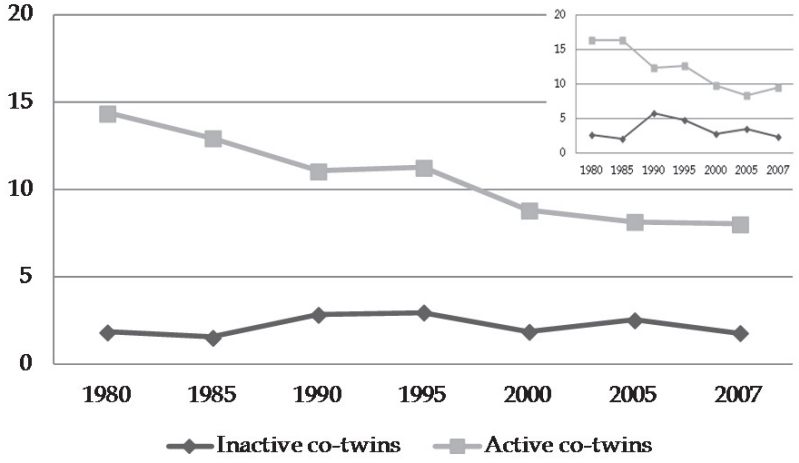

FIGURE 9 Schematic illustration of the follow-up activity among the 16 inactive vs. active co-twins of twin pairs (MET index (MET h/day) in vertical axis). Smaller panel for the seven MZ pairs.

5.2.3 Previous 12-month physical activity

In the assessment of the volume of leisure-time, daily (here gardening, wood works, repairing, berry picking, fishing, and snow works) and commuting activities over the previous 12 months before the laboratory visits significant differences between inactive and active co-twins were found in total (5.2±4.4 vs. 9.6±5.3 MET h/day, p=0.003, respectively) and in leisure-time (2.3±1.8 vs. 7.2±3.8 MET h/day, p=0.002) but not in daily (2.7 vs. 2.1 MET h/day, p=0.84) or in commuting activities (0.2 vs. 0.2 MET h/day, p=0.89). The most common types of physical activities among the active twins were walking (25% of the total 12-month physical activity), jogging (11%) and cross-country skiing (9%). The favorite types of activities among the inactive twins were repairing (27% of the total 12-month physical activity), walking (22%) and wood-working (10%) (Figure 10). Overall 75% of the total activity among the active twins was leisure-time (7.2 MET h/day) and 22% daily activities (2.1 MET h/day) whereas among the inactive twins 44% of the total activity was leisure-time (2.3 MET h/day) and 52% daily activities (2.7 MET h/day) (Figure 11).

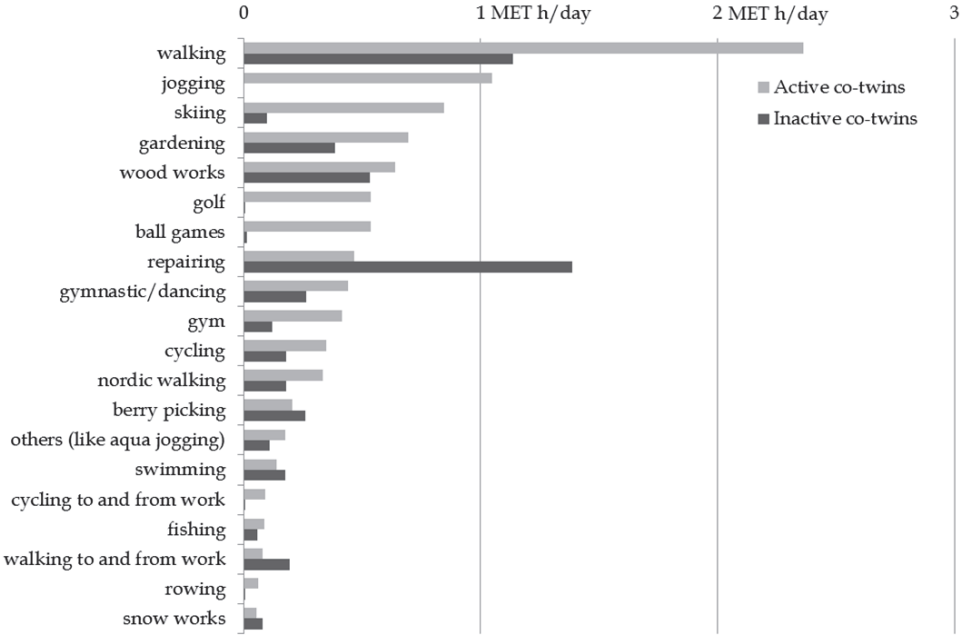

FIGURE 10 List of different types of activities among the inactive and active co-twins according to the 12-months questionnaire (MET h/day). List arranged by the popularity of the activity among the active co-twins.

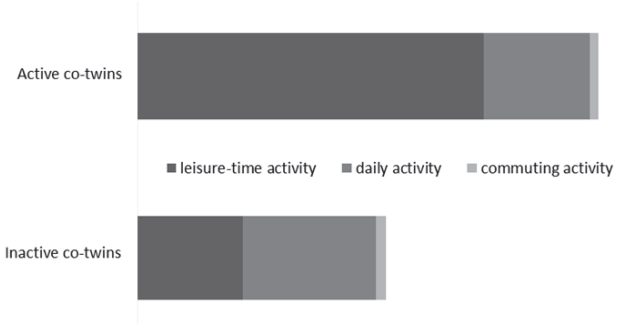

FIGURE 11 Illustration of the distribution of leisure-time, daily and commuting activities among the inactive and active co-twins. The lengths of the bars represent the average absolute volumes of the total activity over the previous 12-months.

5.3 Follow-up end point characteristics

There were no statistical intrapair differences in alcohol use (symmetry test p=0.16), smoking status (p=0.26), work status (p=0.36) or in work-related physical activity (p=0.17) at the end of the follow-up (Table 5). The questionnaire-based leisure-time MET index (2007) was on average 6.9 MET h/day lower among the inactive twins compared to their active co-twins (1.6±1.4 vs. 8.4±4.1 MET h/day, p<0.001, respectively). A similar intrapair difference in leisure-time physical activity was seen in both MZ (2.4±1.4 vs. 9.4±3.8 MET h/day, p=0.001), and DZ pairs (1.0±1.0 vs. 7.7±4.3 MET h/day, p=0.008). No significant intrapair differences in body weight (p=0.12) or in body mass index (p=0.09) were seen at the end of the follow-up (Table 5). The reported daily energy intake, calculated from the 5-day food diary, did not differ significantly between the co-twins (p=0.20) (more details in Rintala et al. 2011).

Physical fitness

There were no significant intrapair differences in resting heart rates (intrapair difference 6 bpm, p=0.083). The active twins achieved 30 watts higher load in the clinical exercise test (active: 168.1±42.0 vs. inactive: 138.9±39.9 W, p=0.003) and performed the test for longer than their inactive co-twins (807.1±201.7 vs. 663.1±194.6 sec, p=0.002). The estimated peak oxygen uptake from the highest load was 23% higher among the active twins compared to their inactive co-twins (intrapair difference 6.1 ml/kg/min, p<0.001) (Table 5). Similar intrapair differences in the estimated VO_{2peak} were found among the MZ (27.4±5.3 vs. 32.2±6.0 ml/kg/min, intrapair difference 4.8 ml/kg/min, p=0.083) and DZ pairs (25.7±4.7 vs. 32.8±5.4 ml/kg/min, intrapair difference 7.1 ml/kg/min, p=0.002) (independent t-test MZ vs. DZ twins, p=0.91). Fourteen out of 16 active twins had a higher estimated peak oxygen uptake than their inactive co-twins.

TABLE 5 Follow-up end point characteristics among 16 twin pairs discordant for physical activity (mean±SD or n). P_{peak}, time-weighted highest load; VO_{2peak}, estimated oxygen uptake at the highest load; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA, the homeostatic model assessment; Hb_{A1C}, glycated hemoglobin. Significant differences bolded.

Follow-up end point characteristics	Inactive	Active	p-value
Age (yrs)	60 (min-max 50-74)		
Body height (cm)	171.8 ± 10.4	171.1 ± 9.9	0.39†
Body weight (kg)	79.5 ± 18.4	72.9 ± 11.9	0.12†
Body mass index (kg/m^2)	26.7 ± 3.5	24.8 ± 2.6	0.09†
Leisure-time MET index (MET h/d)	**1.6 ± 1.4**	**8.4 ± 4.1**	**<0.001**
Reported daily energy intake (kcal)	1539 ± 357	1685 ± 452	0.20
Alcohol (g/day)	6.5 ± 4.7	10.3 ± 10.1	0.16†
Diagnosed type 2 diabetes	3	1	0.16
Screened impaired glucose tolerance	1	1	1.00
Diagnosed coronary heart disease	3	1	0.16
Smoking status			0.26
Current smokers	3	0	
Quitters	7	8	
Never smoked	6	8	
Work status			0.36
Employed	9	9	
Retired	5	5	
Unemployed	0	1	
Other	2	1	
Work-related physical activity			0.17
Sedentary	3	5	
Standing or walking at work	1	3	
Light manual work	5	0	
Heavy manual work	0	1	
Physical fitness			
Resting heart rate (bpm)	66.2 ± 9.7	60.2 ± 6.7	0.083
P_{peak} (W)	**138.9 ± 39.9**	**168.1 ± 42.0**	**0.003**
Estimated VO_{2peak} (ml/kg/min)	**26.4 ± 4.9**	**32.5 ± 5.5**	**<0.001**
Maximal knee extension force[a] (N)	**425.8 ± 87.3**	**507.8 ± 121.4**	**0.006**
Left handgrip strength[b] (N)	422.4 ± 156.1	428.1 ± 126.5	0.72
Cardio-metabolic risk factor levels[c]			
Total cholesterol (mmol/l)	5.61 ± 0.91	5.24 ± 0.93	0.11†
High density lipoprotein (mmol/l)	1.48 ± 0.38	1.58 ± 0.46	0.12
Low density lipoprotein (mmol/l)	3.60 ± 0.74	3.23 ± 0.83	0.13
HDL/LDL ratio	**0.43 ± 0.14**	**0.52 ± 0.19**	**0.023**
Triglycerides (mmol/l)	1.23 ± 0.56	0.95 ± 0.47	0.059
Fasting plasma glucose (mmol/l)	**5.61 ± 1.47**	**5.13 ± 1.03**	**0.041†**
HOMA index	2.95 ± 2.17	2.56 ± 2.95	0.18†
Hb_{A1c} (%)	5.87 ± 0.56	5.70 ± 0.42	0.22
Systolic blood pressure (mm Hg)	143.8 ± 25.1	142.1 ± 18.6	0.77
Diastolic blood pressure (mm Hg)	88.3 ± 13.2	86.9 ± 9.2	0.73
Leptin (pg/ml)	**8322 ± 5052**	**5066 ± 4420**	**0.036**
Hs-CRP	1.41 ± 1.23	1.29 ± 1.91	0.25

†Wilcoxon matched-pair signed-rank test used
[a]n=13 pairs; [b]n=15 pairs; [c]All twins included

The maximal knee extension force was 20% (intrapair difference 82 N, p=0.006) higher among the active twins compared to the inactive co-twins (Table 5). The trend was similar for the MZ (413.7±90.9 vs. 519.8±138.4 N, intrapair difference 106.1 N, p=0.041) and DZ pairs (433.4±90.4 vs. 500.3±118.9 N, 66.9 N, p=0.092) (independent t-test MZ vs. DZ twins, p=0.79). No intrapair differences in hand grip forces were found (Table 5).

Cardio-metabolic risk factor levels

Although this study was not designed or powered (because of the small sample size) to investigate disease, more cases of type 2 diabetes and coronary heart disease were found among the inactive twins than among their active co-twins (Table 5). The inactive twins had a lower HDL/LDL cholesterol ratio than their active co-twins (p=0.023) which remained statictically significant even when all twins taking a cholesterol lowering medication were excluded (p=0.01, n=9 pairs). The fasting plasma glucose levels were higher among the inactive co-twins (5.6 vs. 5.1 mmol/l, p=0.041) and the difference remained significant even when the diabetic co-twins (three inactive and one active) were excluded from the pairwise analysis (5.3 vs. 4.8 mmol/l, p=0.012, n=13 pairs). Significantly lower serum leptin levels were found among the active twins. The remaining differences in the cardio-metabolic risk factors between the co-twins were non-significant (Table 5).

5.4 Body composition

The body weight difference was 6.5 kilograms among all pairs (p=0.12) but among MZ pairs alone it was 1.5 kg (inactive: 77±11.3 kg vs. active: 75.4±13.2, p=0.63) whereas in the DZ pairs it was 10.4 kg (81.4±23.0 vs. 71.0±11.1 kg, p=0.17). There was no intrapair difference in the follow-up weight gain between the co-twins (inactive: +10.2±9.8 kg vs. active: +7.0±7.7 kg, p=0.218). The mean weight gain for all twins (n=32) was 8.6±8.8 kg; this ranged from a weight loss of 13.4 kg to a weight gain of 27.7 kg.

At the follow-up end point, the intrapair difference in fat mass was 6 kg (p=0.015), with the trend being similar for MZ (intrapair difference 4.6 kg, p=0.13) and DZ pairs (7.0 kg, p=0.066) (independent t-test MZ vs. DZ twins, p=0.72) (Table 6). No significant intrapair differences in fat free masses were found. The intrapair difference in body fat percent was around five percent points for all, MZ and DZ pairs. A significant difference in waist circumference was seen among all pairs (intrapair difference 6.3 cm, p=0.050) (Table 6).

TABLE 6 Body composition (InBody 720) and anthropometrical results (mean±SD) in all, MZ and DZ pairs. CI, confidence interval for the mean difference; FM, fat mass; FFM, fat free mass; Fat%, fat percent; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio. Significant differences bolded.

	Inactive	Active	Mean difference *(median*)* (95% CI)	p-value
All pairs (n=16)				
FM (kg)	**21.6 ± 7.7**	**15.6 ± 5.0**	**6.0 (*5.1*) (1.0 to 10.9)**	**0.015†**
FFM (kg)	57.7 ± 12.4	57.2 ± 11.1	0.6 (-4.3 to 5.4)	0.81
Fat%	**27.0 ± 5.3**	**21.5 ± 6.4**	**5.4 (2.0 to 8.8)**	**0.004**
WC (cm)	**96.9 ± 13.1**	**90.6 ± 9.3**	**6.3 (*2.75*) (-0.3 to 12.9)**	**0.050†**
HC (cm)	99.6 ± 7.5	95.8 ± 6.0	3.8 (*0.25*) (-0.4 to 8.0)	0.17†
WHR	0.97 ± 0.09	0.95 ± 0.07	0.03 (*0.02*) (-0.01 to 0.06)	0.15†
WHtR	**0.56 ± 0.05**	**0.53 ± 0.04**	**0.03 (*0.017*) (0.001 to 0.07)**	**0.039†**
MZ pairs (n=7)				
FM (kg)	21.4 ± 5.3	16.8 ± 2.1	4.6 (-1.8 to 11.0)	0.13
FFM (kg)	55.4 ± 9.0	58.5 ± 13.5	-3.1 (-10.0 to 3.9)	0.32
Fat%	27.9 ± 5.5	22.9 ± 4.8	5.0 (-1.4 to 11.3)	0.10
WC (cm)	97.6 ± 7.9	92.9 ± 8.1	4.7 (-1.4 to 10.8)	0.11
HC (cm)	97.7 ± 3.3	96.9 ± 4.5	0.8 (-2.7 to 4.3)	0.60
WHR	1.0 ± 0.07	0.96 ± 0.05	0.04 (-0.007 to 0.09)	0.083
WHtR	0.56 ± 0.02	0.53 ± 0.02	0.03 (-0.007 to 0.07)	0.091
DZ pairs (n=9)				
FM (kg)	21.7 ± 9.5	14.7 ± 6.5	7.0 (*5.5*) (-1.5 to 15.6)	0.066†
FFM (kg)	59.5 ± 14.8	56.1 ± 9.6	3.4 (-4.0 to 10.8)	0.32
Fat%	**26.3 ± 5.4**	**20.5 ± 7.6**	**5.8 (0.9 to 10.7)**	**0.026**
WC (cm)	96.3 ± 16.5	88.8 ± 10.2	7.6 (*3.5*) (-4.6 to 19.7)	0.18†
HC (cm)	101.0 ± 9.5	94.9 ± 7.05	6.1 (-1.3 to 13.5)	0.093
WHR	0.95 ± 0.10	0.94 ± 0.09	0.02 (*-0.005*) (-0.04 to 0.07)	0.86†
WHtR	0.56 ± 0.07	0.52 ± 0.05	0.04 (*0.02*) (-0.02 to 0.09)	0.21†

†Wilcoxon matched-pair signed-rank test

*Median for the non-normally distributed intrapair differences

5.4.1 Abdominal fat distribution

The abdominal MRI showed that the overall abdominal area was greater among the inactive co-twins (p=0.011) (Table 7). In general, the inactive twins had almost 1.5 times higher visceral fat area, measured from the single slice image at the level of L4-L5+5 cm, when compared to their active co-twin (Figure 12). The trend was similar for MZ and DZ pairs (independent t-test, p=0.86). The intrapair difference in subcutaneous fat area was only half that of visceral fat. As an indicator of the accumulation of ectopic fat in the liver, the MRI assessed liver fat score (intensity difference between in and out phase images) was found to be two to three times higher in the inactive co-twins (Table 7). A significant difference was detected in the liver fat score between the inactive and active MZ co-twins.

TABLE 7 Abdominal subcutaneous and visceral fat distribution (mean±SD) among all, MZ and DZ pairs. CI, confidence interval for the mean difference; A_{area}, abdominal area at the level of L4-L5+5 cm; ASAT, area of abdominal subcutaneous adipose tissue; VAT, area of visceral adipose tissue; VAT/ASAT, ratio of visceral-to-subcutaneous adipose tissue area. Significant differences bolded.

	Inactive	Active	Mean difference *(median*)* (95% CI)	p-value
All pairs (n=16)				
A_{area} (cm^2)	**661.6 ± 177.8**	**557.3 ± 104.2**	**104.3 (*75.2*) (13.2 to 195.3)**	**0.011†**
ASAT (cm^2)	195.3 ± 70.0	155.8 ± 49.4	39.5 (-3.0 to 82.0)	0.067
VAT (cm^2)	**170.6 ± 102.8**	**115.1 ± 73.3**	**55.5 (*33.2*) (7.0 to 104.1)**	**0.010†**
VAT/ASAT	0.94 ± 0.56	0.76 ± 0.53	0.18 (-0.001 to 0.36)	0.051
Liver fat score	**21.1 ± 23.2**	**7.9 ± 8.4**	**13.2 (3.5 to 22.8)**	**0.011**
MZ pairs (n=7)				
A_{area} (cm^2)	648.5 ± 112.9	577.9 ± 99.8	70.6 (-8.5 to 149.8)	0.072
ASAT (cm^2)	185.9 ± 40.4	160.9 ± 26.4	25.0 (-12.9 to 63.0)	0.16
VAT (cm^2)	164.7 ± 50.6	127.8 ± 61.8	36.9 (-15.6 to 89.5)	0.14
VAT/ASAT	0.92 ± 0.33	0.81 ± 0.38	0.11 (-0.17 to 0.4)	0.38
Liver fat score	**26.1 ± 21.6**	**6.0 ± 8.2**	**20.1 (6.2 to 34.0)**	**0.012**
DZ pairs (n=9)				
A_{area} (cm^2)	671.7 ± 222.4	541.3 ± 110.6	130.4 (*81.6*) (-37.1 to 298.0)	0.066†
ASAT (cm^2)	202.6 ± 88.4	151.8 ± 63.3	50.8 (-27.5 to 129.0)	0.17
VAT (cm^2)	**175.2 ± 133.6**	**105.3 ± 83.4**	**70.0 (*28.7*) (-16.4 to 156.3)**	**0.021†**
VAT/ASAT	0.96 ± 0.70	0.73 ± 0.65	0.23 (-0.05 to 0.52)	0.095
Liver fat score	17.2 ± 24.9	9.4 ± 8.8	7.8 (-7.1 to 22.6)	0.26

†Wilcoxon matched-pair signed-rank test

*Median for the non-normally distributed intrapair differences

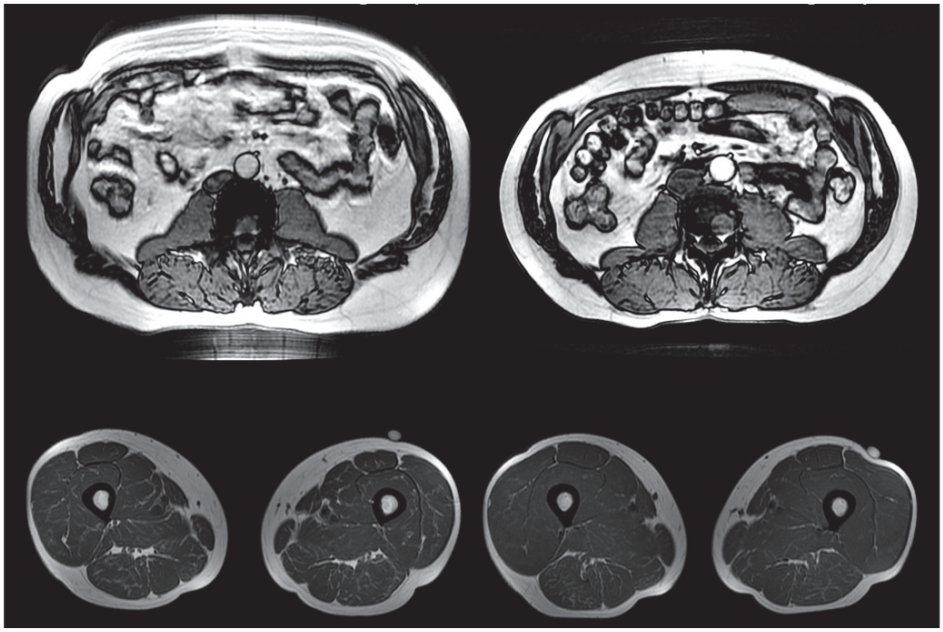

FIGURE 12 An example of the visceral and intramuscular fat accumulation among one of the monozygotic male twin pairs (age 50 yrs). Both co-twins had the same profession and employer. The active co-twin (on the right) had participated regularly in running whereas his inactive co-twin (on the left) had been sedentary throughout the follow-up. Figure adapted from article II.

5.4.2 Midthigh composition

A finding of a significantly (150%) higher intramuscular (extramyocellular) fat among the inactive twins obtained from the midthigh single slice image was found in all, MZ and DZ pairs (Table 8). The inactive twins tended also to have more subcutaneous fat in their midthighs although this was not seen in the MZ pairs. The ratio between intramuscular and subcutaneous thigh fat was significantly higher among the inactive twins in all pairs and in MZ pairs (Table 8). There were no significant intrapair differences in muscle cross-sectional area (when excluding bone, bone marrow and intramuscular fat). Furthermore no significant differences in muscle volumes between the co-twins were seen (Table 8). However, the relative muscle area was significantly higher among the active twins when compared to their inactive co-twin (67% vs. 61% of the total cross-sectional area of the midthigh, $p=0.007$).

TABLE 8 Midthigh single slice assessed composition (mean±SD) among all, MZ and DZ pairs. CI, confidence interval for the mean difference; IMAT, midthigh intramuscular (extramyocellular) adipose tissue area; TSAT, midthigh subcutaneous adipose tissue area. Significant differences bolded.

	Inactive	Active	Mean difference (95% CI)	p-value
All pairs (n=15)				
Midthigh area (cm^2)	196.2 ± 33.5	183.7 ± 22.6	12.5 (-6.0 to 31.0)	0.17
Muscle area[a] (cm^2)	117.2 ± 20.5	122.4 ± 22.1	-5.2 (-10.9 to 0.5)	0.072
Muscle volume[a] (dm^3)	1.88 ± 0.38	1.94 ± 0.38	-0.06 (-0.16 to 0.05)	0.28
IMAT (cm^2)	**14.0 ± 7.0**	**9.1 ± 4.8**	**4.9 (1.9 to 7.9)**	**0.003**
TSAT (cm^2)	**58.7 ± 27.8**	**46.0 ± 19.6**	**12.7 *(8.3*)* (-0.5 to 25.9)**	**0.047†**
IMAT/TSAT	**0.28 ± 0.16**	**0.21 ± 0.12**	**0.07 (0.02 to 0.12)**	**0.007**
MZ pairs (n=6)				
Midthigh area (cm^2)	186.2 ± 24.5	190.6 ± 25.1	-4.3 (-25.6 to 16.9)	0.62
Muscle area[a] (cm^2)	119.1 ± 18.5	129.9 ± 24.6	-10.8 (-23.5 to 1.9)	0.081
Muscle volume[a] (dm^3)	1.92 ± 0.34	2.05 ± 0.42	-0.13 (-0.36 to 0.10)	0.20
IMAT (cm^2)	**13.7 ± 5.6**	**9.1 ± 3.4**	**4.6 (0.1 to 9.0)**	**0.046**
TSAT (cm^2)	47.1 ± 14.9	45.0 ± 9.7	2.1 (-8.3 to 12.4)	0.63
IMAT/TSAT	**0.32 ± 0.15**	**0.21 ± 0.09**	**0.11 (0.02 to 0.19)**	**0.020**
DZ pairs (n=9)				
Midthigh area (cm^2)	202.8 ± 38.2	179.1 ± 21.0	23.7 (-4.5 to 51.8)	0.089
Muscle area[a] (cm^2)	115.9 ± 22.7	117.4 ± 20.2	-1.5 (-7.2 to 4.3)	0.57
Muscle volume[a] (dm^3)	1.86 ± 0.43	1.86 ± 0.35	-0.007 (-0.14 to 0.13)	0.90
IMAT (cm^2)	**14.2 ± 8.2**	**9.1 ± 5.8**	**5.2 (0.3 to 10.0)**	**0.040**
TSAT (cm^2)	66.5 ± 32.3	46.7 ± 24.7	19.8 (-1.9 to 41.4)	0.068
IMAT/TSAT	0.25 ± 0.18	0.20 ± 0.14	0.05 (-0.02 to 0.11)	0.15

†Wilcoxon matched-pair signed-rank test
*Median for non-normally distributed intrapair difference
[a]Bone, bone marrow and intramuscular fat area excluded

Intramuscular fat vs. glucose homeostasis

Moderate associations at best were found between the intramuscular fat area and the markers of glucose homeostasis (HOMA index, fasting glucose, Hb_{A1C}) (r=0.47, r=0.54, r=0.55, respectively). After excluding the diabetic twins (n=4), the associations been even more modest (r=0.28, r=0.30, r=0.32, respectively). Overall, the correlation coefficients between intramuscular fat and the markers of glucose homeostasis were higher among the inactive twins (r=0.59 to 0.69, n=16) when compared to active twins (r=0.30 to 0.39, n=15). However, the most consistent association was found between the intramuscular fat area and Hb_{A1C} (p=0.03 for 31 twins (Figure 13); p=0.06 for non-diabetic twins). This association was also found to be moderate in the intrapair difference correlation analysis (r=0.55, p=0.03, n=15 pairs; r=0.54, p=0.07, n=12 non-diabetic pairs).

