EXERCISE IN WOMEN WITH THE METABOLIC SYNDROME

Molecular mechanisms of exercise-induced changes in insulin resistance and vascular structure

Fleur Poelkens

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ISBN

978-90-9028416-3

Cover design

Hans Scholte in ‘t Hoff (http://www.hansscholteinthoff.nl/)

Printed & Lay Out

Proefschriftmaken.nl || Uitgeverij BOXPress

Published by

Uitgeverij BOXPress, ’s-Hertogenbosch

The research described in this thesis was performed at the departments of Physiology and General Internal Medicine at the Radboud University Nijmegen Medical Centre, the Netherlands. Financial support for this research was provided by an AGIKO-stipend grant from the Netherlands Organisation for Health Research and Development (ZonMw grant nr. 92003409).

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 21 november 2014
om 12.30 uur precies

door

Fleur Poelkens

Geboren op 27 juli 1978
te Hengelo (Ov)

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Voor Koen

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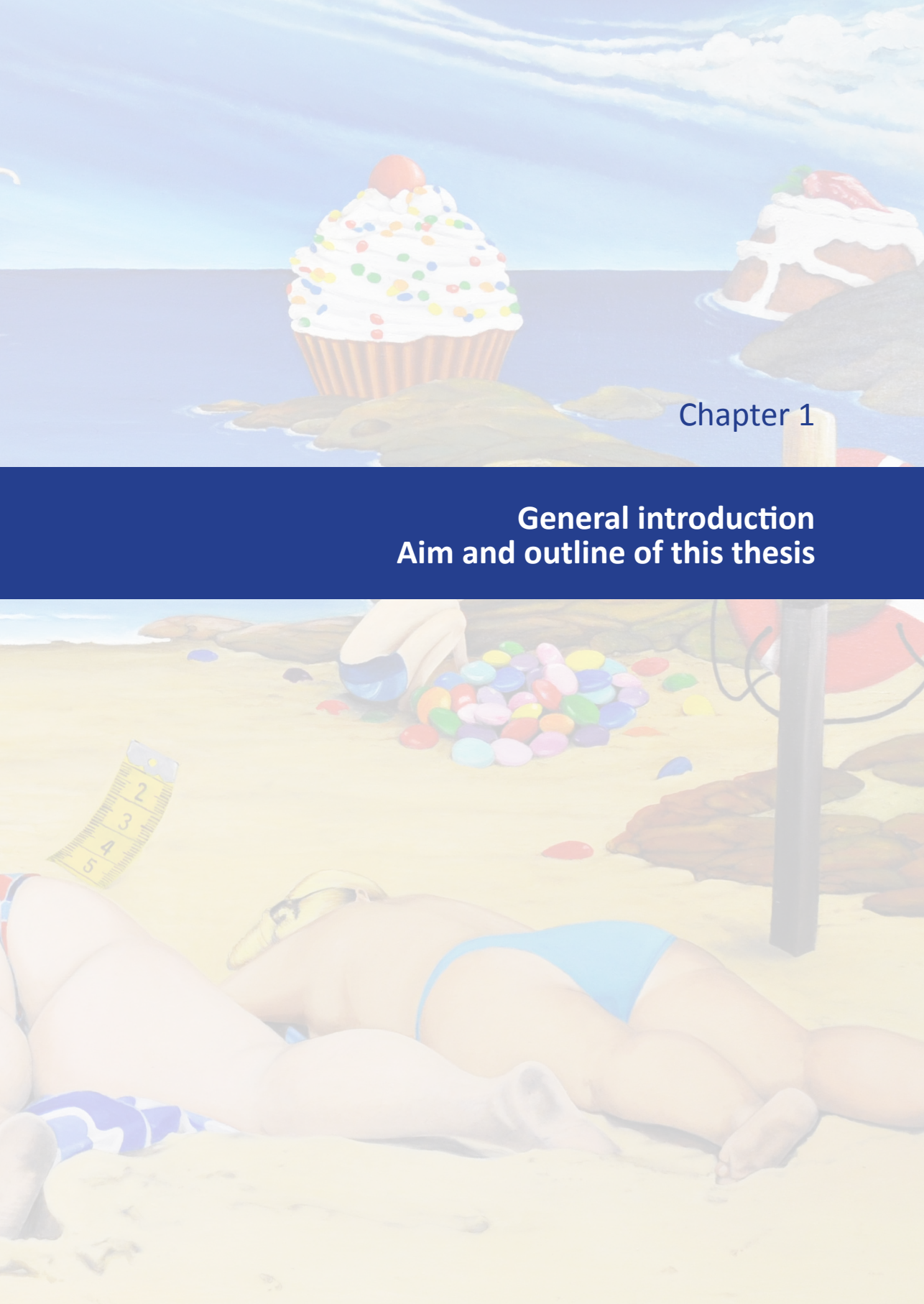