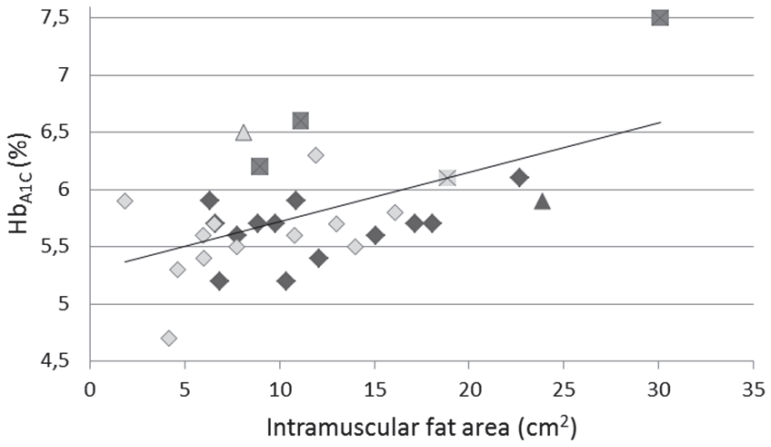

FIGURE 13 Individual-based association between intramuscular fat area and Hb_{A1C}. Inactive twins are dotted diamonds/squares with dark color and active twins those with light grey color. Twins having diagnosed type 2 diabetes are shown as crosses in squares and twins having screen detected impaired fasting plasma glucose are designated by triangles.

5.5 Enriched metabolic and regulatory pathways

Characteristics of the pairs attended to biopsies

Ten pairs agreed to participate in the second visit which included the *m. vastus lateralis* and subcutaneous adipose tissue biopsies. The intrapair weight difference at the end of the follow-up between the co-twins of these ten pairs was 9.3 kilograms (inactive>active, p=0.139), waist circumference difference 7.6 centimeters (inactive>active, p=0.109), fat mass difference 6.6 kg (inactive>active, p=0.047) and fat free mass difference 2.6 kg (inactive>active, p=0.78). The leisure-time physical activity difference at the end of the follow-up was 8.1 MET h/day (inactive: 1.6±1.3 vs. active: 9.8±4.4 MET h/day, p=0.005), it had been 9.4 MET h/day during the whole follow-up (p=0.005). Thus the level of inactivity among the inactive twins corresponded to about 45 minutes brisk walking two times per week, whereas the level of activity among the active co-twins corresponded to about 90 minutes of running four times per week. The intrapair difference in estimated VO_{2peak} was 4.7 ml/kg/min (inactive<active, p=0.023).

Upregulated gene sets among the active twins vs. inactive co-twins

After normalizing the muscle gene expression data within pairs (normalization to the inactive twin) one-sample t-test revealed congruent lists of differentially expressed sequences: 45 sequences at p<0.001, 572 sequences at p<0.01 and 2829 sequences at p<0.05. Of the 45 sequences at p<0.001, 25 sequences were up-

regulated and 20 sequences were down-regulated in the physically active co-twins. In order to investigate the metabolic and expression changes due to physical (in)activity in the adipose tissue the global gene expression profiles were analyzed from the abdominal subcutaneous adipose tissue samples (taken on the same occasion as the muscle biopsies). Forty-seven sequences at $p<0.001$ (one-sample t-test after normalization of data), 401 sequences at $p<0.01$ and 2037 sequences at $p<0.05$ were differentially expressed between active and inactive co-twins. Of the 47 sequences at $p<0.001$, 16 sequences were up-regulated and 31 sequences were down-regulated in the physically active co-twins.

GSEA

Pathway analysis using GSEA utilizing active vs. inactive co-twin gene expression ratios was performed on curated gene sets of canonical pathways containing 639 gene sets. The most enriched gene sets (FDR 5%) in the active members of twin pairs in comparison to the inactive ones in muscle tissue were oxidative phosphorylation, valine, leucine and isoleucine degradation, ubiquinone biosynthesis and fatty acid metabolism (Table 9). Specific genes in the oxidative phosphorylation gene set encode NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c oxidase, H^+ transport and ATP synthase. The most enriched gene sets (FDR 10%) among the active twins vs. inactive co-twins in adipose tissue included valine, leucine and isoleucine degradation (related to aldehyde dehydrogenase activity, branched-chain amino acid catabolic processes and steps of mitochondrial fatty acid beta-oxidation pathway as also in skeletal muscle), polyunsaturated fatty-acid (PUFA) metabolism and inflammatory processes (Table 9).

TABLE 9 Gene sets upregulated and with a significant FDR q-values in skeletal muscle (left panel) and in adipose tissue (right panel) among the active compared to the inactive twins. FDR, False discovery rate.

Gene sets in muscle tissue (FDR 5%)	Gene sets in adipose tissue (FDR 10%)
Oxidative phosphorylation	IL2RB pathway
Valine, leucine and isoleucine degradation	Valine, leucine and isoleucine degradation
Ubiquinone biosynthesis	Polyunsaturated fatty acid biosynthesis
Propanoate metabolism	RECK pathway
Fatty acid metabolism	Prostaglandin synthesis regulation
Butanoate metabolism	T cytotoxic pathway
Tryptophan metabolism	
Fructose and mannose metabolism	
Glycolysis	
Chloroacrylic acid degradation	
Urea cycle and metabolism of amino groups	

Gene set centroids vs. cardio-metabolic risk factors

The leading-edge genes (i.e. genes contributing to the enrichment score of each pathway) were used to calculate the expression centroids (see chapter 4.2.10). A moderate association was found between the estimated peak oxygen uptake and the seven first muscle tissue gene set centroids ranked by active-to-inactive ratio (Figure 14). The majority of the gene sets centroids in muscle tissue correlated significantly with serum HDL cholesterol concentration levels (Figure 15). In addition, the intrapair differences of the oxidative phosphorylation gene set centroid and that of valine, leucine and isoleucine degradation gene set centroid correlated with intrapair difference in the HDL levels (r=0.67, p=0.034 and r=0.69 p=0.028, respectively). Interestingly, the three first gene set centroids most up-regulated in adipose tissue among the active twins had significant and often high at best correlations with visceral fat area (r=-0.86, r=-0.82, r=-0.76, respectively, n=12 individuals) and intramuscular fat area (r=-0.72, r=-0.65, r=-0.63, respectively, n=11 individuals), serum triglyceride concentration levels(r=-0.68, r=-0.76, r=-0.60, respectively, n=12), fasting plasma glucose level (r=-0.91, r=-0.82, r=-0.75, respectively, n=12) and HOMA index (r=-0.87, r=-0.74, r=-0.77, respectively, n=12).

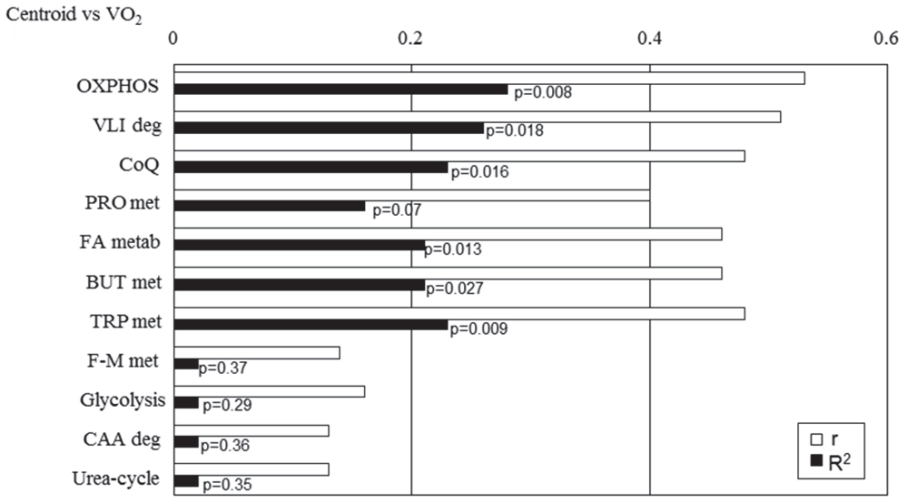

FIGURE 14 Associations between the centroids of gene sets up-regulated in muscle tissue in the active compared to inactive twins and estimated peak oxygen uptake levels. Illustrated as individual-based (n = 20) correlation coefficients (r) and R^2 with its p-value derived from the family cluster regression analysis. OXPHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea-cycle, Urea cycle and metabolism of amino groups.

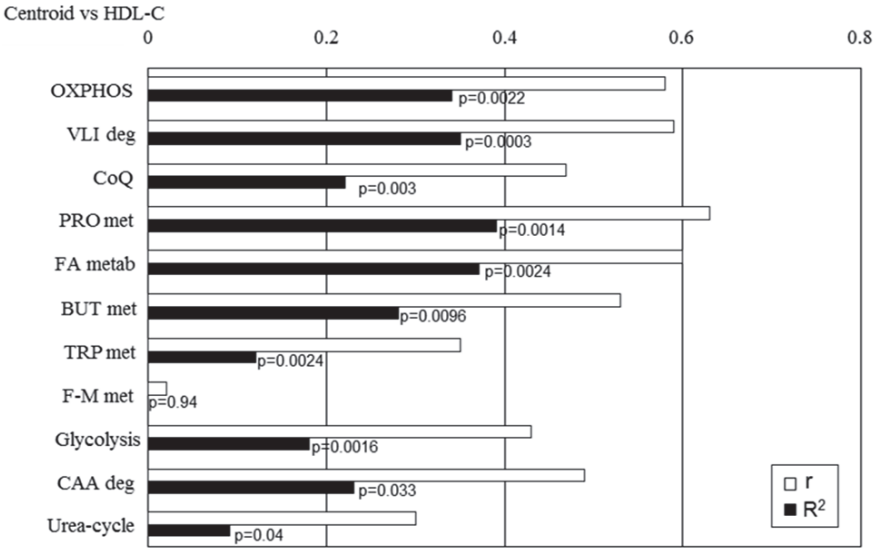

FIGURE 15 Associations between the centroids of gene sets up-regulated in muscle tissue in the active compared to inactive twins and HDL-C levels. Illustrated as individual-based (n = 20) correlation coefficients (r) and R^2 with its p-value derived from the family cluster regression analysis. OXPHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea cycle, Urea cycle and metabolism of amino groups.

6 DISCUSSION

The aim of this thesis (and original articles I-IV) was to clarity the associations of long-term leisure-time physical activity vs. inactivity with health outcomes among 16 (of which seven monozygotic) twin pairs discordant for physical activity habits throughout their adult life. The main findings of this study are that life-long leisure-time activity is associated with better physical fitness and lowered rate of overall, but especially visceral, intramuscular (extramyocellular) and liver fat when compared to long-term inactivity. The association between leisure-time physical activity and smaller levels of ectopic fat were also seen among the monozygotic twin pairs. Furthermore, the long-term physical activity was associated with upregulation of genetic pathways that encode several steps in energy metabolism in both muscle and abdominal subcutaneous fat tissues. The centroids of these metabolic pathways were further associated with cardio-metabolic risk factors.

6.1 Physical activity and physical fitness

The leisure-time activity level of the inactive twins was rather stable throughout the follow-up but in the active twins the dose of leisure-time physical activity seemed to decline with age (see Figure 9). This could be a normal phenomenon as the participation in high-intensity leisure-time physical activities has been found to diminish with age (Talbot, Metter & Fleg 2000, Talbot et al. 2007, Evenson et al. 2002, Orsini et al. 2006). Factors such as body mass index, smoking habits, health and socioeconomic status might be related to maintenance of vigorous activity in middle to old ages (Evenson et al. 2002).

The active twins mostly engaged in aerobic type of activity (walking, skiing, and jogging). In fact, when also walking to and from work and the Nordic walking were considered, then walking seemed to be the most popular type of leisure-time activity in all middle-aged and older twins accounting for one third of the total activity in both sets of co-twins. It is noteworthy, that the total activity among the inactive twins was almost half of that of the active

twins (see Figure 11). According to a meta-analysis, walking lowers the risk for cardiovascular events (HR 0.69) and all-cause mortality (HR 0.68) even in a dose-dependent manner (Hamer & Chida 2008). Observational studies have also indicated that walking, especially at a fast pace, can reduce the risk for suffering cardiovascular disease (Boone-Heinonen et al. 2009).

Cardiorespiratory fitness

In this present study, the intrapair difference in leisure-time physical activity habits reflected to the cardiorespiratory fitness as the active twins had 23% higher estimated VO_{2peak} values and they succeeded better in the symptom-limited ergometer test when compared to their inactive co-twins. The same trend was also seen among the inactive vs. active MZ co-twins although they have similar genetic backgrounds.

When compared to the reference population of nearly 1500 Finnish men and women aged 55 to 74 years, the average fitness level was medium for the inactive female twins and high for the active female twins whereas both the inactive and active male twins were at medium level (Hakola et al. 2011). The average exercise capacity of the active twins almost reached 10 METs (9.3 METs) which is shown to be related to the minimal risk of mortality (Kokkinos & Myers 2010). On the contrary, the aerobic capacity among the inactive twins was 7.5 METs. According to a meta-analysis, subjects with aerobic capacity more than 7.9 METs have been shown to have substantially lower rates of all-cause mortality and cardiovascular events than those with aerobic capacity under 7.9 METs (Kodama et al. 2009).

Lower limb strength and composition

The results of the present study indicate that regular and mostly endurance type of leisure-time activity throughout the adult life is associated with higher lower limb muscle strength (Table 5), irrespective of the rather similar muscle volumes and cross-sectional areas in both active and their inactive the co-twins (Table 8). The genetic background seems to affect the body structure and function (Tiainen et al. 2004, Nguyen et al. 1998, Tiainen et al. 2008), but here even when studying only the MZ pairs, the muscle force-producing system, possibly including their neural factors, was at a higher level in the active individuals as compared to the inactive co-twin. Cross-sectional studies in trained elite athletes also suggest that regular endurance type of training associates with preserved muscle mass, lower rate of fat infiltration (Wroblewski et al. 2011, Louis et al. 2009) and decreased loss of leg strength and muscle morphology (Tarpenning et al. 2004). In addition, a RCT study conducted in elderly people showed that moderate physical activity (mainly walking) could maintain lower limb muscle quality but not the loss of mass (Goodpaster et al. 2008). Furthermore activity prevented almost totally the 18% gain in intramuscular fat seen in control group (Goodpaster et al. 2008). A progressive 18-week-long endurance type of training (walking and step aerobics) has been shown to increase isometric knee extensor muscle strength

(but not mass) similar to strength training in elderly women (Sipilä et al. 1996). Thus the present study findings are in line with previous findings showing that endurance type of activity affects muscle strength but not necessarily mass (Sipilä & Suominen 1995, Goodpaster et al. 2008). However, several RCT studies have revealed that aerobic exercise is important in the maintenance of body fat free mass compared to controls (Nicklas et al. 2009, Ross et al. 2004, Ross & Janssen 2001). However, endurance type of training alone may not preserve the whole body lean mass among aging adults but is enough to maintain muscle function (Tarpenning et al. 2004).

The results of the present study also suggest that mostly aerobic type of training in the active twins (walking and jogging) does not associate with higher muscle cross-sectional area which is in line with previous studies (Tarpenning et al. 2004, Sipilä & Suominen 1995). Resistance type of training is needed to elicit significant gains in lean mass among aging adults (Willis et al. 2012, Peterson, Sen & Gordon 2011, Sillanpää et al. 2009), which naturally has another major advantage since it also achieves a gain in muscle strength (American College of Sports Medicine 2009).

Age-related muscle fat infiltration is shown to be associated with poor muscle strength and reduced lower extremity performance among sedentary elderly persons (Visser et al. 2002). However, the role of muscle fat infiltration in strength loss, muscle weakness and mobility limitations requires to be elucidated (Visser et al. 2005, Delmonico et al. 2009, Goodpaster et al. 2008).

In summary, in contrast to the studies conducted in older sedentary subjects, studies done among consistently active subjects suggest that it is possible to prolong the maintenance of good muscle quality, composition and strength even in the absence of gains in muscle mass. Aerobic type of leisure-time activity, like walking, could be enough for the maintenance. Thus it seems that the muscle force producing system is more vital than its actual mass (e.g. Frontera et al. 2008). This might explain the findings that have shown a disparity between the age-related loss of muscle mass and strength (Hughes et al. 2001, Goodpaster et al. 2006, Frontera et al. 2008). Therefore, retention of muscle strength over mass could be the key factor in the maintenance of muscle function and mobility at older ages (Hughes et al. 2001, Newman et al. 2006, Goodpaster et al. 2006, Visser et al. 2005, Delmonico et al. 2009).

6.2 Weight and body composition

Weight changes

The longitudinal data of this study shows that the inactive twins on average gained ten kilograms and the active twins on average seven kilograms of body weight during their adulthood. However, the data is limited by the self-reported nature of the body weights at the baseline. The intrapair weight difference doubled during the follow-up. Thus it can be stated that the voluntary leisure-time activity among the twins was not enough to prevent

weight gain. This might be due to the decreasing trend in the dose (intensity) of activity among the active twins and due to the insufficient physical activity in the inactive twins. Williams & Wood (2006) showed that vigorous exercise must increase significantly with age if it is to compensate for the expected age-related weight gain. Even in individuals who ran over 64 km/week, there was a significant weight gain over time (Williams & Wood 2006).

However in the present study although the weight gain was not prevented in the physically active twins, it was attenuated which is in line with results from previous population-based follow-up studies (Waller, Kaprio & Kujala 2008, Littman, Kristal & White 2005, Gordon-Larsen et al. 2009). In a previous longitudinal twin study of twin pairs discordant for physical activity, the active twins gained 5.4 kg less weight and 8.4 centimeters less waist girth than their inactive co-twins during their adult life (Waller, Kaprio & Kujala 2008). In the Coronary Artery Risk Development in Young Adults (CARDIA) study among nearly 5000 participants and with a 15-y-long follow-up, regular leisure-time walking was associated with attenuated weight gain (Gordon-Larsen et al. 2009). Furthermore in the same population, those who maintained the highest levels of activity were gaining 2.6 kg (men) and 6.1 kg (women) less weight and increasing 3.1 cm (men) and 3.8 cm (women) less waist circumference over 20 years than those men and women with lower levels of activity (Hankinson et al. 2010).

Confounding factors to the energy balance

Body weight is stably maintained when the energy expenditure equals to the energy intake. Diet (energy intake) and physical activity (energy expenditure) are the major contributors to the weight changes but other factors such as smoking habits, resting metabolic rate, and amount of fat free mass can influence the net energy balance (Thomas et al. 2012, Mozaffarian et al. 2011). There were no significant differences in alcohol and smoking habits or in the occupational groups between the co-twins in baseline (Table 4) or at the end of the follow-up (Table 5). The dietary habits of the co-twins were evaluated by a 5-day food diary and asking the subjects to fill in a questionnaire about their eating habits. The active twins tended to eat more but the quality of the food was rather similar between the co-twins (Rintala et al. 2011). Therefore, it can be speculated that the energy intake among the active twins was not subjected to a decreasing trend in relation to the reduced amount (intensity) of physical activity as they aged and that led to a positive energy balance and weight gain over time.

Fat mass and fat free mass

Based on longitudinal findings, fat mass increases before the age of 60 while fat free mass starts to decrease after 50 years of age. The body weight follows the trends of fat and fat free masses as the weight can increase during adulthood and thereafter decrease with age. However body weight does not necessarily distinguish the exercise-induced changes into its two components which is one

of the major problems in studies of physical activity and weight changes (e.g. Thomas et al. 2012).

The non-significant weight difference between the inactive vs. active MZ co-twins was 1.5 kg. This resulted from a 3.1 kg higher fat free mass and a 4.6 kg lower fat mass in the active MZ twins compared to their inactive MZ co-twins (Table 6). In DZ pairs, the 10.4 kg weight difference was due to the higher fat free mass (3.4 kg) and the much higher fat mass (7 kg) among the inactive twins compared to their active siblings. In summary, the inactive DZ twins were heavier than the inactive MZ twins and the active DZ twins lighter than the active MZ twins but there were no significant differences in fat free masses between the co-twins of either MZ or DZ pairs. However, the intrapair differences in body fat percentages were around five percent points in the combined, MZ and DZ pairs. Also the fat mass difference between the inactive vs. active co-twins was also rather similar in the twins (6.0 kg for all, 4.6 kg for MZ pairs, 7.0 kg for DZ pairs). These findings are in line with previous studies showing that aerobic exercise training is an effective way to reduce fat mass but also to preserve lean mass (Kraemer et al. 1999, Willis et al. 2012, Garrow & Summerbell 1995). For this reason and due to lack of data about the body composition at baseline, it is impossible to know whether the weight gain among the co-twins was only due to an increase in fat mass. In summary, quantitatively similar intrapair differences in the follow-up physical activity (that is follow-up mean MET index) in all, MZ and DZ pairs resulted with quantitatively similar intrapair differences in fat masses and fat percentages but not with that of fat free masses.

6.3 Visceral and ectopic fat stores

Imaging methods are needed if one wishes to study body fat distribution (Hu, Nayak & Goran 2011). It is clearly evident that fat distribution plays a prominent role in the metabolic disturbances linked to both inactivity and obesity. The key drivers for metabolic abnormalities among the sedentary control groups in clinical trials have been found to be the accumulation of visceral, liver and intramuscular fat (Patel, Slentz & Kraus 2011). Therefore it was important to undertake both abdominal and midthigh imagings.

The results showed that the inactive twin had on average 1.5 times higher visceral (intrapair difference 55.5 cm^2) and intramuscular fat area (4.9 cm^2) and 2 to 3 times higher liver fat score than their active co-twin. These findings were consistent for all twins and when subdivided into MZ and DZ pairs and are in agreement with earlier data from observational and short-term intervention studies (Goodpaster et al. 2008, Durheim et al. 2008). Moreover the present study provides compelling evidence that long-term physical activity is associated with low liver and intramuscular fat accumulation also when genetic background is standardized. However, the latter finding should be differentiated from the finding that storing lipids in intramyocellular lipid

droplets may be a beneficial phenomenon among physically active subjects (Goodpaster et al. 2001) as the intramyocellular fat content could not be evaluated from the MR images.

The intrapair differences in fat distribution are quantitatively somewhat similar to those seen in the studies of Ross and co-workers (2000, 2004). Ross et al. (2004) reported that one could achieve ~30% reduction in visceral fat after a 14-week trial with an energy deficit of 500 kcal/day by exercise with a loss of 6.5 cm in waist, 6 kg in body weight and 6.7 kg in fat mass among premenopausal women (Ross et al. 2004). Measurements done in obese men revealed quantitatively similar results in a 3-month intervention with daily exercise equivalent to 700 kcal/day (about 60 min daily) that resulted in a loss of one kilo of visceral fat (area of visceral fat -52 cm^2) and in a loss of 6.5 cm in waist, 7.6 kg in body weight and 6.1 kg in fat mass (Ross et al. 2000). In RCT studies, the reduction of visceral fat has been around 6 to 7 cm^2 per single kilogram of weight loss. In this twin study, the follow-up end point intrapair difference in visceral fat area when proportioned to the intrapair weight difference was 8.7 cm^2. Also the intrapair difference in waist circumference was close to that seen in a previous longitudinal twin study which examined twin pairs discordant for physical activity (Waller, Kaprio & Kujala 2008). The measurement of waist circumference is the easiest way to assess abdominal obesity (Cornier et al. 2011, Balkau et al. 2007, Bigaard et al. 2005).

It has been claimed that it might be possible to lose visceral fat or liver fat independent of total body weight loss (Janiszewski & Ross 2007, Ekelund et al. 2011, Magkos 2010). In support of this claim, this study among the long-term discordant twin pairs suggests that physical activity is associated with smaller ectopic fat accumulation although the weight is gained over time which is in line with the cross-sectional findings among elite athletes (Wroblewski et al. 2011). One of the hypothetical explanations for the phenomenon could be that among physically active persons the subcutaneous fat depot functions as a kind of energy sink for excess fat and in this way protects from the accumulation of visceral and ectopic fat stores (Despres & Lemieux 2006, Despres 2006). In addition, endurance types of activities consume fat as a fuel (e.g. Horowitz & Klein 2000). The results from this twin study add to previous findings (Hannukainen et al. 2011, Magkos 2010) that life-long inactivity is associated with hepatic fat accumulation. A high liver fat content may play a central role in mediating the metabolic perturbations such as insulin resistance (Rector & Thyfault 2011).

Regular physical activity seems to be associated with reduced muscle fat infiltration as the results of this present study seem to also suggest (Wroblewski et al. 2011, Goodpaster et al. 2008). Although fat infiltration is a well-known phenomenon, the mechanisms are still equivocal (e.g. Vettor et al. 2009). Recently much attention has been paid to the link between IMCL and insulin resistance (e.g. Taube, Eckardt & Eckel 2009). However, in this study, the IMCL was not studied separately from the EMCL, instead, MRI was used to quantify the intramuscular fat present within the midthigh muscles. The correlation

analysis revealed only moderate associations between intramuscular fat and glucose homeostasis and that these associations were heavily affected by the diabetic outliers. Also the correlation coefficients of the associations were higher among the physically inactive twins. In the correlation analysis, the moderate relationship between the amount of intramuscular (extramyocellular) ectopic fat and the level of Hb_{A1C}, as illustrated in Figure 13, was also seen in the intrapair difference correlations. Hb_{A1C}, which is a measure of the long-term plasma glucose concentration, is actually one of the factors mediating how exercise decreases the risk for cardiovascular events (Church et al. 2007, Mora et al. 2007). However there were no significant intrapair differences in the fasting Hb_{A1C} levels between the co-twins (Table 5).

Thus, as shown also by the athlete paradox, the hazardousness of IMCL may well be dependent on the level of physical activity and not necessarily depend on the actual amount of IMCL content *per se*. One hypothesis to explain this phenomenon might be the reduced aerobic capacity of inactive (diabetic) individuals. However, although the number of mitochondria has been found to be reduced in the insulin resistant skeletal muscle, the mitochondrial respiratory capacity can be maintained at a normal level (Larsen et al. 2011). Therefore the role of accumulation and function of extramyocellular lipids in contributing to other metabolic disturbances, including insulin resistance, need to be studied more carefully. It does seem as if the increased amounts of extramyocellular fat can be regarded as a marker of impaired metabolism similarly to other high-risk fat depots.

6.4 Metabolic health characteristics

Cardio-metabolic risk factor levels

The HDL/LDL levels and plasma fasting glucose levels were found to be lower among the active twins even after excluding the twins with diabetes and/or cholesterol lowering medication. However, there were no intrapair differences at the follow-up end point in the resting blood pressures. This might be related to the cross-sectional design in which confounding factors other than exercise could well affect the blood pressure levels. It is noteworthy that Barengo et al. (2006) have earlier found a similar finding in the Finnish population (Barengo et al. 2006).

The increased amount of white adipose tissue is related to metabolically triggered inflammation (Hotamisligil 2006). Also various inflammatory pathways are found to be activated in adipose tissue in obese subjects (Pietiläinen et al. 2008b). Exercise has been described to have anti-inflammatory effects (Petersen & Pedersen 2005). However, there were only non-significant differences in CRP levels between the co-twins. A significant intrapair difference in leptin was found. The percentage body fat assessed by multifrequency bioimpedance analysis correlated well with the serum leptin levels ($r=0.72$, $p<0.001$, $n=32$).

Upregulated metabolic and regulatory pathways

Among the physically active members of twin pairs, as compared to their inactive co-twins, gene expression of the central pathways of energy metabolism and supportive metabolic pathways especially those genes related to the processes of oxidative energy production were up-regulated in the skeletal muscle tissue. In fat tissue, the upregulated pathways among the active twins compared to their inactive co-twins were related to branched-chain amino acid degradation and PUFA synthesis. Thus, the data could support the concept that changes in gene expression levels may underlie the muscular and adipose tissue adjustments associated with regular physical activity.

The acquired adaptations in muscle gene expression by habitual activity are to some extent similar to that of higher intrinsic/inherited aerobic capacity detected in high capacity vs. low capacity runner rats (Kivelä et al. 2010) and may indicate that the underlying mechanisms of long-term physical activity on health resemble to some extent those of aerobic capacity. An expression of the oxidative pathway has been previously found to be suppressed in obese subjects having poor fitness (Mustelin et al. 2008). Also a mere nine days bed rest resulted in a considerable decrease in the OXPHOS pathway (Alibegovic et al. 2010).

Intrapair differences in branched-chain amino acid (BCAA) catabolism and in the numbers of mitochondria in adipose tissue have previously been observed among monozygotic twin pairs discordant for obesity (Pietiläinen et al. 2008b). These findings showed down regulated BCAA catabolism among obese twins compared to non-obese co-twins (Pietiläinen et al. 2008b). Dysregulated branched-chain metabolism may make an independent contribution to the development of insulin resistance and glucose intolerance, ultimately leading to type 2 diabetes (Newgard et al. 2009). Thus the findings from the present study could suggest (reversed) BCAA catabolism among consistently active individuals (Table 9).

The present study identified the gene sets most extensively up-regulated among highly and persistently physically active members of the twin pairs compared to their inactive co-twins and gives further information about their association with cardio-metabolic risk (summary in Figure 16). The present study findings also agree with the hypothesis that physical activity-associated increase in the use of skeletal muscles and thus oxidative energy metabolism may contribute to decreased fat accumulation and changes in adipocyte function and to a redistribution of body fat, and further that these changes may influence on the development of e.g. insulin resistance.

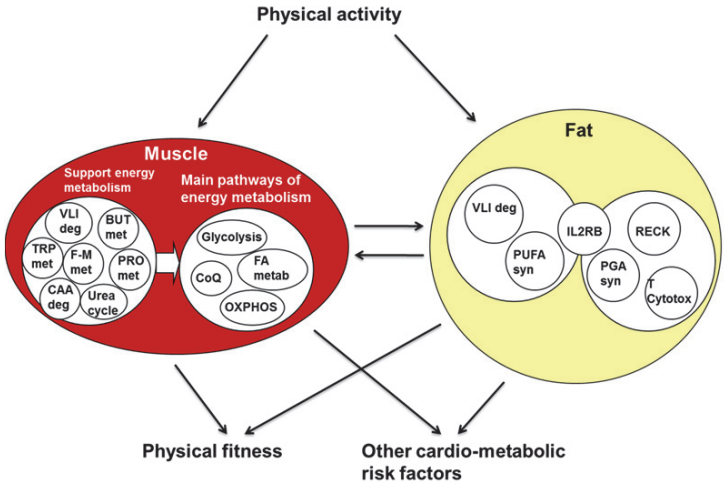

FIGURE 16 Up-regulated gene sets in muscle and fat tissue and their association with cardio-metabolic risk. OXPHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea cycle, Urea cycle and metabolism of amino groups; IL2RB, IL2RB pathway; PUFA syn, Polyunsaturated fatty acid biosynthesis; RECK, RECK pathway; PGA syn, Prostaglandin synthesis regulation; T Cytotox, T cytotoxic pathway. Adapted from article III.

6.5 Strengths and limitations

This study has several strengths. As a result of the complete or close match for genes, age, gender and the intrauterine and childhood environment, the co-twin control study probably represents the best-controlled long-term study design available in humans since adjustment can be made for genetic and familial factors. Despite the strong genetic background in participation in physical activity (e.g. Stubbe et al. 2006), 16 twin pairs with a long-term discordance in leisure-time physical activity habits were found among the baseline healthy 5663 twin pairs (0.28%). Although persistent differences in leisure-time physical activity levels between individuals or siblings are common, it is less common that co-twins of a twin pair have persistently different activity levels. The fact that it was so difficult to identify substantial numbers of twin pairs who were significantly discordant for physical activity emphasizes the importance of a genetic or familial basis in life-long activity patterns. However, this can also be regarded as a limitation as the phenotypic discordance between the co-twins of twin pairs greatly limits the number of twin pairs which could be evaluated in this present study.

This study included an extensive follow-up of leisure-time physical activity habits during adult life (from the subject´s thirties to their sixties). The baseline was the year 1975, when the first physical activity data were available, but it is probable that the discordance in physical activity had begun earlier. In childhood, the inactive and active co-twins were rather similar in terms of their physical activity habits as they reported that eleven of the active and eight of the inactive co-twins participated in competitive sports during childhood (symmetry test, p=0.26). However eight of the active co-twins but none of the inactive co-twins reported of having participated in competitive sports during their adulthood (p=0.005). Motivation could be one of the driving forces that encourage active twins to continue exercise training despite of the environmental changes occurring at the early adulthood (e.g. work, family) (Aaltonen et al. 2012).

A structured physical activity questionnaire was used to measure the dose of leisure-time physical activity at every assessment point. However, the subject´s perception about what leisure-time physical activity is may have changed over time. Therefore the retrospective physical activity assessments may include a greater report bias than the detailed cross-sectional physical activity questionnaires and interviews. Self-reported physical activity can be regarded as a limitation but it would have been impossible to carry out a continuous objective physical activity monitoring throughout decades starting from year 1975. The use of an objective device to capture the total daily activity (instead of only the leisure-time activity dimension) would have been more informative but objective monitoring methods assess only rather short time-periods and the subjects may have abnormal activity levels during these short monitoring periods. There is also seasonal variation in exercise habits in Finland. Moreover, because of the lack of work-related physical activity and physical activity in daily routines in modern life, people should exercise more in their leisure-time. Therefore the use of a physical activity questionnaire to capture lifetime leisure-time physical activity can be considered as adequate and reasonable.

The leisure-time physical activity questionnaire used in the present study has been shown to have high correlations with other questionnaires in previous twin study (Waller, Kaprio & Kujala 2008). Also studies using the similar leisure-time physical activity questionnaire have shown an inverse association with mortality (Kujala et al. 1998, Waller et al. 2010b) and with the occurrence of type 2 diabetes mellitus (Waller et al. 2010a). The inactive twins were also less fit than their active co-twins, a finding which supports the validity of the present activity assessments. By including questions about all the components of leisure-time activity (intensity, duration and frequency) it was possible to calculate the actual dose of activity.

The basics of the discordance was that the inactive twins had leisure-time activity of less than 2 MET h/day and the active twins over 2 MET hours of moderate-to-vigorous activity per day. The 2 MET h/day corresponds to 30-minutes daily walk which is close to the current physical activity

recommendations. Overall the activity level of the inactive members of twin pairs corresponded to that of 50 % of the most physically inactive twin in the whole cohort, and the level of active co-twins was similar to the top 20 % of the active twins in the whole cohort (Kujala et al. 1998), which means that the activity levels utilized in the present study represent clinically relevant population groups. However, the sub-group of discordant MZ pairs were selected with other criteria (intrapair leisure-time activity difference >3 MET h/day). This was done in order to gather a substantial number of healthy physical activity discordant MZ pairs.

It would have been optimal to study MZ twin pairs who have been discordant for physical activity during every time point during the follow-up period, but it was impossible to identify enough such pairs. Therefore, it was decided to include four MZ twin pairs with whom the discordance was not seen at a maximum of one or two of the assessed time points. However, these four MZ pairs had to be discordant at the baseline (in 1975 and 1981) as well as at the physical activity assessments conducted at the end of the follow-up. In this way, the time of being concordant for physical activity was not longer than the time when their activity levels were substantially different.

Childhood family environment and genetic background can be standardized by studying MZ twin pairs. Furthermore by comparing the members of a same-sex twin pair, the effects of age and gender can be controlled. This is important as the response to exercise training varies between non-related individuals, and is influenced by both age and gender. The intrapair differences between inactive and active co-twins were rather similar among the MZ and DZ pairs suggesting that the study findings are not explained by the sequence level genetic differences between the co-twins. However, two confounding factors in twin studies are the epigenetic changes that may be responsible for the phenotypic variation between the co-twins of twin pair (Wong et al. 2010, Talens et al. 2012) and a low birth weight that frequently occurs among twins (Loos et al. 2005). A low birth weight has been shown to be associated with lower levels of conditioning exercise in young adults (Kaseva et al. 2012). A low birth weight has been demonstrated to be associated with type 2 diabetes (Whincup et al. 2008). However, although the lower birth weight is associated with increased disease risk, physical activity has been found to reduce this association among middle-aged men (Laaksonen et al. 2003) and adolescents (Ortega et al. 2011).

Other methodological considerations

Baseline weight and height was self-reported which can be viewed as a study limitation. Body composition at baseline was not measured so that it was not possible to study the changes in body composition over time. The follow-up end point body composition measurements were done using multifrequency impedance plethysmograph body composition analyzer (InBody 720) in a standardized condition after an overnight fast. Although dual-energy X-ray absorptiometry (DXA) is generally regarded as a more valid method of

assessing percentage body fat than bioimpedance, multifrequency bioimpedance analyses have in fact provided rather good assessments of fat mass in healthy subjects and in patients with stable water levels (Völgyi et al. 2008, Pietrobelli et al. 2004). In our laboratory, the correlation coefficients for percentage body fat between the multifrequency bioimpedance analysis and DXA were of a similar magnitude within the different BMI and physical activity groups irrespective of gender (0.55-0.78) (Völgyi et al. 2008). The precision of the repeated measurements during the same morning for percentage body fat was 0.6% (coefficient of variation). A T_1-weighted MRI was acquired to further quantify different fat depots. Previously, MRI has been shown to be a reliable measure for visceral, liver and intramuscular fat contents (Hu, Nayak & Goran 2011). The best correlations between single slice measured visceral adipose tissue area and total VAT volume was found 10 cm and 5 cm higher than the level of L4-L5 in women and men, respectively (Shen et al. 2004, Demerath et al. 2007). Based on this, the single slice at the level of five centimeter above L4-L5 was used for the analysis. However, magnetic resonance spectroscopy would have been more useful since it would have permitted a more specific analysis of the amount of intramyocellular lipids and liver fat content.

Biopsies were taken after an overnight fast at a maximum of one month apart from the first visit. Although all subjects were free from cardio-metabolic diseases and other diseases which would have affected their ability to be physically active in 1975, very long-term physical inactivity may lead to different metabolic consequences, such as insulin resistance, which may influence gene expression in muscle and adipose tissue. The lack of protein-level analyses in addition to the gene expression analyses can be regarded as a defect. However, recently this limitation has been corrected since our group conducted a metabolome analysis in these physical activity discordant twin pairs (Kujala et al. 2013).

6.6 Future directions

The modern lifestyle with its minimal physical activity in daily chores is not necessarily providing the physical stress needed to keep the human body and mind fit and functioning. People spend more and more time sitting in a car, in front of a computer or watching television. The inactive lifestyle may even be risk for health by lowering effort tolerance and energy expenditure resulting in obesity. There is an urgent need to stop the global increase in body weight gain; in fact obesity has now reached the epidemic dimension. More attention should be paid to the net energy balance, that is, daily energy intake vs. daily energy expenditure. The lack of physical effort in work and in routine daily activities calls compensatory activities like leisure-time exercise training as physical activity is the only component of energy expenditure that can be modified behaviorally in a significant manner. To fulfill this at population level as a part

of public health programs one needs to provide evidence based recommendations for non-athletic individuals at different ages.

The main principles of exercise training for non-athletic exercisers are the overload and reversibility principles. This means that the activity performed should be strenuous enough to unbalance basal homeostasis but also that the exercise bouts are performed regularly so that the bouts of different exercises are frequent enough to elicit training responses. The best way to achieve this goal is to ensure that exercise training is adopted as a part of lifestyle habits. If one wishes to fulfill the current physical activity recommendations, then thirty minutes of moderate physical activity in at least five days per week are needed. However, this requires motivation, time and facilities.

Investigations into the reasons for global inactivity and for the consequences of inactivity will be of great interest in the future. Furthermore it is important to determine why so many people are inactive even though there is so much evidence of the health benefits of physical activity. In addition more knowledge of the individual responses to physical activity should be gathered in order to understand why some subjects are so called low responders. A need for individually tailored training programs represents a challenge for the future.

The present study addressed questions and produced further hypothesis related to the underlying mechanisms that mediate exercise training-induced health effects in two respects; i) the pathway analysis revealed rather strong association between long-term physical activity and the genes encoding energy metabolism in muscle and fat tissues (such as OXPHOS, BCAA degradation), and ii) clear associations were found between the cardio-metabolic risk factors and gene cluster expression (Figure 14). More studies on the mechanisms by which physical activity reduces the risk for cardio-metabolic disease are needed as these findings can also benefit drug development. Thus, exercise is medicine.

The present thesis was an observational study. Therefore it was not possible to investigate causality or dose-response. However, one of the messages of this thesis could be that activity-induced health is not always visible on a weight scale; regular exercise may evoke no change in body weight, but cause a gain in muscle properties and decline in ectopic body fat depots. This message should increase the motivation to exercise in order to gain health benefits beyond simply body weight changes. Thus many of the health benefits of exercise could not be seen without sophisticated measurement methods and there is a lot more to found. Therefore more long-term RCT studies using sophisticated methods for physical activity and outcome measures and mechanisms are needed to provide confirmatory evidence of the above questions and to clarify the specific effects of long-term leisure-time physical activity on high-risk body fat depots, especially ectopic fat stores, and related chronic disease risk factors. Also more confirmatory studies utilizing design with a standardized genetic background are needed.

7 MAIN FINDINGS AND CONCLUSIONS

The strong relationship between physical activity and health has been previously identified by epidemiological studies. In this study, the associations were studied among physical activity discordant co-twins of monozygotic and dizygotic twin pairs.

The main findings of the present study can be summarized as follows:

Long-term leisure-time physical activity associates with

- better cardiorespiratory fitness;
- better lower limb muscle strength and composition;
- lower levels of body fat mass;
- reduced levels of visceral, intramuscular (extramyocellular) and liver fat;
- upregulation of metabolic and regulatory pathways in skeletal muscle and subcutaneous adipose tissues which correlated with cardio-metabolic risk

when compared to long-term inactivity.

In conclusion, the results of this study are in line with the findings of earlier epidemiological studies and shorter-term RCT studies that physical activity associates with better physical fitness and lower levels of body fat. The present study however provides compelling evidence that long-term leisure-time physical activity is associated with smaller levels of ectopic fat also when genetic background is standardized. Therefore regular leisure-time physical activity may give a major advance in the prevention of cardio-metabolic diseases. This present study also produces more hypotheses of the mechanisms that may underlie the relationship between long-term physical activity and reduced cardio-metabolic disease risk.

YHTEENVETO (FINNISH SUMMARY)

Kaksosparien jäsenten välisen pitkäaikaisen liikunta-aktiivisuuseron yhteys fyysiseen kuntoon, kehon koostumukseen ja metabolisiin tekijöihin

Fyysisen aktiivisuuden terveyttä edistävistä vaikutuksista on tiedetty jo vuosisatoja, mutta epidemiologista tietoa liikunnan kuntoa parantavista ja terveyttä edistävistä ominaisuuksista on kerätty vasta viimeisten vuosikymmenten ajan. Kuitenkin on niin, että sydän- ja aineenvaihduntasairaudet ovat yhä yleisempiä, sillä fyysinen aktiivisuus ja liikkuminen ihmisten arkipäivässä ei ole enää välttämätöntä.

Krooniset sairaudet kehittyvät vuosikymmenien kuluessa ja siksi on ollut vaikea tieteellisesti osoittaa, mikä on elämäntavan ja toisaalta perimän osuus sairauksien ilmaantumiseen. Tutkimalla suurilla aineistoilla väestöä ja toisaalta pienemmillä aineistoilla liikkujia vs. ei-liikkujia, on pystytty osoittamaan, että liikunta-aktiivisuudella on yhteys sydän- ja aineenvaihduntasairauksien riskitekijöihin ja ilmaantuvuuteen. Lyhytkestoisilla satunnaistetuilla liikuntainterventiolla on voitu selvittää viikkojen tai kuukausien ajan kestäneen harjoitusohjelman tuottamia vaikutuksia elimistön rakenteisiin ja aineenvaihdunnan säätelyyn. Nykyajan liikuntasuositukset perustuvat tällaisiin tutkimuksiin. Niiden tavoite on perustella ja osoittaa, millainen liikunta ja missä määrin on tarpeen terveyden ja toimintakyvyn edistämisessä ja inaktiivisuuteen liittyvien sairauksien ja oireyhtymien ehkäisyssä. Tutkimustieto on myös osoittanut, että säännöllisellä liikunnalla on sekä ennalta ehkäisevää, sairauksien ilmaantumista myöhentävää ja todellista hoitavaa vaikutusta.

Koska elämänaikaista, vuosikymmeniä jatkuvaa liikuntainterventiota ei voi tieteellisenä kokeena järjestää, tässä väitöskirjatutkimuksessa etsittiin suomalaisesta kaksosrekisteristä sellaiset identtiset ja ei-identtiset kaksoset, jotka olivat eläneet liikunnallisilta tottumuksiltaan erilaista elämää vuosikymmenien ajan. Tällainen asetelma ei täysin korvaa tieteelliseen syy-seuraus suhteen analysointiin oikeuttavaa randomisoitua koetta, mutta mahdollistaa myös geneettisesti standardoidussa tilanteessa elämäntavan ja sen seurausten yhteyksien tarkastelun mm. kehon koostumuksen, sairauksien riskitekijöiden ja fyysisen kunnon alueilla. Toisin sanoen tällaisella kaksostutkimusasetelmalla päästään lähemmäksi pitkäaikaisen liikunnan todellista osuutta edellä mainituissa tekijöissä.

Tämä väitöskirjatutkimus käsittelee vuosikymmeniä kestäneen vapaa-ajan liikunta-aktiivisuuden vs. liikkumattomuuden yhteyksiä fyysiseen kuntoon, kehon koostumukseen ja muihin metabolisiin tekijöihin kuudellatoista keskiikäisellä tai vanhemmalla identtisellä (n=7 paria) ja ei-identtisellä (n=9 paria) kaksosparilla, joiden jäsenet eroavat toisistaan pääasiassa vain liikunta-aktiivisuuden suhteen. Tutkimalla liikunnan suhteen eroavia identtisiä kaksosparin jäseniä perimän osuus voidaan standardoida, koska identtiset kaksoset omaavat saman geeniperimän. Tutkittujen kaksosparien välinen ero liikunnan määrässä oli jatkunut 32 vuotta eli koko aikuisiän ajan. Liikunta-aktiivisuutta

mitattiin seuranta-aikana esitettyjen liikuntakyselyjen perusteella muodostetun liikunnan volyymiä kuvaavan MET indeksin avulla.

Seuranta-ajan lopussa, vuonna 2007, kaksosten aerobinen kunto mitattiin epäsuoralla polkupyöräergometritestillä, reiden ojentajien lihasvoima voima-dynamometrillä ja kehon koostumus bioimpedanssimenetelmällä (Inbody 720). Vatsa-alueen rasvakudoksen jakautumista (viskeraalirasva ja maksan rasva) ja reiden lihas- ja rasvakoostumusta mitattiin magneettikuvaleikkeistä. Lisäksi kymmeneltä parilta otettiin lihas- ja rasvakudosnäyte eri geeniryhmien ilmentymistä mittaavaa analyysiä varten.

Tutkimustulokset osoittivat, että aikuisiän kestänyt liikunta-aktiivisuus oli yhteydessä parempaan fyysiseen kuntoon. Aktiivisten kaksosten epäsuoran kuntotestin perusteella arvioitu hapenottokyky oli 23 % korkeampi ($p<0.001$) ja mitattu reiden ojentajien lihasvoima 20 % korkeampi ($p=0.006$) kuin heidän inaktiivisten kaksostensa. Kaksosparien jäsenten välinen painoero kaksinkertaistui seuranta-ajan aikana, mutta se ei ollut merkitsevä seurannan lopussa (inaktiivit: 79.5±18.4 kg vs. aktiivit: 72.9±11.9 kg, $p=0.12$). Inaktiivisten kaksosten rasvamassa oli 6 kg suurempi ($p=0.015$) ja koko kehon rasvaprosentti 5.4 prosenttiyksikköä korkeampi ($p=0.004$) verrattuna aktiivisiin kaksosiin. Kehon rasvattomassa massassa tai reiden lihasten volyymeissa ei ollut tilastollisesti merkitseviä eroja aktiivisten ja inaktiivisten kaksosten välillä. Magneettikuvat osoittivat, että inaktiivisilla kaksosilla oli puolitoista kertaa suurempi viskeraalirasvan pinta-ala ($p=0.010$) ja puolitoista kertaa enemmän reiden lihasten sisäistä rasvaa ($p=0.047$). Lisäksi inaktiivisilla kaksosilla oli jopa yli kaksi kertaa suurempi maksan rasvapitoisuutta kuvaava arvo ($p=0.011$) kuin heidän aktiivisilla kaksossisaruksillaan. Reiden lihasten sisäisen rasva-alan ero ja maksan rasvoittumista kuvaan arvon ero oli merkitsevä myös pelkästään identtisillä kaksosilla tehdyssä parittaisessa analyysissä. Lisäksi lihas- ja rasvakudosnäytteiden geenianalyysit antoivat viitteitä siitä, että aineenvaihduntaa säätelevien geenisettien (mm. oksidatiivinen fosforylaatio, haaraketjuisten aminohappojen pilkkoutuminen) ilmentyminen on yhteydessä pitkäaikaisen liikunnan harrastamiseen, ja että nämä geeniryhmät saattavat välittää säännöllisen liikunnan terveysvaikutuksia.

Tämä väitöskirja vahvistaa aiempia havaintoja ja osoittaa yhtäpitävästi aiempien tutkimusten kanssa, että läpi aikuisiän jatkunut säännöllinen liikunta on myönteisessä yhteydessä fyysisen kuntoon ja terveyttä kuvaaviin kehon koostumuksen ja metabolisen terveyden muuttujiin. Säännöllinen vapaa-ajan liikunta hidasti painonnousua ja suojeli terveydelle haitallisen rasvan kertymiseltä kun taas pitkäaikainen liikkumattomuus oli yhteydessä etenkin ektooppisen rasvan kertymiseen reisilihasten sisälle ja maksaan silloinkin, kun perimän osuus oli standardoitu. Lisäksi aineenvaihduntaan liittyvien geenien ilmentyminen oli aktiivisilla kaksosilla suurempaa kuin inaktiivisilla, ja nämä geenisetit olivat yhteydessä mm. kunto- ja kolesteroliprofiiliin. Näin ollen liikunnallinen elämäntapa saattaa olla merkittävä itsenäinen tekijä aineenvaihduntasairauksien ehkäisyssä.