Chapter 1

General introduction
Aim and outline of this thesis

Obesity, a serious threat to human health, is caused by a complex interplay between genetic susceptibility, physical inactivity and nutrient overconsumption[1]. Since the beginning of the 1980s the prevalence of obesity has more than doubled, with nowadays more than 1.4 billion adults being overweight (BMI > 25 kg/m^2) and more than 500 million being obese (BMI>30 kg/m^2) (WHO Fact sheet N^{0}311, March 2013). Especially in women, the prevalence of obesity has increased dramatically, resulting in 33% more obese women than obese men (WHO Fact sheet N^{0}311, March 2013). Obesity is associated with several metabolic- and cardiovascular risk factors such as insulin resistance, dyslipidemia, low-grade inflammation, and hypertension. Many obese subjects ultimately develop type 2 diabetes mellitus[2] and cardiovascular diseases (CVD)[3]. Interestingly, some 30% of women with obesity have a healthy metabolic and cardiovascular phenotype.[4] They have a cardiovascular disease risk similar to their lean counterparts.[5] The remaining 70% of the obese women have at least one, but often multiple, cardiovascular disease risk factors. Subjects with at least three metabolic or cardiovascular risk factors are classified as people with the metabolic syndrome.[6] Treatment of the metabolic syndrome is of utmost importance. Increasing physical activity levels by an exercise training program improves risk factors associated with the metabolic syndrome and hence reverses the metabolic syndrome.[7-11] However, the molecular mechanisms behind the positive effects of exercise training on e.g. insulin resistance are still largely unknown. With modern technologies in human genetic research, such as the development of high throughput RNA gene expression microarrays, expression levels of virtually all know genes can be analysed in a single experiment. Gene expression microarray analysis is, therefore, a rapid and comprehensive approach for the first identification of exercise-induced molecular changes.

The aim of this thesis is to gain more insight into the molecular mechanism of insulin resistance and the molecular mechanisms of exercise-induced improvements in insulin resistance and arterial remodeling. These studies were performed in women with the metabolic syndrome. This introduction provides some background on the metabolic syndrome in women and the existence of the intriguing metabolically healthy obese phenotype. Furthermore, the effects of exercise training on molecular mechanisms of insulin resistance and arterial remodeling will be described. Finally the aim, outline and methods applied in this thesis are presented.