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ORIGINAL PAPERS

I

EFFECTS OF 32-YEAR LEISURE TIME PHYSICAL ACTIVITY DISCORDANCE IN TWIN PAIRS ON HEALTH (TWINACTIVE STUDY): AIMS, DESIGN AND RESULTS FOR PHYSICAL FITNESS

by

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Effects of 32-Year Leisure Time Physical Activity Discordance in Twin Pairs on Health (TWINACTIVE Study): Aims, Design and Results for Physical Fitness

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The physically active lifestyle is associated with low future morbidity and mortality, but the causality between physical activity and health is not always clear. As some inherited biological characteristics and childhood experiences may cause selection bias in observational studies, we sought to take them into account by identifying 16 twin pairs (7 MZ, 9 DZ, mean age 60 years) discordant for leisure time physical activity habits for thirty years. We conducted detailed health-related examinations among these twin pairs. Our main aims were to study the effects of physical activity and genes on fitness and body composition, with special reference to body fat compartments, metabolic syndrome components and related diseases and risk factor levels, status of arteries, structure and function of the heart, bone properties, and muscle and fat tissue-related mechanisms linked to physical activity and chronic disease development. Our physical activity assessments showed that inactive co-twins were on average 8.8 MET hours/day less active than their active co-twins through out their midlife (2.2 ± 2.3 vs. 11.0 ± 4.1 MET h/day, $p < .001$). Follow-up fitness tests showed that physically inactive co-twins were less fit than their active co-twins (estimated VO_{2peak} 26.4 ± 4.9 vs. 32.5 ± 5.5 ml/kg/min, $p < .001$). Similar differences were found in both MZ and DZ pairs. On the basis of earlier epidemiological observations on nonrelated individuals, these physical activity and fitness differences are large enough to cause differences in many mechanisms and risk factors related to the development of chronic diseases and to permit future analyses.

Keywords: physical activity, fitness, twin study

Various studies have shown that physical fitness and participation in physical activity have a genetic component (Beunen & Thomis, 1999; Bouchard et al., 1986; Stubbe et al., 2006). The genetic component of physical activity may be shared with that of chronic diseases. Some inherited biological characteristics may both make it easier for some individuals to achieve high levels of physical activity or fitness and favor them with low morbidity or with longevity (Kujala et al., 2002; Kujala et al., 2003; Morris et al., 1956). Consequently, it is difficult to estimate the true extent of the effect of physical activity on delaying morbidity or mortality from observational follow-up studies of nonrelated individuals. Randomized controlled trials (RCTs) indicate that exercise is a powerful means of enhancing fitness and reducing disability among elderly subjects with or without chronic diseases; however less is known about the actual progression of diseases (Kujala, 2004).

When studying the associations between physical inactivity/activity and health outcomes we are faced with two specific issues: (1) genetic selection may explain some of the associations between baseline physical activity and morbidity/mortality in observational follow-up studies, and (2) RCTs that investigate the effects of exercise on health outcomes are usually of a too-short duration to document the long-term effects on health. The latter often use proxy measures and biomarkers as risk indicators. To tackle these limitations in

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our study design, we investigated monozygotic (MZ) and dizygotic (DZ) twin pairs, discordant for leisure time physical activity (LTPA) habits, to document the specific effects/associations of long-term physical activity on different health outcomes while controlling for genes and childhood family environment.

This study protocol article reports the aims and design of the TWINACTIVE study and results for physical fitness.

Background and Main Aims of the TWINACTIVE Study

We have previously used the Finnish Twin Cohort (Kaprio et al., 2002) to investigate whether genes or other familial factors cause selection bias in epidemiological studies of the associations between physical activity and future morbidity and mortality. In this cohort results from both individual-based analyses and pairwise analyses of DZ twin pairs discordant for physical activity show that baseline physical activity is associated with reduced future premature mortality (Kujala et al., 1998; Kujala et al., 2002). However, the fact that we were not able to confirm this by studying MZ twin pairs persistently discordant for physical activity (Kujala et al., 2002) suggests that genetic selection may play a role. In relation to specific diseases, we have found that among MZ twin pairs discordant for physical activity type 2 diabetes mellitus develops earlier among physically inactive twins when compared to their physically active co-twins (Kujala et al., 2000). In addition, among male MZ pairs, physically active twins tend to have less coronary heart disease compared to their inactive co-twins (Kaprio et al., 2000).

To obtain a more detailed picture of the effects of physical activity and genetic factors on health, we identified middle-aged MZ and DZ twin pairs discordant for LTPA habits for 30 years and conducted detailed health-related examinations among these twin pairs. We were interested in the effects of physical activity and genes on fitness, body composition, with special reference to body fat compartments, metabolic syndrome components and related diseases and risk factor levels, status of arteries, structure and function of the heart, bone properties, and muscle and fat tissue-related mechanisms linked to physical activity and the development of chronic disease (see Table 1 for measures and outcomes).

We hypothesized that long-term physical activity would be found to have beneficial effects on many of the studied variables and outcomes among the MZ pairs discordant for physical activity. The existence of some intra-pair differences among the discordant DZ pairs, but not among the MZ pairs, would indicate the presence of a genetic component. We also hypothesized that skeletal muscle properties, known to be influenced by genes, would be associated with different metabolic syndrome features and these

Table 1

Timetable of Data Collection During Laboratory Visits in 2007

VISIT 1	
Before	Structured instructions for the measurements. Assessments of 5-d diet and 7-d activity using diaries.
Day 1	
11 am	Blood sample.
12 pm	Standardized interview to assess smoking habits, use of alcohol, dietary habits and exercise attitudes.
1 pm	Echocardiography of cardiac cavity, wall dimensions, systolic and diastolic function of left ventricle.
1.45 pm	Standardized clinical examination. Assessment of medication and health status.
2.15 pm	Resting electrocardiography.
2.30 pm	Symptom-limited maximal clinical exercise test.
3.15 pm	Maximal isometric left knee extensor strength, and left and right hand grip strength measurement.
4 pm	Measure of properties of bone by peripheral quantitative computed tomography.
10 pm	Fast begins.
Day 2	
7.30 am	Anthropometric measurement and assessment of body composition using bioelectrical impedance.
8 am	Fasting blood and DNA sample in order to study atherogenic and metabolic abnormalities.
8.15 am	Oral glucose tolerance test.
8–10 am	Standardized LTPA interview to assess leisure time MET index.
12 pm	MR imaging from abdomen and thigh. MR angiography of macroscopic arteries.
VISIT 2	
Before	Structured instruction of exercise before/after biopsy. Overnight fast.
Day 3	
8–10 am	Muscle and subcutaneous adipose tissue biopsies for histological, biochemical and gene expressional studies.

associations may partly explain why DZ twins are more discordant for physical activity than MZ twin pairs. In agreement with earlier cross-sectional data, we have already documented in longitudinal studies on unrelated individuals that skeletal muscle properties (in particular proportion of type I muscle fibers) predict different components of metabolic syndrome in men (Hernelahti et al., 2005; Karjalainen et al., 2006).

Subjects and Methods

Baseline LTPA Questionnaires

The Finnish Twin Cohort includes same-sex twin pairs born in Finland before 1958 and with both co-twins alive in 1975 (Kaprio et al., 2002). In the cohort, there were 1772 MZ, 3551 DZ and 340 twin pairs with unknown zygosity composing the cohort who were 24 to 60 years old, employed and healthy in 1981 (Kujala

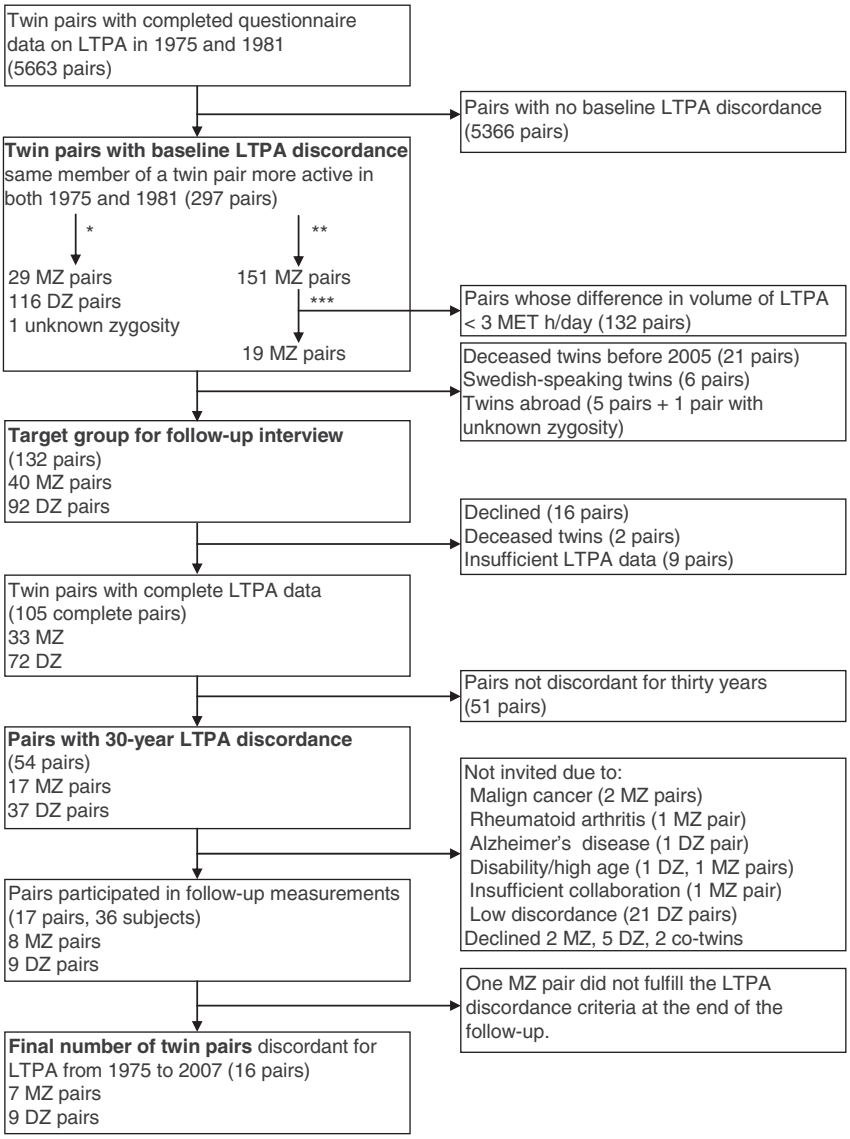

Figure 1

Flow chart of the comprehensive selection of twin pairs discordant for leisure time physical activity (LTPA).

Note: *Baseline discordance in volume (2 MET criteria) and intensity of LTPA (see text)
**Baseline discordance only in volume of LTPA (2 MET criteria) (see text)
***Baseline discordance in volume of LTPA (3 MET criteria) (see text)

et al., 2002). They all had completed the LTPA questionnaire administered in 1975 and in 1981 (Figure 1).

Assessment of LTPA volume (leisure time MET index) was based on a series of structured questions on leisure activity and physical activity during journeys to and from work (Kujala et al., 1998). The leisure time MET index was then calculated by assigning a multiple of the resting metabolic rate to each form of physical activity (intensity of activity x duration of one session x monthly frequency), which was then expressed as a sum score of leisure time MET hours/day. Assessment of the intensity of activity was based on the following question: Is your physical activity during leisure time about as strenuous on average as: (1) walking, (2) alternately walking and jogging, (3) jogging, (4) running? Those who chose 2, 3 or 4 were classified as engaging in vigorous activity (Kujala et al., 1998).

After calculating the leisure time MET indices, we found that 146 pairs (29 MZ, 116 DZ, and 1 unknown zygosity pair) were discordant for LTPA both in participation in vigorous activity and in volume of activity both in 1975 and 1981 (Figure 1). The criterion for the baseline discordance was that one co-twin was physically active (calculated leisure time MET index was > 2 MET h/day corresponding to about 30 min walking per day) while his/her co-twin was less active (leisure time MET index < 2 MET h/day) in both assessments (Kujala et al., 2002; Waller et al., 2008).

To increase the number of LTPA discordant MZ twin pairs, we set up another selection criterion and found that 151 MZ pairs were discordant in volume of activity (2 MET criteria as described above, vigorous activity discordance ignored). Among these MZ pairs, we selected only those 19 MZ pairs whose difference in volume of activity was > 3 MET h/day between the inactive and active co-twin in both 1975 and 1981, while the average intensity of a physical activity session was the same or greater in the active vs. inactive co-twin (Figure 1). This resulted in 165 comprehensively selected twin pairs (48 MZ, 116 DZ, 1 unknown zygosity pair) with baseline discordance for LTPA. These twin pairs constituted a target group for our follow-up interview on midlife LTPA habits. As our aim was to investigate the health effects of physical activity in twin pairs with long-term persistent discordance for LTPA, pairs not persistently discordant for LTPA were excluded during later stages of the study (described later).

Follow-Up LTPA 1980–2005 Interview

Follow-up interviews were carried out during the years 2005–2007 with 132 twin pairs (40 MZ, 92 DZ pairs) as only those pairs were included in which both co-twins were still alive, lived in Finland, and spoke Finnish as their mother tongue. In total, 105 twin pairs (33 MZ, 72 DZ pairs), that is, 80% of available targeted pairs, completed the physical activity assessment section in the follow-up interviews (Figure 1).

The telephone interview included questions on current and past physical activity (for more details see Waller et al., 2008). In brief, physical activity was assessed by two sets of questions. The first shorter retrospective timeline assessment of LTPA volume and intensity (with 5-year intervals) was carried out using the same physical activity questions as in 1975 and 1981 (Figure 2). The mean MET index for all six time points from 1980 to 2005 was then calculated for both the inactive and active co-twins. To aid recall, twins were asked to describe their marital and work status for each year before the retrospective LTPA questions. The second set of questions was a detailed assessment of the volume of leisure time, daily (nonexercise activities such as gardening) and commuting activity over the previous 12 months (12-month MET index) using a modified version of the Kuopio Ischemic Heart Disease Risk Factor Study Questionnaire (Lakka & Salonen, 1997). As a result of these physical activity assessments, we found 17 MZ and 37 DZ twin pairs who, in addition to baseline discordance, were discordant for LTPA habits throughout the follow-up (i.e., 30 years).

Follow-Up Interview and Measurements in 2007

Before issuing an invitation to participate in the laboratory study measurements, we excluded pairs whose health status/medication would severely violate our study aims. Pairs were excluded for the following reasons: one co-twin had malign cancer (2 MZ pairs), oral corticosteroid treatment for rheumatoid arthritis (1 MZ pair), Alzheimer's disease (1 DZ pair), severe

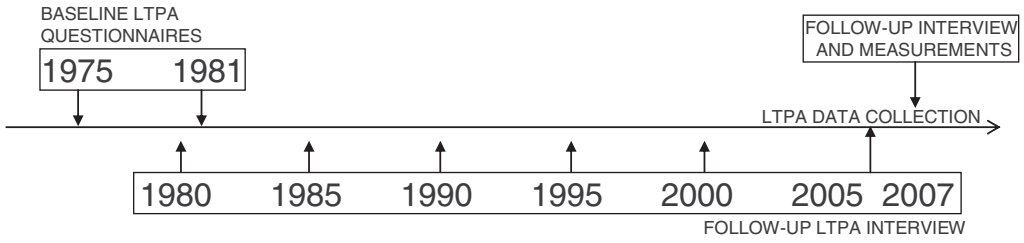

Figure 2

Timetable of physical activity and risk factor data collection.

disability/high age (1 DZ and 1 MZ pair) and insufficient collaboration (1 MZ pair). We also excluded 21 DZ pairs whose LTPA discordance was at the lowest level or not persistent. This procedure left us with 12 MZ and 14 DZ twin pairs. Of these 26 pairs who were invited to the laboratory, 8 MZ and 9 DZ twin pairs (overall 36 twin individuals) underwent our detailed health-related examinations.

Final Number of Twin Pairs

After careful intrapair examination of the leisure time MET indices from 1975 to 2007, we found that 7 MZ (5 male and 2 female pairs) and 9 DZ pairs (6 male and 3 female pairs) fulfilled our discordance criterion. In one MZ pair the leisure activity MET indices were higher among the previously inactive co-twin and lower among his previously active co-twin at the last follow-up assessment. This pair was excluded from the data analysis. The zygosity of the twins was verified at the Paternity Testing laboratory (National Public Health Institute, Helsinki, Finland) using DNA extracted from venous blood sample with a battery of ten highly polymorphic gene markers.

Physical Activity and Fitness Assessments

In the follow-up measurements, LTPA volume (leisure time MET index 2007) and participation in vigorous physical activity were assessed by the same questions as used at the baseline and in the retrospective assessment. Detailed assessment of the volume of leisure time and of daily and commuting activity over the previous 12 months (total 12-month MET index 2007) was also carried out (Lakka & Salonen, 1997). Smoking habits and use of alcohol were collected with diary, questionnaire and interview methods, as used earlier in the cohort (Kujala et al., 1998; Waller et al., 2008).

The symptom-limited maximal clinical exercise test with a cycle ergometer was performed for the assessment of cardiorespiratory fitness using a slightly modified WHO protocol (Lange-Andersen et al., 1971). The testing protocol comprised of 2-minute stages, beginning with a learning stage at 20 W and a warm-up stage at 25 W. Thereafter the increase in workload was 25 W/stage. The recovery stage was performed at 25 W and lasted at least 5 minutes. At the end of each stage, heart rate was recorded from the electrocardiogram, blood pressure was measured and testee was asked to rate their perceived exertion (Borg's scale 6–20). During the recovery stage, these measurements were performed at the end of 1, 3 and 5 minutes. Total exercise time, peak load, and estimated oxygen uptake (for calculation see Table 5) were used as indices of cardiorespiratory fitness.

Ethical Approval

This study was conducted according to good clinical and scientific practice/guidelines and the Declaration of Helsinki. The ethics committee of the Central Hospital of Central Finland approved our study plan on August 15, 2006.

Statistics

We used pairwise analyses to study differences between co-twins of the twin pairs. First we analyzed the results for all the twin pairs and then for MZ and DZ twin pairs separately to find out whether the trends were similar for MZ and DZ pairs. The normality of variables was assessed by the Shapiro-Wilk test. Student's paired t test was used for normally distributed variables. For non-normally distributed variables the Wilcoxon signed rank test was used. The symmetry test (Stata) was used for categorical variables. 95% confidence intervals (CI) were calculated for the absolute mean differences between inactive and active co-twins. The level of significance was set at $p < .05$. Data were analyzed using SPSS 14.0 and Intercooled Stata 8 software.

Results

Subject Characteristics

There were no baseline differences in 1975 for anthropometrics, marital status, alcohol use, smoking habits or work-related physical activity between inactive and active co-twins. Inactive co-twins were less active in their leisure time than their active co-twins (–3.1 MET h/day, 95% CI –4.2 to –1.9 MET h/day, $p < .001$; see Table 2).

There were no statistical differences in alcohol use, smoking habits, or in work-related physical activity between the inactive and active co-twins in 2007. Inactive co-twins weighed 6.5 kg more (95% CI –2.2 to 15.3, $p = .12$) on average and had a 1.9 kg/m^2 higher mean body mass index (95% CI –0.4 to 4.1, $p = .09$) than their active co-twins (Table 3).

Physical Activity Discordance

Physical activity discordance, which showed a decreasing trend with time during the retrospective follow-up (1980–2005), is illustrated in Figure 3. In 2007, the leisure time MET index was on average 6.9 MET h/day lower ($p < .001$) among inactive co-twins compared to their active co-twins, also in both MZ (–7.0 MET h/day, 95% CI –11.4 to –2.6, $p = .018$), and DZ pairs (–6.7 MET h/day, 95% CI –9.9 to –3.6, $p = .008$). During the LTPA follow-up period, from 1980 to 2007, the inactive co-twins were on average 8.8 MET h/day less active than their active co-twins ($p < .001$; see Table 4). The mean differences were similar for both MZ and DZ pairs (Table 4) and for female (–9.0 MET h/day, 95% CI –13.1 to –4.8, $p = .043$) and male (–8.8 MET h/day 95% CI –1.9 to –5.6, $p = .003$) pairs. Eight of the active co-twins, but none of the inactive co-twins, reported having participated in competitive sports during adulthood ($p = .005$).

When we assessed the volume of leisure time, daily and commuting activity over the previous 12 months (Lakka & Salonen, 1997), significant differences between inactive and active co-twins were found in total and leisure time physical activities but not in daily and commuting activities (Table 4). The trends were similar for MZ and DZ pairs. The two question-

Table 2

Baseline Characteristics in 1975

Characteristics	Inactive *N* = 16	Active *N* = 16	*p* value
Age (year; mean, range)	28 (18–42)		
Height (cm) (*N* = 15)	173.7 ± 9.8	172.9 ± 10.1	.57
Weight (kg)	69.3 ± 16.4	66.0 ± 9.4	.57
BMI (kg/m^2) (*N* = 15)	23.0 ± 4.2	22.3 ± 2.0	.88
Ever smoked regularly by 1975 (*N*)	6	9	.25
Leisure time MET index 1975 (h/day)	0.2 ± 0.3	3.3 ± 2.4	<.001
Alcohol (g/day)	6.0 ± 8.3	9.6 ± 12.8	.65
Marital status (*N*)			.37
Single	5	7	
Married	11	8	
Divorced	0	1	
Work related physical activity (*N*)			.23
Sedentary	3	7	
Standing or walking at work	3	4	
Light manual work	10	5	
Heavy manual work	0	0	
Occupational group (*N*)			.43
Upper white-collar	1	3	
Clerical work	5	5	
Skilled workers	4	6	
Unskilled workers	1	0	
Farmers	3	0	
Other	2	2	

Note: Data are mean ± *SD*

Table 3

Follow-up Characteristics Among Twin Pairs Discordant for LTPA in 2007

Characteristics	Inactive *N* = 16	Active *N* = 16	*p* value
Age (year; mean, range)	60 (50–74)		
Height (cm)	171.8 ± 10.4	171.1 ± 9.9	.63
Weight (kg)	79.5 ± 18.4	72.9 ± 11.9	.12
BMI (kg/m^2)	26.7 ± 3.5	24.8 ± 2.6	.09
Alcohol (g/day)	6.5 ± 4.7	10.3 ± 10.1	.16
Smoking (N)			.26
Current smokers	3	0	
Quitters	7	8	
Never smoked	6	8	
Work status (N)			.36
Employed	9	9	
Retired	5	5	
Unemployed	0	1	
Other	2	1	
Work-related physical activity (N)			.17
Sedentary	3	5	
Standing or walking at work	1	3	
Light manual work	5	0	
Heavy manual work	0	1	

Note: Data are mean ± *SD*

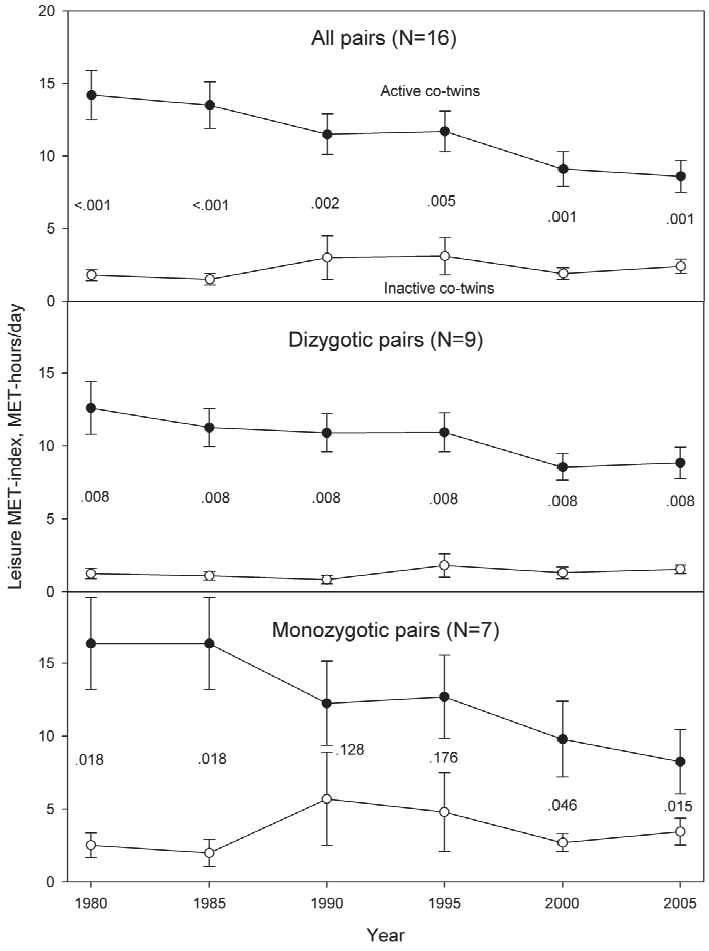

Figure 3

LTPA discordance in 1980–2005 on the basis of the retrospective telephone interview. Upper panel for all 16 pairs, middle panel for 9 DZ pairs, and lower panel for 7 MZ pairs. Results are shown as mean ± SEM. Paired *p* values for statistical difference between inactive and active co-twins by Wilcoxon signed rank test.

naires on the volume of LTPA (the retrospective timeline vs. previous 12-month assessment) showed a good correlation ($r = 0.73$, $p < .001$, $N = 36$).

Physical Fitness

Statistically significant differences in fitness characteristics as measured by the symptom-limited maximal clinical exercise test were found between inactive and active co-twins (Table 5). Total exercise time was 144 seconds (2 min 24 s) shorter ($p = .002$) and achieved peak load 29.3 watts lower ($p = .003$) in inactive co-twins. Estimated peak oxygen uptake was also 6.1 ml/kg/min lower ($p < .001$) in inactive compared to active co-twins. The trends were similar for MZ and DZ pairs (Table 5) and for female (–6.4 ml/kg/min, 95% CI –11.6 to –1.2, $p = .027$) and male (–5.9 ml/kg/min, 95% CI –10.0 to –1.9, $p = .008$) pairs. Fourteen out of 16 active co-twins achieved higher peak oxygen uptake than their inactive co-twins.