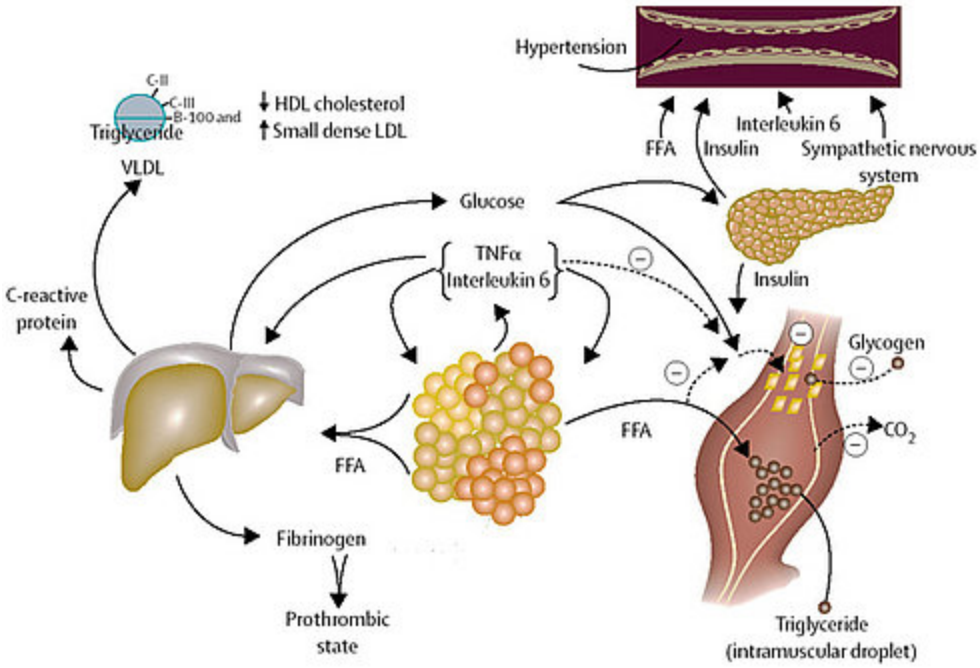
Metabolic syndrome

Already in the 1920s, Kylin noticed clustering of hypertension, hyperglycaemia and gout in some of his patients.[12] In 1988's Banting lecture, Reaven first mentioned the term metabolic syndrome or syndrome-X. But it was not until 1998 before an internationally recognised definition was proposed by the WHO[13] and shortly thereafter by the National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATPIII)[14]. In 2009, a joint interim statement of several leading authorities on the criteria and cut-off points of the metabolic syndrome was proposed, which is currently used as *the* definition of the metabolic syndrome.[6] Women with the metabolic syndrome are defined as having at least three out of five cardiovascular and/or metabolic risk factors including 1) waist circumference above 88 cm (European population), 2) hypertension defined as a blood pressure above 130/85 mmHg and/or use of anti-hypertensive medications, 3) HDL cholesterol levels below 1.3 mmol/l, 4) triglyceride levels above 1.7 mmol/l, and 5) fasting glucose level above 5.6 mmol/l.[6] With the growing pandemic of obesity the prevalence of subjects with the metabolic syndrome is concurrently rising. The latest NHANES (National Health and Nutrition Examination Survey) data from the USA report a prevalence of 32.5% among adult women.[15] Although the prevalence of the metabolic syndrome is lower in Europe compared to the USA, in the Netherlands still one out of six adults (14%) fulfil the metabolic syndrome criteria.[16] The metabolic syndrome is associated with important adverse medical, social, and economic outcomes. Subjects with the metabolic syndrome are not only at risk for developing diabetes (relative risk 5.0)[17-19] and cardiovascular diseases (relative risk 2.5)[3, 20] but are also at increased risk for the development of cancer[21], and even premature death (relative risk 2.8)[22].

The unifying and most accepted hypothesis to describe the pathophysiology of the metabolic syndrome is insulin resistance (Figure 1).[23] Obesity, and more specifically abdominal visceral obesity, seems crucial for the development of insulin resistance and the occurrence of the metabolic syndrome. This explains why waist circumference is one variable of the metabolic syndrome. In subjects with visceral obesity an abundance of circulating fatty acids contribute to the development of insulin resistance. Circulating free fatty acids increase hepatic glucose production and diminish the inhibition of glucose production by insulin. Upon reaching insulin sensitive tissue such as muscle tissue, responsible for disposal of 50-75% of the oral ingested glucose, fatty acids induce insulin resistance by modifying downstream signaling. The major affected downstream signaling pathway is phosphatidylinositol 3-kinase (PI-3K) which normally contributes to the translocation of GLUT4 to the membrane. With increased circulating fatty acid flux to the liver, increased production of triglyceride-rich very low-density lipoproteins (VLDL) occurs. In insulin resistant subjects, insulin subsequently promotes the biosynthesis of triglycerides in the liver

and enhances lipolysis in adipocytes which further increases plasma fatty acid concentration. Due to the hypertriglyceridemia, the HDL particle becomes smaller, resulting in an increased clearance of HDL from the circulation. The relation between insulin resistance and hypertension is well established through several mechanisms including increased plasma catecholamines and enhanced sympathetic nervous system activity. In normal-weight subjects, insulin acts as a vasodilator but in the presence of insulin resistance this effect can be lost, thereby increasing blood pressure. Insulin also increases the renal sodium reabsorption in subjects with the metabolic syndrome inducing plasma volume expansion, and hence, hypertension. Finally, increased adipocyte mass leads to an increased angiotensin production, decreasing natriuresis, leading to hypertension.

Figure 1



Pathophysiology of the metabolic syndrome (insulin resistance).

Adapted from Eckel[23]

In the last decade, the importance of adipose tissue as an endocrine organ secreting numerous bioactive peptides including pro- and anti-inflammatory cytokines has been established. Recent studies have demonstrated a strong link between chronic low-grade inflammation markers such as high-sensitive C-reactive

protein (hs-CRP), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and the inflammasome, and the development of insulin resistance and the metabolic syndrome.[24-27] These important results were obtained in insulin-reactive tissue such as fat. Since skeletal muscle tissue accounts for the majority (50-75%) of insulin-induced glucose uptake, investigating the role of skeletal muscle inflammatory genes that correlate with insulin resistance might generate new insights into the molecular mechanism of insulin resistance.