Comment

The overall aim of the TWINACTIVE study is to investigate the consequences of LTPA in comprehensively selected MZ and DZ twin pairs who have been discordant for LTPA habits during their adult lives. The present study reports the general aims and design of the TWINACTIVE study together with the LTPA and fitness data. LTPA discordance in leisure time activities was determined with repeated standardized questions. The mean MET difference throughout the follow-up was 8.8 MET h/day between inactive and active co-twins. This amount of METs (h/day) is the equivalent of a difference of 612.7 kcal (2611 kJ) in

Table 4

MET Indices (MET hours/day) Among Twin Pairs Discordant for LTPA

Variable	Inactive	Active	Mean difference (95% CI)	*p* value
All 16 pairs				
Mean MET index 1980–2007	2.2 ± 2.3	11.0 ± 4.1	–8.8 (–11.0 to –6.6)	< .001
Leisure time MET index 2007	1.6 ± 1.4	8.4 ± 4.1	–6.9 (–9.1 to –4.6)	< .001
Total 12-month MET index 2007	5.2 ± 4.4	9.6 ± 5.3	–4.4 (–7.0 to –1.8)	.003
Leisure time 12-month MET index	2.3 ± 1.8	7.2 ± 3.8	–5.0 (–7.1 to –2.9)	.002
Daily activity 12-month MET index	2.7 ± 3.8	2.1 ± 3.3	0.6 (–1.7 to 2.8)	.84
Commuting 12-month MET index	0.2 ± 0.4	0.2 ± 0.3	0.03 (–0.3 to 0.3)	.89
7 MZ pairs				
Mean MET index 1980–2007	3.4 ± 3.0	12.2 ± 5.4	–8.8 (–14.1 to –3.5)	.018
Leisure time MET index 2007	2.4 ± 1.4	9.4 ± 3.8	–7.0 (–11.4 to –2.6)	.018
Total 12-month MET index 2007	6.3 ± 6.0	10.5 ± 4.9	–4.2 (–8.2 to –0.2)	.063
Leisure time 12-month MET index	3.0 ± 2.2	9.1 ± 3.9	–6.1 (–9.9 to –2.3)	.028
Daily activity 12-month MET index	3.1 ± 4.9	1.4 ± 1.9	1.7 (–2.6 to 6.0)	.74
Commuting 12-month MET index	0.3 ± 0.6	0.05 ± 0.1	0.24 (–0.2 to 0.7)	.29
9 DZ pairs				
Mean MET index 1980–2007	1.3 ± 0.9	10.1 ± 2.8	–8.9 (–11.0 to –6.7)	.008
Leisure time MET index 2007	1.0 ± 1.0	7.7 ± 4.3	–6.7 (–9.9 to –3.6)	.008
Total 12-month MET index 2007	4.3 ± 2.6	8.8 ± 5.7	–4.6 (–8.9 to –0.2)	.021
Leisure time 12-month MET index	1.7 ± 1.2	5.8 ± 3.1	–4.1 (–6.9 to 1.3)	.021
Daily activity 12-month MET index	2.4 ± 2.8	2.8 ± 4.1	–0.3 (–3.4 to 2.7)	.95
Commuting 12-month MET index	0.1 ± 0.3	0.3 ± 0.4	–0.1 (–0.5 to 0.3)	.50

Note: Data are mean ± *SD*

Mean MET index 1980–2007, mean MET value from follow-up; Leisure time MET index 2007, leisure time MET index in 2007 according to retrospective questionnaire including journeys to and from work; Total 12-month MET index 2007, leisure time + daily (such as gardening) + commuting activities according to 12-month detailed physical activity questionnaire.

leisure time energy expenditure between inactive and active co-twins (173.3 ± 192.5 vs. 794.9 ± 303.3 kcal, 95% CI –783.8 to –459.5, $p < .001$, when 1 MET corresponds to energy expenditure of 1 kcal/kg/h for the average adult). The activity level of inactive members of twin pairs corresponded to that of the most physically inactive 20% of the whole cohort, and the level of active co-twins to that of the active 50% of the whole cohort (Kujala et al., 1998), which means that the activity levels selected for study represent clinically relevant population groups. The inactive twins, again in both MZ and DZ pairs, were also less fit than their active co-twins, a finding that strongly supports the validity of our activity assessments.

On the basis of earlier epidemiological observations on nonrelated individuals, the intrapair differences in the level of physical activity observed in this study are large enough to cause differences in many mechanisms and risk factors related to the development of chronic diseases (Kelley & Goodpaster, 2001; Kohl, 2001) and mortality (Lee & Skerrett, 2001; Kujala et al., 1998). Myers et al. (2004) found that an increase in physical activity of 1000 kcal/week equals to an increase in fitness of 1 MET, and that these both conferred a mortality benefit of 20%. As several earlier studies have indicated, fitness differences also contribute to mortality and morbidity risk (Blair et al., 1989; Ekelund et al., 1988; Gulati et al., 2003; Kokkinos et al., 2008; Lakka et al., 1994; Peters et al., 1983; Wei et al., 1999).

Physical activity interacts with a plethora of metabolic and health-related functions, which, like exercise capacity itself (Bouchard et al., 1998; Bouchard & Rankinen, 2001), are partly genetically regulated. Therefore, some of the associations between physical activity and metabolic and cardiovascular health may be explained by shared genetic factors. Our carefully identified twin pairs discordant for LTPA over 30 years represent a model that can be used to explore the long-term effects and underlying mechanisms of physical activity on metabolic and cardiovascular health, independent by genetic factors. The magnitude of the physical activity discordances found in this study is large enough to lay a foundation for such future analyses.

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Table 5

Physical Fitness Characteristics Among Twin Pairs Discordant for LTPA in 2007

Variable	Inactive	Active	Mean difference (95% CI)	*p* value
All 16 pairs				
Total exercise time (s)	663.1 ± 194.6	807.1 ± 201.7	–144.1 (–225.0 to –63.1)	.002
P_{peak} (W)	138.9 ± 39.9	168.1 ± 42.0	–29.3 (–46.5 to –12.0)	.003
P_{peak}/kg (W/kg)	1.8 ± 0.4	2.3 ± 0.5	–0.6 (–0.8 to –0.3)	< .001
P_{peak}/kg (W/FFM)	2.4 ± 0.5	3.0 ± 0.6	–0.6 (–0.9 to –0.2)	.002
VO_{2peak}[a] (ml/kg/min)	26.4 ± 4.9	32.5 ± 5.5	–6.1 (–9.0 to –3.2)	< .001
VO_{2peak} (ml/FFM/min)	33.6 ± 5.9	39.6 ± 6.3	–6.1 (–9.4 to –2.7)	.002
VO_{2peak} (ml/min)	2086.2 ± 520.0	2362.2 ± 510.4	–276.4 (–485.1 to –67.7)	.013
7 MZ pairs				
Total exercise time (s)	676.1 ± 175.1	830.9 ± 242.4	–154.7 (–303.5 to –5.9)	.044
P_{peak} (W)	140.9 ± 36.5	173.0 ± 50.4	–32.1 (–63.2 to –1.0)	.045
P_{peak}/kg (W/kg)	1.9 ± 0.5	2.3 ± 0.5	–0.4 (–1.0 to 0.1)	.083
P_{peak}/kg (W/FFM)	2.6 ± 0.6	3.0 ± 0.6	–0.4 (–0.9 to –1.9)	.099
VO_{2peak}[a] (ml/kg/min)	27.4 ± 5.3	32.2 ± 6.0	–4.8 (–10.5 to 0.9)	.083
VO_{2peak} (ml/FFM/min)	35.2 ± 6.6	39.7 ± 7.0	–4.5 (–10.0 to 1.1)	.099
VO_{2peak} (ml/min)	2090.5 ± 424.4	2433.9 ± 616.7	–343.4 (–665.4 to –21.4)	.040
9 DZ pairs				
Total exercise time (s)	652.9 ± 218.5	788.7 ± 177.1	–135.8 (–254.1 to –17.5)	.029
P_{peak} (W)	137.3 ± 44.5	164.3 ± 36.9	–27.0 (–52.5 to –1.5)	.041
P_{peak}/kg (W/kg)	1.7 ± 0.4	2.3 ± 0.5	–0.6 (–1.0 to –0.3)	.002
P_{peak}/kg (W/FFM)	2.3 ± 0.5	3.0 ± 0.6	–0.7 (–1.1 to –0.2)	.010
VO_{2peak}[a] (ml/kg/min)	25.7 ± 4.7	32.8 ± 5.4	–7.1 (–10.8 to –3.4)	.002
VO_{2peak} (ml/FFM/min)	32.3 ± 5.3	39.6 ± 6.2	–7.3 (–12.3 to –2.3)	.010
VO_{2peak} (ml/min)	2082.9 ± 609.8	2307.2 ± 442.0	–224.3 (–559.2 to 110.6)	.161

Note: Data are mean ± *SD*; P_{peak}, load weighted for time exercised at highest load; FFM, fat free mass (kg); [a](11.016 · P_{peak} / body mass) + 7 (ACSM, 2000).

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II

LEISURE-TIME PHYSICAL ACTIVITY AND HIGH-RISK FAT: A LONGITUDINAL POPULATION-BASED TWIN STUDY

by

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ORIGINAL ARTICLE

Leisure-time physical activity and high-risk fat: a longitudinal population-based twin study

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Background and Objective: Exercise is thought to reduce high-risk body fat, but intervention studies are frequently limited by short follow-ups and observational studies by genetic selection. Therefore, we studied the effects of a physically inactive vs active lifestyle on high-risk (visceral, liver and intramuscular) fat in twin pairs discordant for leisure-time physical activity habits for over 30 years.
Design: A longitudinal population-based twin study.
Subjects: Sixteen middle-aged (50–74 years) same-sex twin pairs (seven monozygotic (MZ), nine dizygotic (DZ)) with long-term discordance for physical activity habits were comprehensively identified from the Finnish Twin Cohort (TWINACTIVE study). Discordance was initially defined in 1975 and the same co-twin remained significantly more active during the 32-year-long follow-up.
Main Outcome Measures: Magnetic resonance imaging-assessed visceral, liver and intramuscular fat.
Results: In within-pair analyses carried out after the adult life-long discordance in physical activity habits, the physically inactive co-twins had 50% greater visceral fat area compared with the active co-twins (mean difference 55.5 cm^2, 95% confidence interval (CI) 7.0–104.1, $P=0.010$). The liver fat score was 170% higher (13.2, 95% CI 3.5–22.8, $P=0.030$) and the intramuscular fat area 54% higher (4.9 cm^2, 95% CI 1.9–7.9, $P=0.002$) among the inactive co-twins. All the trends were similar for MZ and DZ pairs. Peak oxygen uptake was inversely associated with visceral ($r=-0.46$, $P=0.012$) and intramuscular fat area ($r=-0.48$, $P=0.028$), with similar trends in intrapair difference correlations ($r=-0.57$, $P=0.021$ and $r=-0.50$, $P=0.056$, respectively). The intrapair difference correlation between visceral and intramuscular fat was also high ($r=0.65$, $P=0.009$).
Conclusion: Regular physical activity seems to be an important factor in preventing the accumulation of high-risk fat over time, even after controlling for genetic liability and childhood environment. Therefore, the prevention and treatment of obesity should emphasize the role of regular leisure-time physical activity.
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Keywords: physical activity; body fat; twins; visceral fat; ectopic fat

Introduction

Some people may inherit a tendency for high body fat.[1] However, the main causes of the obesity epidemic seem to be environmental, as the genetic pool changes slowly.[2,3] Abdominal obesity in particular is now recognized as a risk factor for cardiovascular and metabolic diseases as well as death.[4,5] Trends toward higher waist circumferences are frequently reported around the world.[6–8] Visceral obesity is strongly linked to an altered metabolic profile, possibly along with the ectopic fat deposition.[9] Deposition of fat in the liver and muscle has a major role in the development of obesity-related health risks.[10]

A physically active lifestyle is assumed to have a role in the prevention of obesity, although the results on the effect of physical activity on weight gain are somewhat conflicting.[11] The effect of relatively short-term exercise training in reducing total and abdominal fat is well documented by randomized controlled trials,[12–15] but less is known regarding the effects of long-term physical activity on different

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body fat compartments.[10,16,17] Research in this area is challenging as, on the one hand, it is difficult to carry out long-term randomized controlled exercise trials, whereas, on the other hand, observational population-based follow-ups may include genetic selection bias.[18] Therefore, we carried out within-pair analyses in middle-aged same-sex twin pairs identified on the basis of their long-term discordance for physical activity and blinded to body composition data. By studying both monozygotic (MZ) and dizygotic (DZ) twin pairs, we were able to control for childhood environment and partially for genetic liability, as MZ co-twins share all and DZ co-twins share half of their segregating genes. Thus, in this study with a 32-year-long follow-up, we analyzed the effects of a physically inactive vs active lifestyle on body fat, in particular high-risk (that is, visceral, liver and intramuscular) fat, in twin pairs persistently discordant for physical activity.

Materials and methods

Subjects

Sixteen same-sex twin pairs (7 MZ pairs and 9 DZ pairs; 11 male and 5 female pairs, mean age 60 years) were comprehensively identified from the Finnish Twin Cohort ($N = 5663$ pairs defined as healthy in 1981[18]). The identification process was primarily carried out on the basis of physical activity data and the researchers were blinded to data on height, weight and other body composition characteristics.[19] Discordance was based on a series of structured questions on leisure activity and physical activity during journeys to and from work. The leisure time (metabolic equivalent (MET)) index was calculated by assigning a multiple of the resting metabolic rate (intensity × duration × frequency) and expressed as a sum score of leisure time MET hours day^{-1}.[17,19–21]

The discordance for physical activity was initially identified in the assessment carried out in 1975. Although we refer to the year 1975 as baseline, the discordance had most probably started earlier. The follow-up discordance for leisure-time activity was assessed in the following three stages. First, twin pairs discordant for physical activity in 1975 and also in the assessment carried out in 1981 were identified (165 out of 5663 pairs). Second, a retrospective follow-up interview on leisure activity (covering the years from 1980 to 2005 in 5-year intervals) was carried out. All the twin pairs for whom the discordance between the co-twins was found in at least four out of six follow-up time points were included for further studies (54 out of the 165 pairs). Finally, 16 twin pairs fulfilled all our study inclusion criteria, volunteered to participate in the study measurements and were discordant for leisure-time physical activity on the basis of the detailed physical activity interview conducted in 2007.[19] Validity data of physical activity questionnaire and interviews are available in our earlier reports.[17,19] Altogether, there were four cross-sectional (1975, 1981, 2005 and 2007) and five retrospective time-points at which the active co-twins reported to have a higher physical activity level compared with the inactive co-twins ($P < 0.005$ for all time-points for the leisure MET index difference between inactive and active co-twins) (Figure 1).

The average mean MET difference in leisure-time activity between the co-twins (from 1980 to 2007 documented by the retrospective interview) was 8.8 MET h day^{-1} (2.2 ± 2.3 vs 11.0 ± 4.1 MET h day^{-1}, $P < 0.001$). The difference was similar in both MZ and DZ pairs. At the end of the follow-up, we administered symptom-limited maximal clinical exercise tests with a cycle ergometer to these co-twins and found, when we estimated VO_{2peak} from the highest load achieved in the test, that the discordance for physical activity was also reflected in the co-twins' cardiorespiratory fitness levels (26.4 ± 4.9 vs 32.5 ± 5.5 ml kg^{-1} min^{-1}, $P < 0.001$).[19] In addition, the effects of the difference in long-term loading were seen in tibial bone properties.[20]

Smoking habits and use of alcohol, together with other confounders, were collected with diary, questionnaire and interview methods as described earlier.[17,21] Energy intake was assessed using a 5-day food diary. Zygosity was confirmed at the National Public Health Institute, Helsinki, Finland, by the genotyping of 10 informative genetic markers.

This study was conducted according to the guidelines for good clinical and scientific practice laid down by the Declaration of Helsinki. The subjects were invited to attend a clinical examination and measurements in Jyväskylä, Finland, and traveled there so as to arrive the previous day. The body fat-related measurements were conducted the next day. The study was approved by the Ethics Committee of the Central Finland Health Care District, and all participants gave written informed consent.

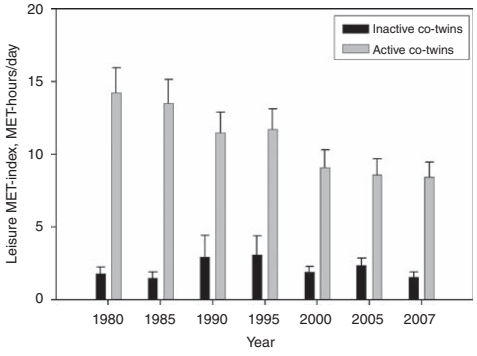

Figure 1 Follow-up leisure-time physical activity recorded by the retrospective interview among the inactive and active co-twins. The mean age of twins was 33 years (range 23–47 years) in 1980 and 60 years in 2007. Data are mean ± s.e.m. $P = 0.005$ to < 0.001 for comparisons between the inactive and active co-twins (for more details, see Leskinen *et al.*[19]).

Magnetic resonance imaging of abdomen
The T1-weighted MR scans of the abdomen were performed using a 1.5-T scanner (GE Signa Excite HD CVI (General Electric Healthcare, Milwaukee, WI, USA)) with a torso phase-array coil using a matrix of 256×192, field of view of 40×30 cm and gradient echo sequence with a repetition time/echo time of 150/2.16 ms with a flip angle of 90° in the out-phase and 150/4.97 ms with a flip angle of 90° in the in-phase images (FSPGR PulsSeg.). Areas of abdominal subcutaneous and visceral fat were manually segmented from a single slice image 5 cm above the L4–L5 intervertebral disc using the open-source image analysis software OsiriX (OsiriX Foundation, Geneva, Switzerland). The level of visceral fat area used in this study has shown high correlations with the volume of abdominal visceral fat in both men and women ($r = 0.95$ and $r = 0.97$, respectively).[22]

Assessment of liver fat score
The liver fat score was calculated from a single slice image as the difference in mean signal intensity of six regions of interest between the in-phase and out-phase images.[23] The regions of interest, 1 cm^2 each in size, were placed in the following segments of the liver parenchyma: lobus caudatus (segment I), superior subsegment of the lateral segment of the left lobe (II), left medial segment of the left lobe (IV), superior subsegment of the anterior segment of the right lobe (VIII) and superior subsegment of the posterior segment of the right lobe (VII).[24] MRI has been shown to be a reliable method for assessing the liver fat content when compared with magnetic resonance spectroscopy ($r = 0.85$, $P < 0.001$).[25]

Magnetic resonance imaging of thigh
Magnetic resonance imaging from the thigh was carried out with a matrix of 384×256, field of view of 40×28 cm, and T1-weighted fast spin echo sequence with a repetition time/echo time of 540/15.18 ms with 90° flip angle (FSE-XL PulsSeg.). A single axial image of the thigh was positioned at the midpoint of the femur lengthwise using the greater trochanter and head of the tibia as anatomical landmarks. Following the imaging, the areas of muscle, subcutaneous and intramuscular fat were segmented using OsiriX software (OsiriX Foundation). The midthigh intermuscular fat area has been shown to be associated with total intermuscular fat volume ($r = 0.60$–0.72)[26] and insulin resistance risk.[27] In our study, a high correlation between intramuscular fat area and fasting blood glucose was found ($r = 0.54$, $P = 0.031$ and $N = 31$).

Anthropometrics
The weight and height were measured barefoot in light clothing to the nearest 100 g and 0.5 cm, respectively. Waist circumference was measured midway between the spina iliaca superior and the lower rib margin and hip circumference at the level of the greater trochanters, both to the nearest half centimeter.[28] Body composition was determined after an overnight fast using an InBody (720) (Biospace, Seoul, Korea) eight-point tactile electrode multifrequency impedance plethysmograph body composition analyzer. Although dual-energy X-ray absorptiometry is generally regarded as a more valid method of assessing the percentage of body fat than bioimpedance, multifrequency bioimpedance analyses have in fact provided rather good assessments of fat mass in healthy subjects and in patients with stable water levels.[29–31] In our laboratory, the correlation coefficients for percentage of body fat between the multifrequency bioimpedance analysis and dual-energy X-ray absorptiometry were of a similar magnitude (0.55–0.78) within the different body mass index and physical activity groups irrespective of gender.[31] The precision of the repeated measurements during the same morning for percentage of body fat was 0.6% coefficient of variation (CV). In addition, percentage of body fat assessed by multifrequency bioimpedance analysis correlated well with measured serum leptin levels ($r = 0.72$, $P < 0.001$ and $N = 32$).

Blood studies
Ten-h fasting plasma and blood samples for DNA analyses were collected by venipuncture after an overnight fast and after 15-min rest in a supine position.

Statistical analysis
Pairwise analyses were used to study differences between co-twins. First, we analyzed the results for all the twin pairs and then for MZ and DZ twin pairs separately to find out whether the trends were similar. The normality of variables was assessed by the Shapiro–Wilk test. Student's paired *t*-test was used for normally distributed variables. For non-normally distributed variables the Wilcoxon signed rank test was used. The symmetry test (STATA) was used for the categorical variables. In all, 95% confidence intervals (95% CIs) were calculated for the absolute mean differences between the inactive and active co-twins. Pearson's correlation coefficient was used for the intrapair difference correlations ($N = 16$ pairs). When calculating individual-based correlations ($N = 32$), the within-pair dependency of twin individuals was taken into account using the cluster option[32] of STATA.[33] The level of significance was set at $P < 0.05$. Data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA) and STATA 8 (StataCorp LP, College Station, TX, USA) software.

Results

At baseline (1975), the active co-twins engaged in more leisure-time activity than the inactive co-twins ($P < 0.001$), although the co-twins did not differ in anthropometrics or in work-related physical activity (Table 1). After the follow-up,

Table 1 Baseline and follow-up characteristics of 16 twin pairs

Characteristic	*Inactive (N = 16)*	*Active (N = 16)*	P-*value*
Baseline 1975			
Sex (*N*, female:male)	5:11		
Age (years)	28 (range 18–42)		
Body height (cm) (*N* = 15)	173.7 ± 9.8	172.9 ± 10.1	0.57
Body weight (kg)	69.3 ± 16.4	66.0 ± 9.4	0.57
BMI (kg m^{-2}) (*N* = 15)	23.0 ± 4.2	22.3 ± 2.0	0.88
Leisure-time MET index (MET h day^{-1})	0.2 ± 0.3	3.3 ± 2.4	<0.001
Work-related physical activity (N)			0.23
Sedentary	3	7	
Standing or walking at work	3	4	
Light manual work	10	5	
Heavy manual work	0	0	
Follow-up 2007			
Age (years)	60 (range 50–74)		
Leisure time MET index (MET h day^{-1})	1.6 ± 1.4	8.4 ± 4.1	< 0.001
VO_{2peak} (ml kg^{-1} min^{-1})	26.4 ± 4.9	32.5 ± 5.5	< 0.001
Diagnosed type 2 diabetes *(N)*	3	1	0.16
Diagnosed coronary heart disease *(N)*	3	1	0.16
Daily energy intake (kcal)	1538.6 ± 356.6	1685.4 ± 452.0	0.20
Alcohol intake (g day^{-1})	6.5 ± 4.7	10.3 ± 10.1	0.16
Smoking status (N)			0.26
Current smokers	3	0	
Former smokers	7	8	
Never smoked	6	8	
Work status (N)			0.36
Employed	9	9	
Retired	5	5	
Unemployed	0	1	
Other	2	1	
Work-related physical activity (N)			0.17
Sedentary	3	5	
Standing or walking at work	1	3	
Light manual work	5	0	
Heavy manual work	0	1	

Data are mean ± s.d.

Table 2 Follow-up body fat distribution in 16 twin pairs

Characteristics	*Inactive (N = 16)*	*Active (N = 16)*	P-*value*
Anthropometrics			
Body height (cm)	171.8 ± 10.4	171.1 ± 9.9	0.63
Body weight (kg)	79.5 ± 18.4	72.9 ± 11.9	0.121
Body mass index (kg m^{-2})	26.7 ± 3.5	24.8 ± 2.6	0.088
Waist circumference (cm)	96.9 ± 13.1	90.6 ± 9.3	0.059
Hip circumference (cm)	99.6 ± 7.5	95.8 ± 6.0	0.17
Waist-to-hip ratio	0.97 ± 0.09	0.95 ± 0.07	0.103
Waist-to-height ratio	0.56 ± 0.05	0.53 ± 0.04	0.044
Fat percent (%)	27.0 ± 5.3	21.5 ± 6.4	0.004
Fat mass (kg)	21.6 ± 7.7	15.6 ± 5.0	0.015
Fat-free mass (kg)	57.7 ± 12.4	57.2 ± 11.1	1.000
Abdomen[a]			
Abdominal area (cm^2)	661.6 ± 177.8	557.3 ± 104.2	0.011
ASAT (cm^2)	195.3 ± 70.0	155.8 ± 49.4	0.079
VAT (cm^2)	170.6 ± 102.8	115.1 ± 73.3	0.010
VAT/ASAT ratio	0.94 ± 0.56	0.76 ± 0.53	0.051
Liver fat score[b]	21.1 ± 23.2	7.9 ± 8.4	0.030
Thigh (N = 15 pairs)			
Thigh area (cm^2)	196.2 ± 33.5	183.7 ± 22.6	0.13
IMAT (cm^2)	14.0 ± 7.0	9.1 ± 4.8	0.002
TSAT (cm^2)	58.7 ± 27.8	46.0 ± 19.6	0.047
IMAT/TSAT	0.28 ± 0.16	0.21 ± 0.12	0.015

Abbreviations: ASAT, abdominal subcutaneous adipose tissue area; IMAT, midthigh intramuscular adipose tissue area; TSAT, midthigh subcutaneous adipose tissue area; VAT, visceral adipose tissue area. Data are mean ± s.d.
[a]From the level of 5 cm above the L4–L5. [b]$SI_{inphase}$–$SI_{outphase}$ (see text).

no significant differences in daily energy intake, alcohol or tobacco use or in work-related physical activity were found between the inactive and active co-twins, although the significant difference in leisure time activity persisted (leisure time MET index difference 6.9 MET h day^{-1}, 95% CI −9.1 to 4.6, $P<0.001$). We also found that the inactive co-twins were less fit than their active co-twins (peak oxygen uptake difference 6.1 ml kg^{-1} min^{-1}, 95% CI −9.0 to 2.7, $P<0.001$) (Table 1).