Metabolically healthy obese phenotype

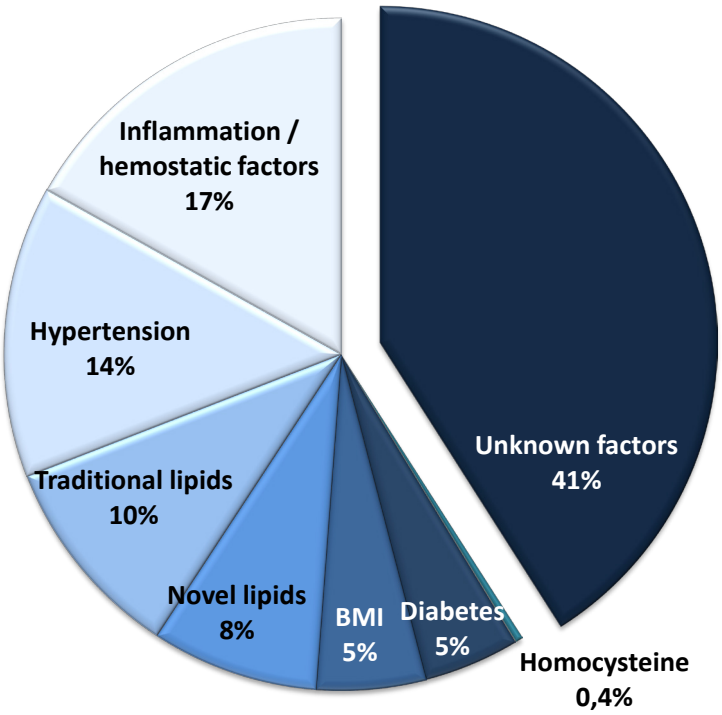
Although obesity is strongly associated with the development of several metabolic- and cardiovascular risk factors, interestingly some 30% of obese women have a 'metabolically healthy obese phenotype'.[4] Despite their obesity, they have a similar 10 years cardiovascular disease risk compared to their lean counterparts[5] in contrast to obese women with the metabolic syndrome, who have an almost 3-fold increased risk for cardiovascular disease events [3]. In recent years, several hypotheses have been postulated regarding this metabolic healthy obese phenotype including higher physical fitness levels[28], different fat tissue distribution and fat metabolic activity[29, 30,] higher birth weight[31], and early onset of obesity[32]. Large epidemiological studies have shown that obese individuals who are in the higher range of physical fitness or physical activity levels, are more likely to have a metabolic healthy phenotype [4, 33, 34]. Bouchard et al. reported similar findings in women with the metabolically healthy obese phenotype who performed a faster and 6-min walk test over a longer distance compared to obese women with risk factors[35]. Unfortunately, almost all of these studies used subjective self-reported questionnaires or general prediction models to determine physical activity levels and physical fitness, respectively. Besides physical fitness, the metabolic activity of fat tissue, including the inflammatory cytokine response [29, 30] and the specific location of fat tissue, have been proposed as possible explanations for the metabolically healthy obese phenotype [32, 36]. Since there seems to be an association between fitness level and subclinical inflammatory profile in obese subjects [37], investigating the role of physical fitness simultaneous with the inflammation profile in metabolically healthy obese women and in obese women with the metabolic syndrome might improve our understanding of the metabolically healthy obese phenotype. Understanding of the physiological mechanisms why some obese women seem to be protected against the detrimental effects of obesity might help to optimize strategies to prevend obesity-associated risk factors.

Exercise training in women with the metabolic syndrome

Having the metabolic syndrome has major consequences, so treatment of the metabolic syndrome is clearly of utmost importance. Increasing physical activity levels with an exercise training program can improve the individual risk factors (insulin resistance, blood pressure, lipid profile) associated with the metabolic syndrome[38-41] and hence reverse the metabolic syndrome.[7-11, 42] Relatively few studies have investigated the effects of exercise training in metabolic syndrome per se. Already brisk walking for 30 minutes a day, which is the current international physical activity guideline/recommendation for adults[43], can prevent the metabolic syndrome.[44, 45] A few weeks of intensive endurance exercise training even reverses the metabolic syndrome in approximately 40% of the subjects.[7, 8] Although the beneficial effects of exercise training on the human metabolic and cardiovascular system are strongly established, the mechanisms behind these positive effects of exercise training are still partly unknown. In a prospective study of apparently healthy women, the beneficial effects of exercise training on cardiovascular disease risk could only partly be explained by improvements of traditional cardiovascular risk factors such as haemostatic and inflammation factors, hypertension, lipid profile, body weight and insulin resistance (Figure 2).[46] Approximately 40% of the beneficial effects of exercise could not be explained by changes in the traditional risk factors: the so called 'risk factor gap'. Several hypothesis have been postulated to explain this risk factor gap. The vascular endothelium emerged as a key factor since a blunted endothelial function is a risk factor for cardiovascular disease and an enhanced endothelial function appears to be protective against cardiovascular disease.[47, 48] Another hypothesis postulated to explain this risk factor gap is related to the autonomic nervous system. There is a variety of evidence that an altered autonomic function can have a profound effect on cardiovascular disease, but this hypothesis is beyond the scope of this thesis.[49]

With emerging new technologies in human genetic research, such as high throughput RNA microarrays, expression levels of virtually all know genes can be analysed in a single experiment. This provides the opportunity to identify whole genome transcriptional responses to stimuli such as exercise training. Exercise training induces chronic adaptations in i.a. skeletal muscle tissue such as an increase in aerobic ATP generation, less glucose utilization (more fats) and improved insulin sensitivity[50]. Therefore, comparing skeletal muscle RNA gene expression before and after an exercise training program may lead to the identification of novel genes and molecular signaling pathways. This will hopefully generate new hypothesis regarding the beneficial effects of exercise training that can explain the 'risk factor gap'.

Figure 2.