The most pronounced differences between the inactive and active co-twins' follow-up body fat distributions were seen in the ectopic and visceral fat depots (Table 2). The magnetic resonance imaging-assessed liver fat score was 170% higher among the inactive co-twins (mean difference 13.2, 95% CI 3.5–22.8, $P=0.030$). Among MZ pairs alone, the inactive co-twins had as much as 435% higher liver fat score as compared with the active co-twins (26.1 ± 21.6 vs 6.0 ± 8.2, $P=0.028$) (Figure 2a). In addition, the inactive co-twins had 54% higher intramuscular fat area (4.9 cm^2, 95% CI 1.9–7.9, $P=0.002$), 28% higher subcutaneous fat area (12.7 cm^2, 95% CI −0.5 to 25.9, $P=0.047$) and a higher intramuscular-to-thigh subcutaneous fat ratio (0.07, 95% CI 0.02–0.12, $P=0.015$) in their midthigh. Again, the intramuscular fat difference was statistically significant among MZ pairs alone (13.7 ± 5.6 vs 9.1 ± 4.3 cm^2, $P=0.028$) (Figure 2b).

The visceral fat area was 50% (mean difference 55.5 cm^2, 95% CI 7.0–104.1, $P=0.010$) and abdominal subcutaneous fat area 25% (39.5 cm^2, 95% CI −3.0 to 82.0, $P=0.079$) higher among the inactive co-twins than in their active co-twins (Figure 2c). A trend toward a higher visceral-to-abdominal subcutaneous fat ratio among the inactive co-twins was also found (0.18, 95% CI −0.001 to 0.36, $P=0.051$) (Table 2).

The inactive co-twins tended to have higher body weight than their active co-twins; the 6.5-kg weight difference between co-twins (95% CI −2.2 to 15.3, $P=0.12$) matched the 6.0-kg difference in fat mass (95% CI 1.0–10.9, $P=0.015$), although no difference in fat-free mass was found. The inactive co-twins had also a higher percentage of body fat (5.4%-units, 95% CI 2.0–8.8, $P=0.004$), with a similar trend obtained for MZ and DZ pairs

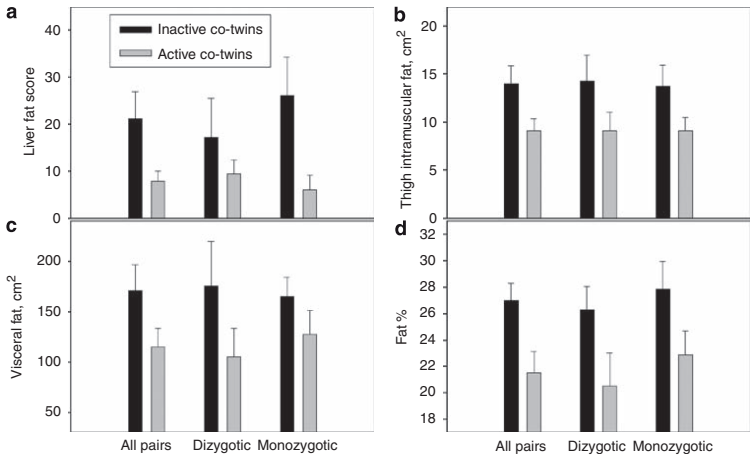

Figure 2 Follow-up body fat measures in twin pairs discordant for physical activity. Panel **a**, liver fat score; **b**, thigh intramuscular fat; **c**, visceral fat; **d**, fat percent. All 16 pairs (left bars), 9 DZ pairs (middle bars) and 7 MZ pairs (right bars). Data are mean ± s.e.m. $P<0.05$ for comparisons between inactive and active co-twins (all pairs).

(Figure 2d), a 6.3-cm higher waist circumference (95% CI −0.3 to 12.9, $P=0.059$) and a higher waist-to-height ratio (0.03, 95% CI 0.001–0.07, $P=0.044$) than their active co-twins (Table 2).

Visceral and intramuscular fat areas were strongly correlated both in individual twins ($N=31$) and using intrapair differences ($N=15$ full pairs) ($r=0.55$, $P=0.007$ and $r=0.65$, $P=0.009$, respectively). Similarly, liver fat score and visceral-to-subcutaneous fat ratio were positively correlated in individuals ($r=0.47$, $P=0.025$ and $N=32$) and within pairs ($r=0.49$, $P=0.057$ and $N=16$ pairs). Physical fitness as estimated by peak oxygen uptake was inversely associated with visceral ($r=-0.46$, $P=0.012$) and intramuscular fat area ($r=-0.48$, $P=0.028$), with similar trends in intrapair difference correlations ($r=-0.57$, $P=0.021$ and $r=-0.50$, $P=0.056$, respectively).

Discussion

Our twin study marks a new step in efforts to understand how long-term physical activity affects obesity-related high-risk fat. Our carefully phenotyped twin pairs persistently discordant for physical activity represent an ideal model with which to examine the effects of an inactive vs active lifestyle on high-risk fat independent of (in MZ twins) or partially adjusted for genetic factors (DZ twins). Across a follow-up of more than 30 years, we showed that habitual physical activity could potentially prevent accumulation of high-risk (that is, visceral and ectopic) fat, even after controlling for genetic liability and childhood environment (Figure 3). This is noteworthy because both participation in exercise and the accumulation of body fat may be genetically influenced. Our findings also support the idea of a causal link between leisure-time inactivity and the accumulation of metabolically high-risk fat.

Some, but not all, epidemiological studies have shown that physically active individuals are less likely to gain weight compared with inactive subjects.[11] However, according to randomized controlled trials, physical activity alone without diet control is associated with only modest weight reduction.[34] Importantly, it seems to be possible to reduce visceral fat by exercise without losing the weight.[12,14,16,35,36] Thus, it should be noted that physical activity seems to have beneficial effects on the accumulation of high-risk fat also in the absence of changes in body mass index.[10]

Our study supports earlier findings on ectopic fat deposition in a state of positive energy balance.[9,37,38] Physical activity, by decreasing visceral fat, has been shown to decrease hepatic fatty free acid uptake.[39] Evidence has been obtained recently that reduced physical activity increases intramuscular fat.[40] Overall, physical activity is known to enhance muscle metabolism and insulin sensitivity,[41] thereby leading to improved muscle-fat crosstalk.[42]

Our study gives compelling new evidence that long-term physical activity is associated with low liver and intramuscular fat accumulation independent of genes (Figure 2). This is in agreement with earlier data from observational and short-term intervention studies.[43–45] However, this finding should be differentiated from the finding that storing lipids in intramyocellular lipid droplets may be a beneficial phenomenon among physically active subjects,[46] as we did not measure muscle intramyocellular fat.

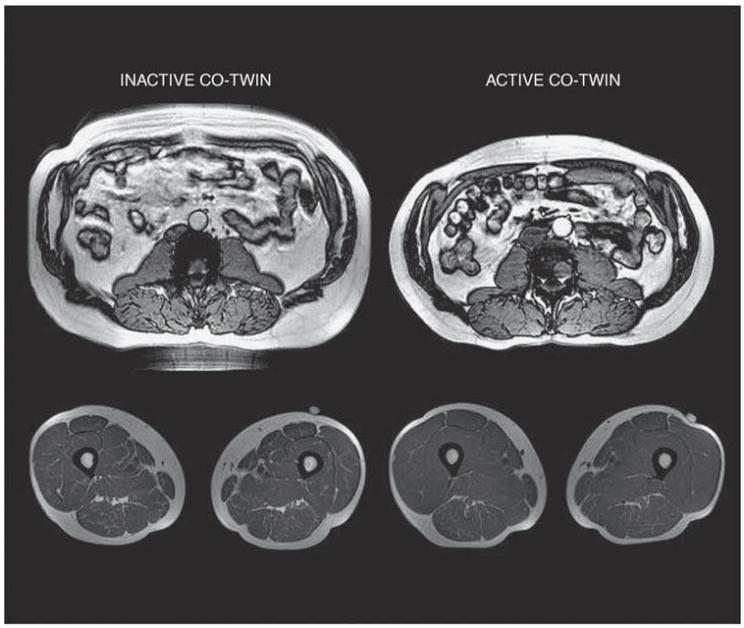

Figure 3 Illustrative example of high-risk fat accumulation in a physical activity discordant monozygotic (MZ) male twin pair. The active co-twin (on the right) had participated regularly in running, whereas his inactive co-twin (on the left) had been sedentary throughout the follow-up. Both co-twins had the same profession and employer. The inactive co-twin had a 74% higher visceral fat area, 150% higher intramuscular fat area and 63% higher fat mass.

Two large prospective cohort studies have found that higher waist circumference is associated with increased risk for death independently of body mass index.[47,48] Regular physical activity and normal weight have been shown to be associated with a reduced risk of all-cause mortality, cardiovascular disease and cancer.[49] Increased fitness has been shown to have similar effects independently of body weight status,[50] further underlining the importance of the long-term health effects of habitual physical activity.

Our findings suggest that the lowering of the morbidity and mortality risk by active lifestyle may be because of the disproportionately large reductions in high-risk fat as compared with overall or subcutaneous fatness. In long-term physical activity, compared with inactivity, the body uses large amounts of fat as fuel in energy metabolism, and persistently active lifestyle seems to effectively reduce the accumulation of high-risk fat in the body.

Although our study was not designed or powered (because of the small sample size) to analyze the outcomes, more cases of type 2 diabetes and coronary heart disease were found among the inactive twins than among their active co-twins (see Table 1). We found that inactive twins had a lower high-density lipoprotein-to-low-density lipoprotein cholesterol ratio ($P=0.023$) and higher fasting plasma glucose ($P=0.041$) compared with their active co-twins (detailed results not shown), again showing the metabolic benefits of a physically active lifestyle.

Strengths and limitations

This study has several strengths. As a result of the complete or close match for genes, age, gender and the intrauterine and childhood environment, the co-twin control study probably represents the best-controlled long-term study design available in humans in which adjustment can be made for genetic and familial factors. Our overall follow-up period was extremely long (32 years), covering most of the participants' mid-adult life. However, as observational follow-ups never truly start at the chosen baseline, we assume that the discordance for physical activity is of longer duration than our follow-up, and covers most of adulthood. Although we used validated methods to document leisure-time physical activity,[17,19] the subject's understanding on what is leisure physical activity may have changed over time. Therefore, our retrospective physical activity assessments may include more limitations than our detailed cross-sectional physical activity questionnaires and interviews. However, all of our physical activity questionnaires and

interviews clearly differentiated inactive and active co-twins in the intrapair analyses. At the end of the follow-up, we also carried out cardiorespiratory fitness tests.

We were able to measure body fat compartments with modern methods, allowing us to estimate the deposition of the high-risk (visceral, liver and intramuscular) fat components also independent of genes (Figure 3). For reasons related to our overall study protocol,[19] we used the bioimpedance method to assess body composition; this we regard as one of the limitations of our study despite the fact that the measurements were carried out in standardized conditions after an overnight fast. Baseline body fat was not measured apart from overall self-reported weight and height.

Another limitation of the study is the small sample size, which was because of our strict criterion for activity discordance. Despite the fact that different persistent leisure-time physical activity levels are common, it is less common that co-twins of a twin pair have persistently different activity levels. Furthermore, the fact that it was difficult to observe substantial numbers of twin pairs significantly discordant for physical activity itself speaks for a genetic or familial basis for lifetime activity patterns. Owing to the small sample size of discordant pairs, it was not possible to analyze what is the minimum difference in the activity volume that has an effect on high-risk body fat. In addition, continuous objective physical activity volume monitoring throughout decades would have been impossible. More studies are needed to show the dose–response effects of leisure physical activity on high-risk body fat, related chronic diseases and mortality independent of genetic selection.

Conclusion

In conclusion, habitual physical activity seems to be an important factor in preventing fatness and, in particular, the accumulation of high-risk fat, even after controlling for genetic liability and childhood environment. Therefore, the prevention and management of obesity should include a strong component on the long-term maintenance of adequate levels of leisure-time physical activity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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III

DIFFERENCES IN MUSCLE AND ADIPOSE TISSUE GENE EXPRESSION AND CARDIO-METABOLIC RISK FACTORS IN THE MEMBERS OF PHYSICAL ACTIVITY DISCORDANT TWIN PAIRS

by

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Differences in Muscle and Adipose Tissue Gene Expression and Cardio-Metabolic Risk Factors in the Members of Physical Activity Discordant Twin Pairs

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Abstract

High physical activity/aerobic fitness predicts low morbidity and mortality. Our aim was to identify the most up-regulated gene sets related to long-term physical activity vs. inactivity in skeletal muscle and adipose tissues and to obtain further information about their link with cardio-metabolic risk factors. We studied ten same-sex twin pairs (age range 50–74 years) who had been discordant for leisure-time physical activity for 30 years. The examinations included biopsies from *m. vastus lateralis* and abdominal subcutaneous adipose tissue. RNA was analyzed with the genome-wide Illumina Human WG-6 v3.0 Expression BeadChip. For pathway analysis we used Gene Set Enrichment Analysis utilizing active vs. inactive co-twin gene expression ratios. Our findings showed that among the physically active members of twin pairs, as compared to their inactive co-twins, gene expression in the muscle tissue samples was chronically up-regulated for the central pathways related to energy metabolism, including oxidative phosphorylation, lipid metabolism and supportive metabolic pathways. Up-regulation of these pathways was associated in particular with aerobic fitness and high HDL cholesterol levels. In fat tissue we found physical activity-associated increases in the expression of polyunsaturated fatty acid metabolism and branched-chain amino acid degradation gene sets both of which associated with decreased 'high-risk' ectopic body fat and plasma glucose levels. Consistent with other findings, plasma lipidomics analysis showed up-regulation of the triacylglycerols containing the polyunsaturated fatty acids. Our findings identified skeletal muscle and fat tissue pathways which are associated with the long-term physical activity and reduced cardio-metabolic disease risk, including increased aerobic fitness. In particular, improved skeletal muscle oxidative energy and lipid metabolism as well as changes in adipocyte function and redistribution of body fat are associated with reduced cardio-metabolic risk.

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Introduction

Increased or reduced risks for common chronic cardio-metabolic diseases are the result of complex molecular networks responding to genetic and environmental factors [1,2]. A phenotype characterized by high physical activity and/or aerobic fitness predicts low cardio-metabolic morbidity and mortality more strongly than any other known biological risk factor [3–9]. These associations seem to be explained mechanistically via a complex network of pathways, including changes in body composition and serum cardio-metabolic risk factor levels [9], such as the high high-density lipoprotein cholesterol (HDL-C) among highly physically active individuals [10].

Skeletal muscles represent more than one-third of the body mass of a normal weight person and play an important role in the whole-body energy metabolism. When working vigorously, skeletal muscles strongly increase their oxidative activity from that of the resting level. The skeletal muscles also have a high capacity to adapt to changes in metabolic demand [11]. While many studies have been published on the effects of exercise training on athletic performance, the health-related effects of skeletal muscle metabolism has also received much attention, with findings indicating that molecular mechanistic networks in skeletal muscle may have an influence on cardio-metabolic risk factors [6,12–14]. Fat tissue is an important energy store for endurance-type physical activities and its metabolism is linked to the aerobic metabolism of skeletal muscle and cardio-metabolic risk [13]. However, physical activity-induced changes in muscle and fat tissue-related complex molecular networks and their links with cardio-metabolic disease risk are not comprehensively understood [14].

As it is difficult to carry out very long-term randomized controlled exercise trials, and as observational population-based

follow-ups may include genetic selection bias, we carried out within-pair analyses in middle-aged same-sex twin pairs identified on the basis of their long-term discordance for physical activity [15–17]. By studying twin pairs, we were able to control for childhood environment and partially for genetic liability. The aim of our co-twin control study with a 32-year-long follow-up was to investigate how gene expression profiles of skeletal muscle and fat tissue differ between physically inactive and active members of twin pairs and how these gene expression differences are associated with physical fitness and other cardio-metabolic risk factors. Our findings identify skeletal muscle as well as fat tissue pathways which are associated with the long-term physical activity and reduced cardio-metabolic disease risk, including the increase in aerobic fitness.

Results

Sixteen middle-aged (50–74 yrs) same-sex twin pairs discordant for physical activity for more than 30 years were identified from the *Finnish Twin Cohort* [15]. Ten twin pairs (Table 1, Figure 1, Table S1) volunteered to give muscle and fat biopsies for this study as in three pairs at least one twin had a chronic disease and in three pairs one or both co-twins refused.

The active compared to inactive co-twins had higher peak oxygen uptake, a lower whole body fat percentage with lower ectopic 'high-risk' fat accumulation, higher HDL-C levels and lower fasting glucose and triglyceride levels (Table 1).

After normalizing the muscle gene expression data within pairs (normalization to the inactive twin) one-sample t-test discovered congruent lists of differentially expressed sequences: 45 sequences at $P<0.001$, 572 sequences at $P<0.01$ and 2829 sequences at $P<0.05$. Of the 45 sequences at $P<0.001$, 25 sequences were up-regulated and 20 sequences were down-regulated in the physically active co-twins (Table S2).

Pathway analysis using Gene Set Enrichment Analysis (GSEA) utilizing active vs. inactive co-twin gene expression ratios was performed on curated gene sets of canonical pathways containing 639 gene sets. For skeletal muscle the analysis yielded ten enriched gene sets with a FDR q-value <0.01. The most enriched gene sets in the active members of twin pairs were oxidative phosphorylation, valine, leucine and isoleucine degradation, ubiquinone biosynthesis and fatty acid metabolism (Table 2). Specific genes in the oxidative phosphorylation gene set encode NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochome c oxidase, H^+ transport and ATP synthase (Figure S1). Genes in the valine, leucine and isoleucine degradation group were related to aldehyde dehydrogenase activity, branched-chain amino acid catabolic processes and steps of the mitochondrial fatty acid beta-oxidation pathway. The "leading-edge" genes (i.e. genes contributing to the enrichment scores of GSEA analysis) that were used to calculate the expression centroids are shown in Table S3. Many genes or gene sets encoding the steps of the mitochondrial electron transport chain were up-regulated in active compared to inactive co-twins (Table S2, Table S3).

High correlation coefficients (r) and high coefficients of determinations (R^2) were found between peak oxygen uptake and gene set centroids ranked by active-to-inactive ratio (Figure S2). Percentage of the total measured area of muscle cross-section graded to be most oxidative according to succinate dehydrogenase (SDH) staining (Figure S3) were also positively correlated with centroids ranked by the active-to-inactive ratio (Figure S2).

HDL-C was higher in the active compared to inactive co-twins (Table 1) and was significantly correlated with 9 out of 10 centroids of gene sets upregulated in active co-twins (Figure S4).

Table 1. Characteristics of 10 twin pairs discordant for physical activity.

Characteristics	Inactive N = 10	Active N = 10	Mean Difference (95% CI)	p-Value
Baseline (1975; self-reported)				
Body height (cm) *(N = 9)*	172.7±9.4	170.3±9.2	2.3 (−2.6 to 7.3)	0.31
Body weight (kg)	67.8±18.6	63.7±10.0	4.1 (−7.6 to 15.8)	0.48
BMI (kg/m^2) *(N = 9)*	22.6±3.7	22.2±1.8	0.4 (−2.7 to 3.5)	0.67
Follow-up (2007; measured)				
Body height (cm)	170.7±9.8	168.6±8.9	2.1 (−2.0 to 6.3)	0.28
Body weight (kg)	78.4±23.0	69.1±11.7	9.3 (−4.9 to 23.6)	0.14
BMI (kg/m^2)	26.5±4.3	24.2±2.8	2.3 (−1.4 to 6.0)	0.20
Whole body fat percent (%) [a]	25.5±5.6	19.9±5.9	5.6 (1.2 to 10.1)	0.019
Visceral fat area (cm^2) [b]	158.4±122.7	90.4±70.0	68.0 (−9.3 to 145.4)	0.037
IMAT area (cm^2) [c]	11.4±5.7	7.5±4.2	3.9 (−0.7 to 8.6)	0.038
Estimated VO_{2peak} (ml/kg/min) [d]	28.3±3.6	33.0±5.0	−4.7 (−8.6 to −0.8)	0.023
Fasting plasma glucose (mmol/L)	5.3±1.3	4.7±0.6	0.6 (−0.3 to 1.4)	0.022
HOMA index	2.34±1.57	1.37±0.85	0.97 (−0.34 to 2.28)	0.059
Total cholesterol (mmol/L)	5.8±0.8	5.3±1.1	0.5 (−0.3 to 1.2)	0.24
HDL-C (mmol/L)	1.6±0.4	1.8±0.5	−0.2 (−0.3 to −0.01)	0.037
Triglycerides (mmol/L)	1.1±0.7	0.8±0.4	0.3 (−0.04 to 0.7)	0.059

BMI, Body mass index; IMAT, Intramuscular (extra myocellular) fat; HOMA index, (Fasting plasma glucose x Fasting plasma insulin)/22.5; HDL-C, High-density lipoprotein cholesterol.
[a]Measured by InBody (720) (Biospace, Korea) body composition analyzer [16].
[b]Measured by MRI [16].
[c]Cross-sectional intramuscular fat area of midthigh measured from MR-image [16].
[d]Calculated from symptom-limited maximal exercise test [15].
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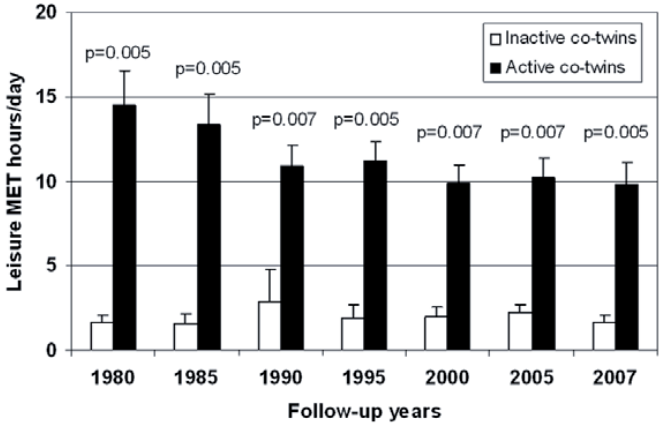

Figure 1. Follow-up physical activity discordance in the 10 twin pairs. Data is Mean ± SD. Calculation of mean MET discordance was based on a series of structured questions on leisure time physical activity and was quantified by calculation of the leisure activity metabolic equivalent [intensity x duration x frequency] expressed as a sum score of leisure time MET hours/day [3,17].
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Adjustment for gender changed the correlations only minimally. Each of the centroids of the first five gene sets explained the variation in HDL-C levels with coefficients of determination from 0.22 to 0.39. Also, intrapair differences of the gene set centroids correlated well with those of the HDL-C levels (Figure S4).

In order to investigate the metabolic and expression changes due to physical (in)activity in the adipose tissue as well, the global gene expression profiles were analyzed from the abdominal subcutaneous adipose tissue samples (taken on the same occasion as the muscle biopsies). Forty-seven sequences at $P<0.001$ (one-sample t-test after normalization of data), 401 sequences at $P<0.01$ and 2037 sequences at $P<0.05$ were differentially expressed between active and inactive. Of the 47 sequences at $P<0.001$, 16 sequences were up-regulated and 31 sequences were down-regulated in the physically active co-twins (Table S4). The most enriched gene sets in the active co-twins (Table 3) included valine, leucine and isoleucine degradation (related to aldehyde dehydrogenase activity, branched-chain amino acid catabolic processes and steps of the mitochondrial fatty acid beta-oxidation pathway, as found also in skeletal muscle), polyunsaturated fatty-acid (PUFA) metabolism and inflammatory processes. The "leading-edge" genes used to calculate expression centroids in fat tissue, are shown in Table S5. Interestingly, the gene set centroids most up-regulated in the active co-twins also had a very high correlation with reduced BMI, reduced visceral fat, reduced intramuscular but extracellular fat accumulation, reduced serum triglycerides, reduced plasma glucose and increased HOMA index (Table S6).

In order to link the findings from tissue-specific gene expression with systemic lipid metabolism, plasma lipidomics analysis was performed. Among all 16 twin pairs [15] nominally statistically significant differences between active compared to inactive co-

Table 2. Gene sets up-regulated in skeletal muscle among active compared to inactive co-twins (GSEA analysis).

Gene Set Name	Up-regulated/Size	ES	NOM p-Value	FDR q-Value
HSA00190 Oxidative phosphorylation	51/111	0.272	<0.0001	<0.0001
HSA00280 Valine, leucine and isoleucine degradation	27/44	0.383	<0.0001	0.00036
Valine, leucine and isoleucine degradation	24/35	0.425	<0.0001	0.00047
HSA00130 Ubiquinone biosynthesis	7/8	0.786	<0.0001	0.0015
Propanoate metabolism	21/30	0.415	<0.0001	0.00015
HSA00071 Fatty acid metabolism	21/47	0.335	<0.0001	0.0021
HSA00650 Butanoate metabolism	20/45	0.329	<0.0001	0.0044
HSA00380 Tryptophan metabolism	32/60	0.277	0.0016	0.009
Fructose and mannose metabolism	11/24	0.422	<0.0001	0.0085
Glycolysis	17/52	0.285	0.0016	0.0091
HSA00641_3 Chloroacrylic acid degradation	9/15	0.496	<0.0001	0.013
HSA00220 Urea cycle and metabolism of amino groups	14/30	0.369	0.0017	0.013

ES, Enrichment score (the primary outcome of GSEA analysis); NOM p-value, Nominal p-value; FDR q-value, False discovery rate q-value
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Table 3. Gene sets up-regulated (with FDR q-values ≤0.10) in subcutaneous abdominal fat tissue among active compared to inactive co-twins (GSEA analysis).