Exercise-induced improvements in risk factors associated with a reduction in CVD events.
Adapted from Mora[46]

Effects of physical activity on insulin resistance

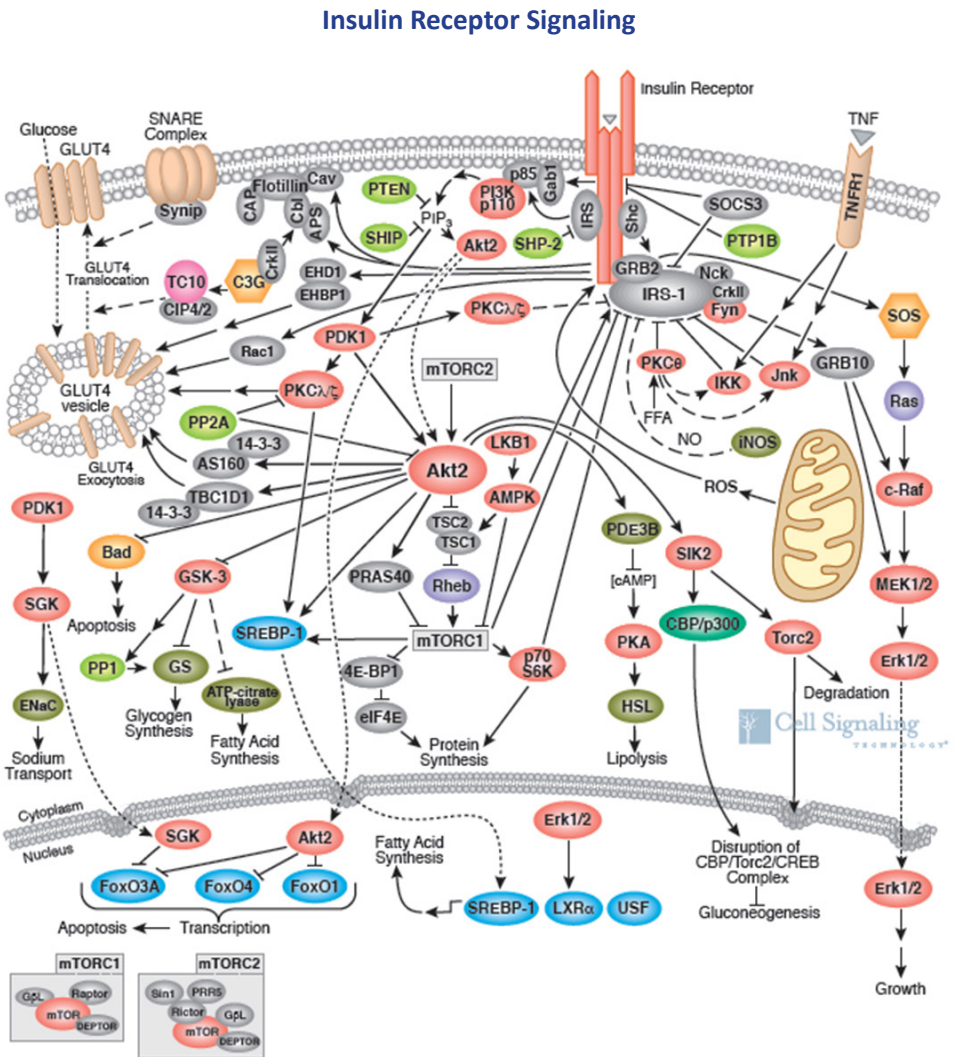
Regular physical activity leads to a number of adaptations in skeletal muscle tissue that allow the muscle to utilize substrates for ATP production more efficiently and to become more responsive to insulin. Three major skeletal muscle adaptations are 1) a switch in muscle fiber type IIb to type IIx and IIa, and in some cases to slow-twitch oxidative fiber type I, 2) an increase in mitochondrial activity and content, and 3) an increase in GLUT4 protein expression.[51] Although the effects of exercise training on whole body insulin sensitivity have been extensively studied over the last decades[7, 52, 53], the molecular mechanisms that underlie this important beneficial effect are only partly understood. Exercise, just like insulin, increases glucose uptake in skeletal muscle through translocation of glucose transporter proteins (i.a.

GLUT4) from the intracellular compartment to the surface of the cell.[54] However, the intracellular molecular signaling pathways that lead to this translocation of GLUT4 to the membrane are distinct between insulin and exercise. Insulin binds to the α-subunit of the insulin receptor, resulting in auto-phosphorylation of tyrosine residues in the receptor β-subunit, tyrosine phosphorylation of the insulin receptor substrates IRS-1 and ISR-2, and activation of phosphatidylinositol 3-kinase which subsequently activates the Akt/PKB and PKCλ cascades leading to GLUT4 translocation (Figure 3). Exercise does not seem to activate these proximal signaling steps, and other signaling molecules have been proposed to explain the beneficial effects of exercise, such as AMP-activated protein kinase (AMPK), peroxisome-proliferator-activated receptor-γ coactivator 1α (PGC-1α), calcineurin, AS160 and p38 MAPK seem to be involved.[54, 55] There is probably no single factor that accounts for the enhanced muscle insulin sensitivity for glucose uptake after exercise training. Most probably, a combination of plasma factors, muscle fibre type composition, mitochondrial capacity, and intracellular pathways are involved. Microarrays are a powerful tool to study these molecular mechanisms comprehensively. Previous microarray studies have provided differentially expressed genes and gene cluster analysis (i.a. Gene Ontology terms) when comparing healthy insulin sensitive subjects with insulin resistant subjects[56-58] or comparing strength trained versus endurance trained athletes[59]. Few studies have focused on skeletal muscle gene expression after an exercise training program but either these were performed in healthy insulin sensitive individuals[60] or following a relatively short training period (weeks)[61]. Focusing on differential gene expression in skeletal muscle of insulin resistant women with the metabolic syndrome and in insulin sensitive lean women after a prolonged exercise training (months), taking into account their improvement in insulin sensitivity, might identify novel function of genes and molecular signaling pathways.

Effect of physical activity on arterial remodeling

The beneficial effects of exercise training on the human cardiovascular system have also strongly been established. Exercise training decreases the risk for cardiovascular diseases[62, 63], and a higher physical fitness confers cardioprotection.[64] Approximately 60% of this decreased risk can be explained by the exercise-induced improvements in traditional cardiovascular risk factors.[46] One hypothesis postulated as an explanation for the remaining 40%, the so called 'risk factor gap', is a direct effects of exercise on the vessel wall. The intima-media thickness (IMT), the thickness of the tunica intima and tunica media – the two innermost layers of the arterial wall, is a validated surrogate marker for atherosclerosis[65] and an increased carotid IMT has been associated with increased risk for both cardiac[66] and peripheral

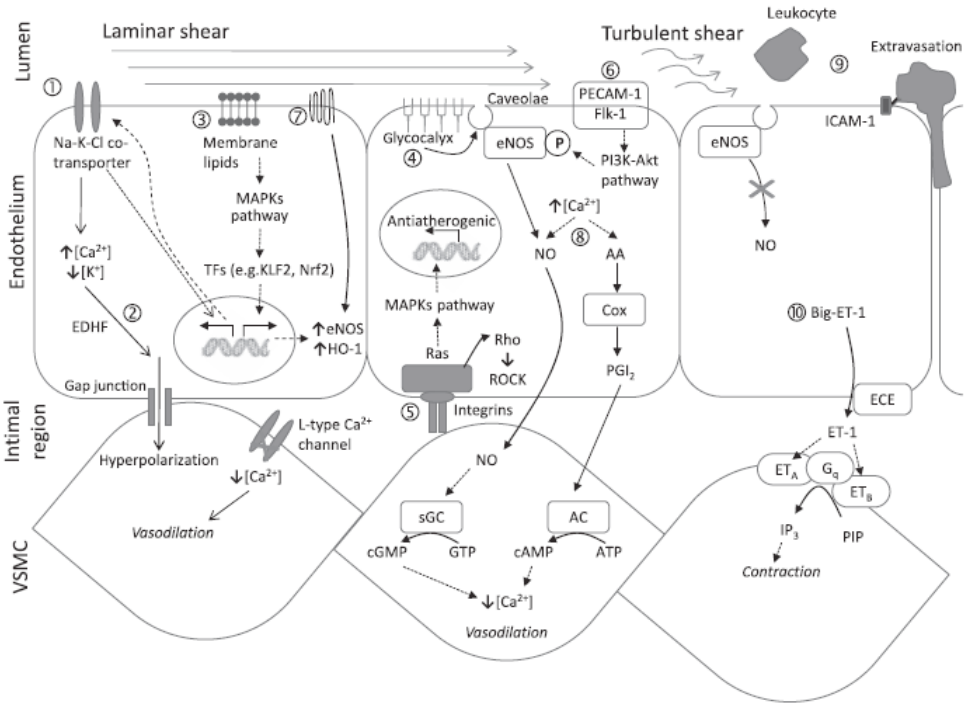
Figure 3.



Schematic overview of insulin receptor signaling pathway depicting current complex knowledge about insulin signaling. Insulin activates numerous signaling partners and cascades ultimately leading to GLUT4 translocation to the plasma membrane enabling glucose uptake. Insulin signaling also has growth and mitogenic effects, insulin signaling inhibits gluconeogenesis in the liver and, promotes fatty acid synthesis. Adapted from https://www.cellsignal.com/pathways/glucose-metabolism.jsp

vascular events.[67] Subjects with the metabolic syndrome are known to have an increased arterial wall thickness[68, 69], which seems more pronounced in women.[70] Already a few weeks of exercise training can decrease arterial wall thickness in healthy men[71, 72] and in young obese subjects.[73, 74] The exercise-induced arterial

Figure 4.