Gene set name	Up-regulated/Size	ES	NOM p-Value	FDR q-Value
IL2RB pathway	12/34	0.61	<0.0001	0.073
Valine, leucine and isoleucine degradation	26/35	0.58	<0.0001	0.10
HSA01040 Polyunsaturated fatty acid biosynthesis	10/14	0.67	<0.0001	0.077
HSA00280 Valine, leucine and isoleucine degradation	29/44	0.56	<0.0001	0.099
RECK pathway	4/9	0.72	0.001	0.089
Prostaglandin synthesis regulation	14/28	0.58	<0.0001	0.085
T cytotoxic pathway	2/11	0.68	0.002	0.087

ES, Enrichment score; NOM p-value, Nominal p-value; FDR q-value, False discovery rate q-value.
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twins were found in 14 of the 215 lipids identified (Table 4). Seven of the centroids of gene sets up-regulated in skeletal muscle samples in the active compared to inactive co-twins associated statistically significantly with ChoE (18:2) with coefficients of determination from 0.15 to 0.46 (Table S7).

Discussion

Our study identifies the gene sets most up-regulated among highly and persistently physically active members of twin pairs compared to their inactive co-twins in skeletal muscle and fat tissue and gives further information about their association with cardio-metabolic risk factors (Figure 2).

Studying scattered individual genes and their function can only account for a small part of the phenomena underlying complex traits. Obesity, insulin resistance and advanced age as well as many chronic diseases are suggested to be related to reduced muscle mitochondrial function [18]. In our study the up-regulated gene sets in muscle and adipose tissue were partially the same and partially different although related to the same mechanisms.

Table 4. Lipids differing in plasma lipidomics between active and inactive co-twins[a].

Lipid Name	FC	p-Value	FDR q-Value
ChoE (18:2)	1.47	0.0053	0.10
TG (58:10)	2.32	0.0056	0.10
TG (56:9)	2.03	0.0069	0.10
TG (50:5)	1.73	0.010	0.13
TG (58:7)	1.60	0.013	0.14
TG (58:9)	2.33	0.014	0.14
TG (49:3)	1.53	0.017	0.14
TG (47:0)	1.84	0.018	0.14
TG (56:8)	2.16	0.025	0.15
TG (58:8)	2.15	0.027	0.15
TG (53:5)	1.52	0.034	0.17
TG (54:6)	1.60	0.046	0.20
TG (56:7)	2.63	0.048	0.21
TG (54:7)	1.64	0.049	0.21

[a]n = 16 twin pairs.
FC, fold change (increased active/inactive ratio); FDR, False discovery rate.
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Together, our results show that long-term physical activity is associated with high oxidative capacity of skeletal muscle as seen by up-regulation of the genes encoding energy metabolism, oxidative phosphorylation and lipid metabolism. In particular, up-regulation of genes encoding the steps of the mitochondrial electron transport chain, which suggests enhanced oxidation capacity of the skeletal muscle (i.e. mitochondrial biogenesis) of the active co-twins, was seen [11,19].

The complex mechanisms underlying the association between up-regulated skeletal muscle pathways and high HDL-C, which we observed at the systemic level, are to some extent unknown. It is worth noting that HDL-C has vasodilatatory effects [20] further enhanced by specific steroid-fatty acid components of HDL-C [21] which may contribute to the increased oxygen supply to muscles. Also, increase in the oxidative capacity of the mitochondrial apparatus switches fuel preference towards fatty acids which seems to accompany with increased cholesterol export.

Mechanistically, our study also supports earlier findings that increased branched-chain amino acid catabolism is linked to increased oxidative energy production, lower ectopic fat accumulation and lower insulin resistance [22–24]. Dysregulated branched-chain metabolism may make an independent contribution to development of insulin resistance and glucose intolerance, ultimately leading to type 2 diabetes [25]. In adipose tissue, expression of the oxidative pathway was previously found to be suppressed in the obese and poor fitness phenotype [26]. In monozygotic twin pairs discordant for obesity, differences in branched-chain amino acid catabolism and adipose tissue mitochondria count have also been observed [24].

Increased PUFA synthesis may contribute to increased fitness and reduced cardio-metabolic risk by contributing to membrane functions and by increasing peroxisomal beta-oxidation and further oxidative phosphorylation, both of which were seen to be higher among physically active co-twins compared to inactive on the basis of muscle gene expression.

In plasma lipidomics analysis the up-regulation of triacylglycerols containing the polyunsaturated fatty acids is consistent with increased PUFA synthesis in adipose tissue (Table 3) and elevated HDL-C (Table 1). These triglycerides were also positively associated with improved insulin sensitivity in an earlier study [27].

Interestingly, the gene set expression centroids most up-regulated in the adipose tissue of the active co-twins also had a very high correlation with reduced BMI, reduced visceral fat, reduced intramuscular but extracellular fat accumulation, reduced serum triglycerides, reduced plasma glucose and reduced HOMA

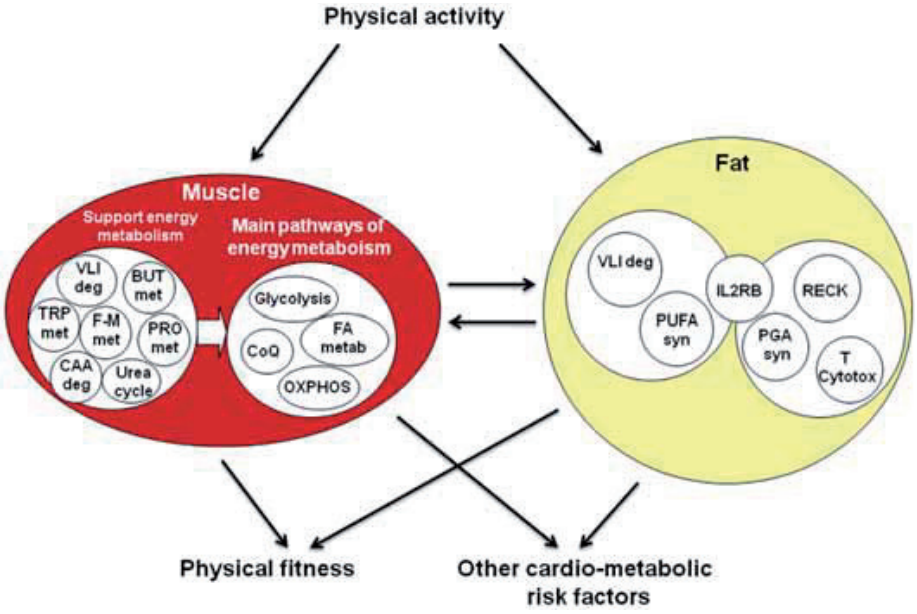

Figure 2. Up-regulated gene sets in muscle and fat tissue and their association with cardio-metabolic risk. Among the physically active members of twin pairs, as compared to their inactive co-twins, gene expression in the skeletal muscle was up-regulated for the central pathways of energy metabolism and supportive metabolic pathways related especially to the processes of oxidative energy production. In fat tissue the pathways were related e.g. to branched-chain amino acid degradation and PUFA synthesis. These metabolic changes were associated with decreased cardio-metabolic risk, including an increase in aerobic fitness. Centroids: OXPHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea cycle, Urea cycle and metabolism of amino groups; IL2RB, IL2RB pathway; PUFA syn, Polyunsaturated fatty acid biosynthesis; RECK, RECK pathway; PGA syn, Prostaglandin synthesis regulation; T Cytotox, T cytotoxic pathway.
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index (Table S6). This is in line with the known effects of exercise training [9]. Physically inactive subjects were less insulin sensitive than active co-twins as the HOMA index tended to be higher in inactive co-twins. It is to note that muscle contraction stimulates translocation of Glut4 glucose transporter via an insulin-independent mechanism [28] but this insulin sensitizing mechanism of contractile activity is evident only during 24 hours after exercise [14]. Thus, our study investigated other non-acute mechanisms.

Our study also suggested physical-activity associated up-regulation of some other gene sets in adipose tissue (Table 4). Some of the genes up-regulated in the IL2RB pathway also seem to be linked to better membrane functions and insulin sensitivity. The RECK pathway, prostaglandin synthesis regulation and the T cytotoxic pathway may contribute among other things to regulation of inflammation, cell proliferation and vasoconstriction/dilatation (Table S5), but these findings need further confirmation.

Strengths and limitations

In our study we employed a co-twin control design using twin pairs with 30-year discordance for physical activity habits. However, only moderate statistical power in our study means that there is a risk for type II error in particular in analyses of adipose tissue gene expression data. Comparing members of same-sex twin pairs takes into account effects of age and gender but it is to note that our material included both MZ and DZ pairs. Concerning individual-based correlations we have analyzed that the presented associations were not explained by gender. Dietary factors may also influence the findings of our study, but as there were only minor differences in nutrient intakes between active and inactive co-twins [29] it is very unlikely that dietary differences explain our findings. There were no significant differences in the smoking habits or use of alcohol between the active and inactive co-twins [15]. Also, as the trends between active and inactive co-twins usually were rather similar among DZ and MZ pairs, our findings seem not to be explained by sequence level genetic differences between co-twins. The carefully documented discordance in physical activity between co-twins was further confirmed by the differences in the co-twins tibial bone properties [30]. Related to the gene expression studies lack of protein-level analyses is a limitation. Although all subjects were free from cardio-metabolic diseases and other diseases affecting the ability to be physically active in 1975, very long-term physical activity may lead to different metabolic consequences, such as insulin resistance, which may have an effect on gene expression in muscle and adipose tissue. On the basis of our analyses on the associations between physical activity, gene expression and cardio-metabolic risk factors it seems that the observed up-regulations in skeletal muscle gene expression are not directly related to all of the cardio-metabolic risk factors but decreased fat accumulation and

differences in body fat distribution may mediate the association between high physical activity and many of the other cardio-metabolic risk factors, including insulin resistance.

Clinical conclusions

To make clinical conclusions a limitation of our study is the small sample size, which was because of our strict criterion for activity discordance to demonstrate reliably the effects of physical activity. Despite the fact that the studied persistent leisure-time physical activity levels (inactivity or activity) are more common in the population, it is less common that co-twins of a twin pair have persistently different activity levels. Unfortunately no data exists from large population samples to exactly describe how big proportion of individuals are persistently physically inactive or active during leisure time over a 30-year period according to our criteria. Thus, our study can be regarded as a model giving evidence on the associations between long-term physical activity vs. inactivity, gene expression and cardio-metabolic risk factors. Also, our study shows the associations between mitochondrial function, PUFA and branched-chain amino acid metabolism and occurrence of metabolic disorders. Interestingly, very similar associations were found when rats with high intrinsic aerobic capacity were compared to those with low capacity [31] suggesting that both inherited and acquired properties contribute to metabolic disease risk factors. So, they are good targets for future bio-marker research and possibly for drug development research. Our findings agree on the hypothesis that physical activity-associated increased skeletal muscle use and oxidative energy metabolism may contribute to decreased fat accumulation and changes in adipocyte function and redistribution of body fat, and further that these consequent changes in adipose tissue may have an effect on the development of insulin resistance.

Methods

Subjects

Sixteen middle-aged and older (50–74 yrs) same-sex twin pairs discordant for physical activity for more than 30 years were identified (TWINACTIVE study) from the *Finnish Twin Cohort*. For detailed subject recruitment and clinical assessments see Leskinen et al. [15]: http://www.atypon-link.com/AAP/doi/pdf/10.1375/twin.12.1.108. In brief, ten twin pairs of which 3 were monozygotic (2 female) and 7 dizygotic (2 female) pairs (Table 1) volunteered to give muscle and fat biopsies for this study as in three pairs at least one twin had a chronic disease and in three pairs one or both co-twins refused. Gene expression analyses on muscle tissue samples were successfully carried out for all of these twin pairs and on fat tissue samples for six complete pairs (2 monozygotic and 4 dizygotic pairs).

The identification process of participants was primarily carried out on the basis of physical activity data and the researchers were blinded to data on height, weight and other body composition characteristics [15,17]. Discordance was based on a series of structured questions on leisure activity and physical activity during journeys to and from work. The leisure time (metabolic equivalent (MET)) index was calculated by assigning a multiple of the resting metabolic rate (intensity x duration x frequency) and expressed as a sum score of leisure time MET hours per day [3,15,17]. The discordance for physical activity was initially identified in the assessment carried out in 1975. The discordance for leisure-time activity was assessed in the following three stages. First, twin pairs discordant for physical activity in 1975 and also in the assessment carried out in 1981 were identified (165 out of 5663 twin pairs defined as healthy in 1981). Second, a retrospective follow-up interview on leisure activity (covering the years from 1980 to 2005 in 5-year intervals) was carried out. On the basis of these assessments 54 out of the 165 pairs were included for further studies [15]. Finally, 16 twin pairs fulfilled all the TWINACTIVE study inclusion criteria, volunteered to participate in the TWINACTIVE study measurements and were discordant for leisure-time physical activity on the basis of the detailed physical activity interview conducted in 2007 [15]. The ICC between the shorter MET index and the detailed 12-month physical activity MET index was 0.68 ($P<0.001$) for leisure time physical activity and 0.93 ($P<0.001$) for work journey.

Physical activity discordance during the follow-up period for the ten twin pairs included in this study is shown in Figure 1 and Table S1. Leisure time physical activity between the inactive and active members of the twin pairs differed in 1975, 1981 and at each of the 7 follow-up occasions, the mean difference between the co-twins amounting to 9.4 MET h/day (2.0 ± 1.9 vs. 11.4 ± 3.0, 95% CI 7.6 to 11.2, $p = 0.005$). However, the subjects were advised not to exercise vigorously (except for walking) during the morning and two days before both of their laboratory visit (one visit for clinical examinations including exercise tests and one visit for biopsy studies) as we investigated long-term adaptations to exercise [15].

Other health habits

Smoking habits and use of alcohol, together with other confounders, were collected with diary, questionnaire and interview methods as described earlier [3,15,17]. Energy intake was assessed using a 5-day food diary [29].

Muscle and adipose tissue needle biopsies

Tissue samples were taken after an overnight fast between 8 am and 10 am under local anaesthesia after skin cooling and disinfection. The muscle biopsy was taken from the mid-part of *m. vastus lateralis* defined as the midpoint between the greater trochanter and the lateral joint line of the knee using Bergström's needle (ø 5 mm) biopsy technique with suction, and a needle biopsy (12 G needle, ø 2 mm) of subcutaneous abdominal adipose tissue was taken at the level of the umbilicus. The samples were cleaned of any visible connective tissue and muscle samples were cleaned of any visible adipose tissue. One part of the biopsies was frozen in liquid nitrogen immediately after withdrawing from the needle and stored at -80°C until used for mRNA analysis. The second part of the muscle biopsy used for succinate dehydrogenase analysis was mounted transversely on a cork with Optimal Cutting Temperature compound (Tissue Tek^{TM}, Miles, Elkhart, In, USA; Sakura, Cat. # 4583), and frozen rapidly (10-15 sec) in 2-Methylbutane (isopentane) (Fluka, Cat. # 59080) precooled to -160°C in liquid nitrogen and stored at -80°C.

Succinate dehydrogenase (SDH) staining

The activity of SDH in muscle cryosections was assessed histochemically [32]. The converted 8-bit images (range of gray-levels 0–255) from the stained sections were processed and analyzed using ImageJ software (NIH). An intensity threshold representing minimal intensity values corresponding to SDH activity was set manually and uniformly for all images (least oxidative 18–56; most oxidative 137–206). Finally, three intensity scaled fractions representing different level of oxidative capacities were expressed as the percentage of the total measured area.

Gene-expression array

The RNA preparation, cRNA generation and microarray hybridization procedures were used as previously described [33].

In brief, Trizol-reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from muscle biopsy samples of *m. vastus lateralis* homogenized on FastPrep FP120 apparatus (MP Biomedicals, Illkirch, France). From adipose tissue total RNA was isolated following needle suspension with Ambion's RNAqueous -Micro Kit (AM 1931, Applied Biosystems) according to manufacturer's instructions. Experion (Bio-Rad Laboratories, Hercules, CA) was used to inspect RNA concentration and quality. Only pure, good-quality RNA was used in the further analyses (260/280 ratio >1.8). An Illumina RNA amplification kit (Ambion, Austin, TX) was used according to the manufacturer's instructions to obtain biotinlabeled cRNA from 500 ng of total RNA. Experion was used to perform quality control after amplification. Hybridizations (one array per tissue) to Illumina HumanWG-6 v3.0 Expression BeadChips (Illumina Inc., San Diego, CA, USA) containing probes for 48803 transcripts, were performed by the Finnish DNA Microarray Center at Turku Center for Biotechnology according to the Illumina BeadStation 500x manual (Revision C). Six samples were hybridized on the same chip with twin and co-twin always on the same chip. Hybridized probes were detected with Cyanin-3-streptavidin (1 μg/ml, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) using Illumina BeadArray Reader (Illumina Inc.) and BeadStudio v3 software (Illumina Inc.). Raw data (= average probe signals) were extracted using the numerical results with Illumina Bead Studio v3.0.19 software with default settings without any additional normalization. The background for each bead was estimated by calculating the average of the 5 dimmest pixels in the area around the bead in question, outliers of transcript replicates greater than 3 deviations from the replicate median were removed, unexpressed genes were not removed and no log-transformations were performed. Initial data analyses were performed with R software environment for statistical computing (http://www.R-project.org), including Bioconductor development software (http://www.bioconductor.org). The raw data of each chip were quantile-normalized with affy package of Bioconductor [34]. Data quality was assessed by calculating Pearson correlations and clustering. For pairwise analysis normalized data was exported to Excel and SPSS statistical package. Fold change (FC) between twin-pairs was calculated by dividing the normalized expression value (of each gene) of the active twin with the respective value of the inactive twin. Statistical analysis of this data was done using one-sample t-test (FC vs. 1). In both analyses, lists of genes at different significance levels ($P<0.05$, $P<0.01$ and $P<0.001$) were created. The gene expression data and the raw data sets have been deposited in the GEO database, accession number GSE20319 for skeletal muscle data and GSE20536 for adipose tissue data. MIAME guidelines were followed during array data generation, preprocessing, and analysis. The clustering of differentially expressed genes into functional groups and significance of their distribution among groups was estimated with Gene Set Enrichment Analysis (version 2.0; GSEA, http://www.broad.mit.edu/gsea/) [35]. A list of all transcripts on the chip ranked according to the inter-pair expression ratio was utilized in the GSEA analysis with 1000 "gene set" permutations. The "leading-edge" genes (i.e. genes contributing to enrichment scores of GSEA analysis) were used to calculate expression centroids. The mean centroid of each leading-edge subset was computed by normalizing the expression levels of all subset genes to a mean of zero (0) [36].

Lipid risk factor and lipidomics analyses from serum and plasma

After overnight fast, a blood sample was drawn from an antecubital vein. Total cholesterol, triglycerides, and HDL-C were analyzed using VITROS DT60 (Chemistry System Ortho-Clinical Diagnostics, Inc., Rochester, NY, USA). Plasma glucose was determined using Biosen C-line (EKF-diagnostic, Magdeburg, Germany). Plasma lipidomics analysis was performed using ultra-performance liquid chromatography coupled to electrospray ionization mass spectrometry (UPLC-ESI-MS) as previously described in detail [37] with data processing using MZmine software version 0.60 [38].

Ethical approval

This study was conducted according to good clinical and scientific practice/guidelines and the Declaration of Helsinki. All subjects provided written informed consent. The ethics committee of the Central Hospital of Central Finland approved our study plan on August 15, 2006.

Other statistical analyses

Pairwise analyses were used to study differences between co-twins. The normality of variables was assessed by the Shapiro-Wilk test. Student's paired *t*-test was used for normally distributed variables and the Wilcoxon signed rank test for non-normally distributed variables. The symmetry tests (Stata version 8.0, www.stata.com) was used for the categorical variables. In the GSEA (1000 "gene set" permutations) and lipidomics analysis p-values were adjusted using False Discovery Rate (FDR) [35,39]. Ninety-five percent confidence intervals (95% CI) were calculated for the absolute mean differences between the inactive and active co-twins. The Pearson correlation coefficient was used for the intrapair difference (absolute differences between pairs) correlations and for individual-based correlations between gene set centroids and cardio-vascular risk factors (supplementary files) when the number of observations of continuous variables was ≥ 10 and the examination of distributions were suggestive of normal distribution (all skewness values for cardio-vascular risk factors <1 and for gene set centroids <2.2). When calculating individual-based coefficient of determination, the within-pair dependency of twin individuals was taken into account using the cluster option of Stata [40]. The level of significance was set at $p<0.05$. Data were analyzed using SPSS 14.0, Stata 8.0 and R software [41].

Supporting Information

Figure S1 Example of the enrichment plot of oxidative phosphorylation. Core enrichment genes on the right. Genes are presented in the order they were situated in the GSEA ranking list and affected to the enrichment score (the most up-regulated gene first, etc.).
Found at: doi:10.1371/journal.pone.0012609.s001 (2.51 MB TIF)

Figure S2 Associations between the centroids of gene sets up-regulated in muscle in the active compared to inactive co-twins and maximal oxygen uptake levels (A) and the proportion of most oxidative muscle cross-section as determined by succinate dehydrogenase staining (B). r, Correlation coefficient; R2 and p-value from family cluster regression analysis. Centroids: OX-PHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea cycle, Urea cycle and metabolism of amino groups.
Found at: doi:10.1371/journal.pone.0012609.s002 (0.07 MB TIF)

Figure S3 Percentage distribution of the total measured area of muscle cross-section of different oxidative capacities according to succinate dehydrogenase staining. Data is Mean ± SD. The inactive vs. active differences in succinate dehydrogenase staining muscle cross-section percentages were statistically non-significant; least oxidative ($p = 0.31$), intermediate oxidative ($p = 0.24$) and most oxidative ($p = 0.24$) muscle cross-section percentage.
Found at: doi:10.1371/journal.pone.0012609.s003 (0.07 MB TIF)

Figure S4 Associations between the centroids of gene sets up-regulated in the active compared to inactive co-twins and HDL-C levels. Individual-based ($n = 20$) correlation coefficients (r) and R2 and p-values from family cluster regression analysis are shown in panel A. Correlation plot between intrapair differences (IPD) in HDL-C and in centroid of oxidative phosphorylation ($n = 10$ pairs; panel B) and valine, leucine and isoleucine degradation (panel C). Centroids: OXPHOS, Oxidative phosphorylation; VLI deg, Valine, leucine and isoleucine degradation; CoQ, Ubiquinone biosynthesis; PRO met, Propanoate metabolism; FA metab, Fatty acid metabolism including mitochondrial β-oxidation and peroxisomal β-oxidation; BUT met, Butanoate metabolism; TRP met, Tryptophan metabolism; F-M met, Fructose and mannose metabolism; CAA deg, Chloroacrylic acid degradation; Urea cycle, Urea cycle and metabolism of amino groups.
Found at: doi:10.1371/journal.pone.0012609.s004 (0.06 MB TIF)

Table S1 Physical activity MET-indices of the inactive and active members of the twin pairs during follow-up.
Found at: doi:10.1371/journal.pone.0012609.s005 (0.07 MB DOC)

Table S2 Significantly regulated genes in muscle tissue with one-sample t-test $p<0.001$.
Found at: doi:10.1371/journal.pone.0012609.s006 (0.08 MB DOC)

Table S3 Genes contributing to enrichment scores and calculation of expression centroids in muscle tissue.
Found at: doi:10.1371/journal.pone.0012609.s007 (0.05 MB DOC)

Table S4 Significantly regulated genes in fat tissue with one-sample t-test $p<0.001$.
Found at: doi:10.1371/journal.pone.0012609.s008 (0.08 MB DOC)

Table S5 Genes contributing to enrichment scores and calculation of expression centroids in fat tissue.
Found at: doi:10.1371/journal.pone.0012609.s009 (0.05 MB DOC)

Table S6 Associations between the centroids of gene sets up-regulated in active vs. inactive co-twins in fat tissue and cardio-metabolic risk factors.
Found at: doi:10.1371/journal.pone.0012609.s010 (0.05 MB DOC)

Table S7 Associations between the centroids of gene sets up-regulated in muscle tissue in the active compared to inactive co-twins and ChoE (18:2).
Found at: doi:10.1371/journal.pone.0012609.s011 (0.05 MB DOC)

Author Contributions

Conceived and designed the experiments: TL JK HK UMK. Performed the experiments: TL RRT TSL EP MA SS VK PR MO HK UMK. Analyzed the data: TL RRT MR EP MO HK UMK. Contributed reagents/materials/analysis tools: VK UMK. Wrote the paper: TL RRT MR TSL EP MA SS JK VK PR MO HK UMK.