Schematic overview of current knowledge about the molecular effects of exercise-induced shear stress regulating vascular structure and function.

Adapted from Whyte-Laughlin[76]

remodeling is probably induced by increased vascular shear stress during repeated bouts of exercise.[75] The blood flow-generated endothelial shear stress induces altered gene expression in endothelial cells and induces the release of several factors which can influence the vascular endothelium and the underlying vascular smooth muscles (Figure 4). The molecular mechanisms that cause these vascular changes are gradually elucidated.[76] One key factor is nitric oxide release induced by shear stress via phosphorylation of endothelial NO Synthase by AKT (V-AKT murine thymoma viral oncogene homolog 1).[77] Nitric oxide diffuses to the underlying vascular smooth muscle cell and subsequently induces vasodilation. Also a role for growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGFβ-1), extracellular matrix proteins, and anti-oxidative enzymes in response to exercise has been established.[78-80]

Gene expression microarrays are a powerful tool to study these molecular mechanisms comprehensively. Microarrays have previously been used to study changes in vascular gene expression after exercise in animal models.[81-84] In humans,

gene expression microarrays have been used to study changes in muscular gene expression after exercise.[61, 85, 86] But only few studies in humans have linked results of microarrays with vascular remodeling after exercise training.[78] Identification of differentially expressed vascular-related genes after an exercise training program may increase the understanding of arterial wall remodeling.

Outline of this thesis

The aim of this thesis is to obtain more insight into the molecular mechanism of insulin resistance and the molecular mechanisms of exercise-induced improvements in insulin resistance and arterial remodeling in humans, with a special interest in women with the metabolic syndrome. To answer these study questions, a number of human-translational studies will be performed.

Women with the metabolic syndrome often display insulin resistance. This obesity-induced insulin resistance is thought to arise from a defective signal transduction along the insulin signaling cascade, and a strong link with chronic low-grade inflammation has been established. Since insulin resistance is predominantly located in skeletal muscle tissue, the aim of **chapter 2** is to identify differentially expressed inflammatory genes in skeletal muscle tissue of insulin resistant women with the metabolic syndrome compared to healthy controls. Identification of these key players may increase our understanding of the link between obesity induced low-grade skeletal muscle inflammation and insulin resistance in the context of the metabolic syndrome.

Interestingly, 30% of the women with obesity have a healthy metabolic phenotype. Despite a high body fat percentage they are insulin sensitive and they have a 10 years cardiovascular disease risk similar to their lean counterparts.5 Several hypotheses have been postulated regarding this metabolically healthy obese phenotype. In **chapter 3** we investigate whether physical fitness and a low-grade inflammatory profile can explain the metabolically healthy obese phenotype.

While physical inactivity is associated with the development of insulin resistance, exercise training effectively counteracts this development by stimulating the insulin signaling cascade. To gain more insight into the molecular mechanisms behind the development and prevention of insulin resistance, the aim of the studies described in **chapter 4** is to assess muscular gene expression after physical deconditioning compared to muscular gene expression after an exercise training program. Genes that are downregulated after deconditioning and upregulated after

exercise training are the most interesting, because such genes may play a role in the detrimental effects of physical inactivity as well as in the beneficial effects of regular exercise.

Exercise training can reverse the metabolic syndrome and the accompanying insulin resistance. In **chapter 5a** the use of HOMA-IR, in assessing insulin resistance after an exercise training program, is discussed. Although the beneficial effects of regular exercise on insulin sensitivity are well established, the molecular mechanisms are only partly understood. In **chapter 5b** we study the effects of an exercise training program on insulin sensitivity in insulin resistant women with the metabolic syndrome and in insulin sensitive lean women. By comparing the exercise-induced changes in skeletal muscle gene expression between insulin resistant and insulin sensitive women, we aim to identify key genes responsible for the exercise-induced improvement in insulin sensitivity.

Women with the metabolic syndrome are prone to develop atherosclerosis and cardiovascular disease. Exercise training induces arterial remodeling, such as a decrease in IMT, and can hence reverse the atherosclerotic process. This arterial remodeling is believed to result from increased vascular shear stress during repeated bouts of exercise. The blood flow-generated shear stress induces altered gene expression in endothelial cells enabling arterial remodeling to occur. In **chapter 6** the effects of exercise training on central and peripheral conduit artery intima-media thickness and diameter will be investigated. To identify important genes and molecular pathways involved in exercise-induced arterial remodeling, we will perform muscle gene expression microarrays and relate the results to functional changes in vascular function.

Finally a general discussion of the findings and future perspectives will be provided in **chapter 7** and a summary in **chapter 8**.