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IV

PHYSICALLY ACTIVE VS. INACTIVE LIFESTYLE, MUSCLE PROPERTIES AND GLUCOSE HOMEOSTASIS IN MIDDLE-AGED AND OLDER TWINS

by

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Physically active vs. inactive lifestyle, muscle properties, and glucose homeostasis in middle-aged and older twins

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Abstract Exercise-induced positive changes in skeletal muscle properties and metabolism decrease the risk for disability, cardiometabolic diseases and mortality. Here, we studied muscle properties and glucose homeostasis in a non-exercise stage in twin pairs with co-twins discordant for physical activity habits for at least 32 years of their adult lives. Isometric knee extension force, MR imaging of midthigh tissue composition and muscle volume, and fasting blood samples were acquired from 16 same-sex (seven monozygotic, nine dizygotic) middle-aged and older twin pairs. The consistently active twins had 20 % higher knee extension forces than their inactive co-twins (p=0.006) although the active twins had only 4 % higher midthigh muscle cross-sectional areas (p=0.072). These results were similar in intrapair analysis in which only the seven identical twin pairs were included. The ratio between the area of midthigh fat and muscle tissues was significantly lower among the active twins (0.65 vs. 0.48, p=0.006). The active twins had also lower fasting plasma glucose levels (5.1 vs 5.6 mmol/l, p=0.041). The area of midthigh intramuscular (extramyocellular) fat was associated with the markers of glucose homeostasis, especially with glycated hemoglobin, and these associations were emphasized by the diabetic and inactive twins. Regular exercise throughout the adult life retains muscle strength and quality but not necessarily mass. The regular use of muscles also prevents from the accumulation of intramuscular fat which might be related to maintained glucose metabolism and, thus, prevention of metabolic disorders.

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Introduction

In humans, muscle tissue starts to decrease around the fifth decade of life. The loss of muscle mass is linked to loss of muscle fiber number and size, muscle strength, power, and endurance (Deschenes 2004; Faulkner et al. 2007). Regular physical activity can preserve as well as increase skeletal muscle mass and strength at all ages (PAGAC 2008). Maintenance of muscle properties has notable benefit for the prevention of age-related disability (Rantanen et al. 1999; Janssen 2006), and high muscle strength is associated with lower mortality (Newman et al. 2006; Ruiz et al. 2008). Moreover, exercise-induced positive changes in skeletal muscle structure, function, and metabolism are linked to delay in the progression of multiple diseases (Wolfe 2006; Kujala 2009).

Skeletal muscle volume accounts for the majority of glucose uptake. In individuals with type 2 diabetes mellitus, glucose uptake is reduced by the insulin resistance of skeletal muscle. Exercise training improves the skeletal muscle glucose transport system, but the effects are rather short-lived if they are not followed by another bout of exercise (Henriksen 2002; Boule et al. 2005; Hawley and Lessard 2008). Thus, regular physical activity offers a beneficial way to enhance glucose homeostasis also in insulin resistant muscle. Here, we studied the effects of long-term leisure-time physical activity vs. inactivity on skeletal muscle properties and glucose homeostasis in a non-exercise stage in middle-aged and older co-twins of twin pairs discordant for physical activity habits.

Methods

Subjects

Twin pairs discordant for physical activity were initially identified from the Finnish twin cohort (N=5,663 healthy pairs) by self-reported physical activity assessments conducted in 1975 and 1981 (Kujala et al. 2002). All pairs who were discordant for physical activity in both assessments were selected for a retrospective physical activity follow-up in which their physical activity history was obtained for each 5-year period, from 2005 back to 1980, by means of a telephone interview (Waller et al. 2008). Recall was anchored to relevant life-events. Twin pairs with consistent discordance in physical activity habits throughout the follow-up were invited to participate in the TWINACTIVE study measurements held in 2007 (Leskinen et al. 2009a). Overall, 16 same-sex middle-aged and older twin pairs (seven monozygotic (MZ) and nine dizygotic (DZ) pairs, five female and 11 male pairs, age range 50–74 years) were able to participate in the measurements and were discordant for physical activity at every time point at which their physical activity habits were assessed. The baseline was set to year 1975 when the physical activity discordance was detected for the first time. Thus, overall, the 16 twin pairs were documented of having continuous physical activity discordance for 32 years of their adult lives. The follow-up's mean metabolic equivalence task (MET) difference, that is, the average difference in leisure-time activity habits between the inactive and active co-twins, was 8.8 MET h/day (2.2±2.3 vs. 11.0 ±4.1 MET h/day, p<0.001, respectively). This is the equivalent of, for example, the exercise volume of a 2-h walk. The most common types of physical activity engaged in by the active twins during the previous 12 months were walking (30 % of total physical activity volume), jogging (11 %), and cross-country skiing (9 %). At the end of the follow-up, the co-twins did not differ in work-related physical activity and alcohol or tobacco use, and they had only minimal difference in their daily energy intake (Leskinen et al. 2009a; Rintala et al. 2011).

Physical activity assessment

Discordance for physical activity was defined on the basis of a series of structured questions on leisure-time activity and physical activity during journeys to and from work. Leisure-time activity was quantified as metabolic equivalent units (average intensity of activity (MET) × average duration of one session (in hours) × (monthly frequency / 30 days)) and expressed as the sum score of leisure-time MET hours per day (Kujala et al. 1998; Waller et al. 2008; Leskinen et al. 2009a).

Muscle force measurements

Maximal isometric left knee extensor force was measured in a sitting position using an adjustable dynamometer chair (Good Strength, Metitur, Palokka, Finland) as

described earlier (Sipilä et al. 1996). Briefly, the left knee was set at an angle of 60° from full extension. Overall, four maximal efforts with a 30-s pause between each were conducted. The best performance was accepted as a result. In addition to maximal force, the maximal rate of force development over an interval of 10 ms was recorded. Knee extensor force was measured in 27 co-twins (13 complete pairs). Co-twins with multiple diseases (n=2), long-standing diabetes (n=2), and polio (n=1) were excluded. Left maximal handgrip force was measured with the elbow flexed at 90° using the same adjustable dynamometer chair and the same protocol as described above. Left hand grip force was measured in 31 co-twins (15 complete pairs), excluding the co-twin with polio affecting the left hand.

Midthigh muscle tissue composition acquisition

Nine axial T1-weighted MR images of 10-mm thickness and with a 20-mm slice interval were acquired from the left midthigh using 1.5 T GE-Signa Exite HD CVi with a matrix of 384×256, field of view 40×28 cm, and gradient echo sequence with TR/TE 550/15.2 ms (FSE-XL PulsSeg). The midslice (fifth image) was positioned at the midpoint lengthwise of the left femur. The midpoint of the femur was skin-marked using the greater trochanter and join line of the knee as anatomical landmarks. The midslice was used for the detailed analyses of the total midthigh cross-sectional area, muscle cross-sectional area, knee extensor (musculus quadriceps femoris) cross-sectional area, and the intramuscular (extramyocellular) and subcutaneous fat areas. Segmentation was made manually using OsiriX software (OsiriX Foundation, Geneva, Switzerland). Muscle tissue was detected by setting a density threshold and using the automatic segmentation parameters. The midthigh volume was calculated from the part of the thigh which all nine slices covered (lengthwise 16 cm) using automatic volume calculation. Fifteen complete pairs were included in the MR analysis because one co-twin was not imaged.

Blood studies

Ten-hour fasting plasma samples were collected by venipuncture after 10 min of supine rest. Plasma glucose was determined using Biosen C-line (EKF-diagnostic, Magdeburg, Germany) and serum insulin by IMMULITE® 1000 Analyzer (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The HOMA index was calculated using the formula: (fasting plasma glucose × fasting plasma insulin) / 22.5 (Muniyappa et al. 2008). Glycated hemoglobin (Hb_{A1C}) was analyzed using HPLC Variant II (Bio-Rad Laboratories, Munich, Germany). Subjects were advised not to exercise vigorously (except for walking and other routine activities) during the 2 days before their laboratory visit as we were investigating long-term adaptations to exercise.

Other measures

Body composition was determined after an overnight fast using an InBody (720) (Biospace, Korea) eight-point tactile electrode multifrequency impedance plethysmograph body composition analyzer. We also administered symptom-limited maximal clinical exercise tests with a cycle ergometer and estimated VO_{2peak} from the highest load achieved in the test. The zygosity of the co-twins was verified at the Paternity Testing Laboratory (National Public Health Institute, Helsinki, Finland) using DNA extracted from a venous blood sample with a battery of ten highly polymorphic gene markers.

Ethics

The TWINACTIVE study was conducted according to the guidelines for good clinical and scientific practice laid down by the Declaration of Helsinki. The study was approved by the Ethics Committee of the Central Finland Health Care District, and all the participants gave their written informed consent.

Data analysis

Pairwise analyses were used to study differences between the co-twins of the twin pairs. Normality of the means was assessed with Shapiro–Wilk test. Student's paired t test was used for normally distributed data and the Wilcoxon matched-pair signed-rank test for non-normally distributed data. Ninety-five percent confidence intervals were calculated for the absolute mean differences between the inactive and active co-twins. By studying same-sex twin pairs, all the pairwise analyses were age- and sex-adjusted. The Pearson correlation coefficient was used for the correlation analyses. When calculating individual-based coefficients of determination

(R^2), the within-pair dependency of twin individuals was taken into account using the cluster option of Stata (svy: regress) (Williams 2000). The level of significance was set at $p<0.05$. Data were analyzed using IBM SPSS Statistics 19 and Stata 8.0 software.

Results

At the end of the follow-up, the active twins were more active, more fit, and leaner than their inactive co-twins (Table 1). The maximal knee extension force was 20 % higher among the active twins ($p=0.006$). This finding was consistent for the MZ and DZ pairs (Fig. 1a). The rate of force development was rather similar among the co-twins (pairwise analysis, $p=0.71$). No differences in hand grip forces were found (inactive twins, 422.4 N (SD 156.1) vs. active co-twins, 428.1 N (SD 126.5), $p=0.72$). The difference in midthigh muscle cross-sectional area between the co-twins was nonsignificant (Fig. 1b), but the active twins had substantially lower fat-to-muscle tissue ratio

Table 1 Baseline and follow-up characteristics in twin pairs discordant for physical activity

Characteristic	Inactive $N=16$	Active $N=16$	P value
Baseline 1975			
Sex (n, female:male)	5:11		
Age (years)	28 (range 18–42)		
Leisure-time MET index[a] (MET h/day)	0.2±0.3	3.3±2.4	<0.001
Body height[a] (cm) ($n=15$)	173.7±9.8	172.9±10.1	0.96
Body weight[a] (kg)	69.3±16.4	66.0±9.4	0.57
BMI (kg/m^2) ($n=15$)	23.0±4.2	22.3±2.0	0.88
Follow-up end point 2007 (measured)			
Age (years)	60 (range 50-74)		
Leisure-time MET index (MET h/day)	1.6±1.4	8.4±4.1	<0.001[b]
Estimated VO_{2peak} (ml/kg/min)	26.4±4.9	32.5±5.5	<0.001
Knee extension force (N) ($n=13$ pairs)	425.8±87.3	507.8±121.4	0.006[b]
Body height (cm)	171.8±10.4	171.1±9.9	0.39
Body weight (kg)	79.5±18.4	72.9±11.9	0.121
BMI (kg/m^2)	26.7±3.5	24.8±2.6	0.09
Total body fat percent (%)	27.0±5.3	21.5±6.4	0.004[b]
Total body fat mass (kg)	21.6±7.7	15.6±5.0	0.015
Total body fat-free mass (kg)	57.7±12.4	57.2±11.1	1.00
Fasting plasma glucose[c] (mmol/l)	5.6±1.5	5.1±1.0	0.041
Fasting serum insulin[c] (μlU/ml)	10.9±4.8	10.6±12.2	0.18
HOMA index[c]	2.9±2.2	2.6±3.0	0.18
Hb_{A1C}[c] (%)	5.9[d]±0.6	5.7[e]±0.4	0.22
Diagnosed type 2 diabetes	3	1	0.16[f]
Impaired fasting plasma glucose	1	1	1.00[f]

Values are means ± SD or frequencies. P values from Wilcoxon matched-pair signed-rank test

BMI body mass index, *HOMA* the homeostatic model assessment, Hb_{A1C} glycated hemoglobin

[a] Self-reported

[b] P value from paired t test (normal distribution)

[c] Diabetic twins included

[d] 41 mmol/mol

[e] 39 mmol/mol

[f] Symmetry test, Stata

within their midthighs (Fig. 1c). There was a rather small difference in midthigh muscle volumes between the co-twins (pairwise analysis, p=0.28) (Table 2).

Fasting plasma glucose levels were higher among the inactive co-twins (5.6 vs. 5.1 mmol/l, p=0.041) and the difference remained significant when the diabetic co-twins (three inactive and one active) were excluded from the pairwise analysis (5.3 vs. 4.8 mmol/l, p=0.012, n=13 pairs). The remaining differences in the markers of glucose homeostasis

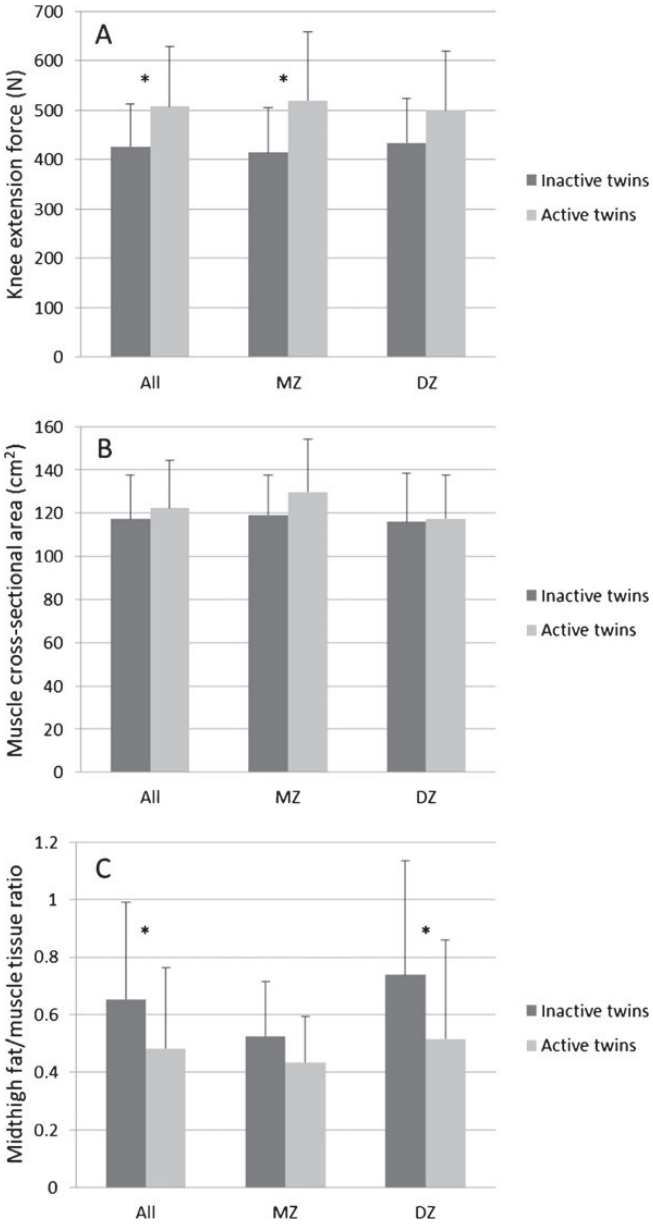

Fig. 1 **a** Knee extensor force, **b** muscle cross-sectional area, **c** midthigh fat-to-muscle tissue ratio among inactive and active members for all, MZ, and DZ pairs. Values are means ± SD. The *asterisks* indicate $p<0.05$

between the co-twins were nonsignificant (see Table 1). We found high associations between the intramuscular (extramyocellular) fat area per se and the markers of glucose homeostasis (R^2=0.22–0.30), although after excluding the diabetic twins (n=4), the relationships were more modest (R^2=0.08–0.10). Furthermore, the inactivity seemed to highlight the association as the correlations coefficients between intramuscular fat and the markers of glucose homeostasis were higher among the inactive twins (r=0.59–0.69, n=16) compared to active twins (r=0.30–0.39, n=15). The most constant association was found between the intramuscular fat area and Hb_{A1C} (R^2=0.30, p=0.03, n=31 twins; R^2=0.10, p=0.06, n=27 non-diabetic twins) (Fig. 2a). This association was also found to be high in the intrapair difference correlation analysis (r=0.55, p=0.03, n=15 pairs; r=0.54, p=0.07, n=12 non-diabetic pairs) (Fig. 2b). The correlation coefficient between intramuscular fat area and Hb_{A1C} among the inactive twins was 0.591 (p=0.016, n=16) (Fig. 2c).

Discussion

Physical activity seems to be an efficient way to slow down the loss of muscle strength and muscle atrophy in middle to old age (Sipilä and Suominen 1995; Sipilä et al. 1996; Tarpenning et al. 2004; Goodpaster et al. 2008). Our results add to the existing findings by suggesting that long-term physical activity preserves muscle strength independently of genes (Tiainen et al. 2004). However, we found only rather small intrapair differences in midthigh muscle volumes (intraclass correlation of 0.93 in the 15 pairs) between the co-twins. This is consistent with the idea that genes make a major contribution to body structure and muscle function (Nguyen et al. 1998; Tiainen et al. 2008). Moreover, this finding only reflects the fact that aerobic-type training (e.g., walking and jogging) does not affect lower limb muscle mass or handgrip strength. The difference observed in knee extensor force between co-twins could therefore be explained by a better muscle force-producing system, including neural factors, and by the better muscle quality among the active twins (Tarpenning et al. 2004; Faulkner et al. 2007; Frontera et al. 2008). Hence, exercise-induced retention of muscle strength rather than that of muscle mass could be the key factor in the maintenance of muscle function to avoid age-related mobility limitations (Hughes et al. 2001; Visser et al. 2005; Goodpaster et al. 2006; Newman et al. 2006; Delmonico et al. 2009). Furthermore, regular physical activity is an effective way to slow down the age- and/or inactivity-related infiltration of muscle fat (Goodpaster et al. 2008; Delmonico et al. 2009; Leskinen et al. 2009b). However, the role of muscle fat infiltration in strength loss, muscle weakness, and mobility limitations needs further study (Visser et al. 2005; Goodpaster et al. 2008; Delmonico et al. 2009).

Regular physical activity maintains glucose homeostasis (Henriksen 2002; Boule et al. 2005; Petersen and Shulman 2006; Church et al. 2010; Mikus et al. 2012). Moderate-intensity exercise decreases the risk for type 2 diabetes mellitus (Henriksen 2002; Jeon et al. 2007; PAGAC 2008), even at low levels of activity (<150 min/week) (Waller et al. 2010). Disuse of muscles

Table 2 Left midthigh muscle properties in twin pairs discordant for physical activity

Variable	Inactive N=15	Active N=15	Mean Diff.	95 % CI	P value
Total midthigh cross-sectional area (cm^2)	196.2±33.5	183.7±22.6	12.5	−6.0 to 31.0	0.17
Muscle cross-sectional area[a] (cm^2)	117.2±20.5	122.4±22.1	−5.2	−10.9 to 0.5	0.072
Quadriceps Femoris area[b] (cm^2)	58.7±11.0	60.3±11.0	−1.7	−4.2 to 0.9	0.19
Midthigh fat/muscle ratio	0.65±0.34	0.48±0.28	0.17	0.04 to 0.30	0.006[c]
Muscle volume, left midthigh[a] (dm^3)	1.88±0.38	1.94±0.38	−0.06	−0.16 to 0.05	0.28

Values are means ± SD. P values from paired t test

95 % CI 95 % confidence interval for the mean difference

[a] Bone and bone marrow area and intramuscular fat area excluded

[b] Intramuscular fat included (manual delineation of the muscle group)

[c] P value from Wilcoxon matched-pair signed-rank test (non-normal distribution)

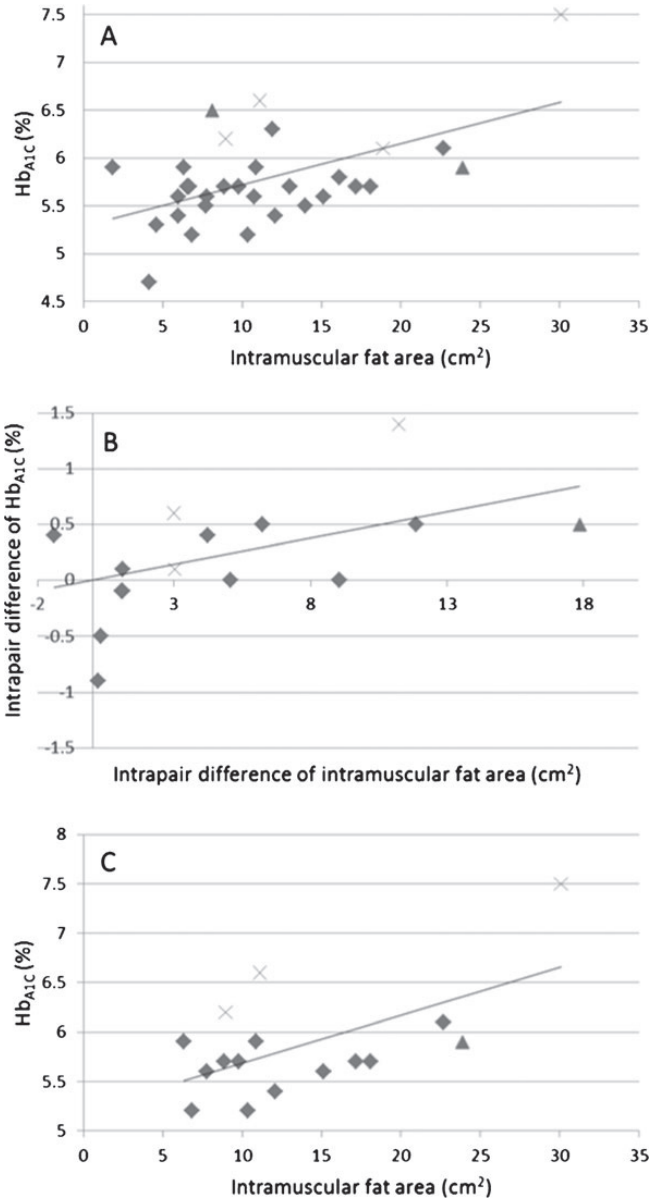

Fig. 2 **a** Individual-based association between intramuscular fat area and Hb_{A1C} (r=0.54, p=0.03, n=31 twins), **b** correlation between the intrapair difference of intramuscular fat area and Hb_{A1C} (r=0.055, p=0.03, n=15 pairs), **c** association between intramuscular fat area and Hb_{A1C} among inactive twins (r=0.59, p=0.016, n=16 twins). Twins having diagnosed type 2 diabetes (marked with a *multiplication sign*); twins having screen detected impaired fasting plasma glucose (marked with *black triangle*)

(due to inactivity) decreases metabolic flexibility and induces lipotoxity, that is, muscles start to fill with fat (Eckardt et al. 2011). This muscle fat infiltration is frequently linked to the insulin resistance (Taube et al. 2009), excluding the findings from endurance athletes who may have high amounts of intramyocellular fat in their insulin sensitive muscles (Goodpaster et al. 2001). In this study, we found the plasma glucose levels to be significantly higher among the persistently inactive co-twins (Table 1). The markers of glucose homeostasis

were correlated with the amount of infiltrated fat, and the associations were emphasized when the diabetic and inactive twins were included. Our correlation analysis highlighted the relationship between the amount of intramuscular (extramyocellular) ectopic fat and the level of Hb_{A1C}, as shown in Fig. 2. Hb_{A1C}, which is the measure of long-term plasma glucose concentration, is one of the mediating factors by which exercise decreases the risk for cardiovascular events (Thomas et al. 2006; Mora et al. 2007; Church et al. 2010). However, the link between the intramuscular lipid accumulation and insulin resistance is not yet fully understood (Goodpaster et al. 2001; Goodpaster and Brown 2005; Dube et al. 2008; Hawley and Lessard 2008; Taube et al. 2009; Eckardt et al. 2011). One of the suggestions for the association could be the reduced aerobic capacity among the inactive co-twins (Leskinen et al. 2010). However, although the number of mitochondria might be reduced in an insulin resistant skeletal muscle, the mitochondrial respiratory capacity can be maintained normal (Larsen et al. 2011).

As such, the accumulation of ectopic "high-risk" fat and adipocyte function are important mechanisms in mediating the prolonged effects of life-long regular exercise on glucose metabolism (Goodpaster et al. 2000; Boule et al. 2005; Petersen and Shulman 2006; Dube et al. 2008; Leskinen et al. 2009b; Taube et al. 2009; Rector and Thyfault 2011). Notably, the co-twins did not differ significantly in either body weight or BMI (see Table 1), and there were no differences in their fat intakes (Rintala et al. 2011), yet the inactive co-twins had significantly more visceral, intramuscular, and liver fat, as we have reported earlier (Leskinen et al. 2009b). Therefore, we can also conclude that the disadvantage of prolonged inactivity is not necessarily seen in body weight status but rather in specific risk factors of metabolic deterioration (Patel et al. 2011).

Our study included an extensive follow-up of leisure-time physical activity habits during adult life (from the thirties to sixties). The baseline was the year 1975, when the first physical activity data were available, but it is probable that the discordance in physical activity had begun earlier. We used structured physical activity questionnaire which has shown high reliability in previous studies (Kujala et al. 1998; Kujala et al. 2002; Waller et al. 2008; Leskinen et al. 2009a). Although we used validated methods to document leisure-time physical activity, subject's understanding on what leisure-time physical activity is may have changed over time. However, all of our physical activity assessments clearly differentiated inactive and active co-twins in the intrapair analyses. Due to our strict criteria for the physical activity discordance, we ended up with rather a small sample size. However, we are dealing with very unique phenomenon. Despite the fact that different persistent activity levels are common, it is less common that co-twins of a twin pair have persistently different activity levels. According to the classic twin design, MZ co-twins are genetically identical at the sequence level, and the intrapair differences (discordance) in MZ twins are due to environmental factors, taken very broadly. Therefore, we were also able to control for shared genes and childhood environment that may confound the traits studied.

In conclusion, our co-twin control study showed that long-term physical activity preserves knee extensor muscle strength. This finding underlines the role of voluntary, mostly aerobic type of physical activity in maintaining muscle quality, but not necessarily mass. These results were similar in intrapair analysis in which only the seven identical twin pairs were included. Regular exercise, namely, use of muscles, prevents also from the accumulation of intramuscular fat which might be related to maintained glucose metabolism and, thus, prevention of metabolic disorders.

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