Methods applied in this thesis

Physical inactivity model and exercise interventions

Comparing physical deconditioning to exercise training

To exclude whole-body systemic effects and to identify specifically effects on skeletal muscle, we have used in the thesis unique human in vivo models for local inactivity and for local exercise training. Unilateral lower limb suspension is a proven model for local deconditioning[87, 88]. One leg of the subjects is suspended

and unloaded from all weight bearing for a period of three weeks. The effectiveness of this model is monitored by measuring skin temperature and calf circumference. The model for local exercise training used in this thesis was training of subjects with a spinal cord lesion above thoracic level 12. These subjects underwent twenty 30-minute sessions of functional electrical stimulation exercise training using a computer-controlled leg cycle ergometer over an eight weeks' time period.

Exercise training protocol

The aims of the exercise interventions applied in this thesis were to induce improvements in cardio-respiratory fitness, in the metabolic profile including insulin resistance, to decrease low-grade inflammatory status, and to induce arterial remodeling. Therefore, women trained for six months, three times a week under the supervision of a professional trainer. Training consisted of cycling exercise on a ergometer (Lode, Groningen, the Netherlands) starting with a 10 minute warming-up, followed by 30 minutes of exercise at 65% of the individual heart rate reserve and finished off with a cooling-down of 5 minutes. As exercise tolerance improved, the training intensity was increased by 5% to a maximum of 85% of the heart rate reserve.

Methods

Cardio-respiratory fitness test

Women performed a maximal exercise test on an electrically braked leg-cycling ergo meter (Lode, Angio 300, Groningen, the Netherlands) using an incremental protocol to assess their cardio-respiratory fitness. Workload increased by 10 W per minute, starting at 10 W, until exhaustion. A gas-analyzer was used to measure oxygen consumption continuously (Jaeger Benelux BV, Breda, the Netherlands). Maximal oxygen consumption (VO2max) was analyzed as the mean of the last minute of the exercise bout.

Hyperinsulinaemic euglycaemic clamp

Peripheral tissue sensitivity to exogenous insulin was measured using a 120 minutes hyperinsulinaemic euglycaemic clamp.[89, 90] Insulin was infused intravenously in a dose of 430 pmol · m-2 · min-1 (60 mU · m^{-2} · min^{-1}) and arterialized plasma glucose concentrations were clamped at 5 mmol·l-1 by a variable glucose 20% (≈ 200mg × ml^{-1}) infusion rate, adjusted depending on plasma glucose level measurements at 5-minute intervals. Plasma glucose was measured in duplicate by the glucose oxidation method (Beckman Glucose Analyzer 2, Beckman Instruments Inc, Fullerton, CA 92634, USA). Insulin was measured in duplicate against

International Standard 83/500 by an in-house radio-immunoassay (RIA) using an anti-human insulin antiserum raised in guinea pig and radio-iodinated human insulin as a tracer. Whole body glucose disposal during the euglycaemic clamp was expressed as M-value:

= Mean glucose infusion $T_{90\text{-}120\ min}$ ($mg \times min^{-1}$)/weight (kg)

Dexa scan

Total body mass, body fat percentage, and body fat distribution was measured by total body Dexa scan (QDR 4500 densitometer, Hologic Inc. Waltham, MA).

Echo-Doppler ultrasound

Baseline diameter of the common carotid artery, brachial artery, common femoral artery and superficial femoral artery and intima-media thickness (IMT) of the common carotid artery and common femoral artery were measured using high resolution echo ultrasonography (Picus, Pie Medical Benelux, Maastricht, the Netherlands or T3000, Terason, Burlington, MA, USA). Baseline blood flow (i.e. red blood cell velocity) was measured by the Doppler technique (WAKI e, Atys, France or Terason). IMT was defined as the distance from lumen-intima interface to media-adventitia interface[91] and normalized for lumen diameter size (IMT/lumen).[91, 92]

Muscle biopsies

A percutaneous needle muscle biopsy was obtained from the right vastus lateralis muscle using the Bergström technique. On the day of the skeletal muscle biopsy, subjects consumed a standardized breakfast consisting of 250 kcal energy (79 % carbohydrates, 11.2 % protein, 9.8 % fat). After local anesthesia of the skin with lidocaine 1%, a small incision was made through the skin and fascia and ~100 mg of muscle tissue taken via the biopsy needle. The removed muscle tissue was immediately frozen in liquid nitrogen and will be stored at -800C until further analysis.

Microarray gene expression

From the muscle biopsies total RNA was isolated and purified. RNA gene expression profiling was performed using Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix Inc., Santa Clara, CA, USA). The average fluorescence intensity of all genes was calculated using the Robust Multiarray Analysis (RMA) Algorithm[93]. The Affymetrix CEL-files were imported into Partek (Genomic Suite Software version 6.4, Partek Inc., St Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA logarithm with GC background correction. Transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative gene expression levels.

The Database for Annotation Visualization and Integrated Discovery functional annotation tool (DAVID, http:david.abcc.ncifcrf.gov)) was used as an integrated biological knowledgebase and analytical tool to systematically extract biological meaning from large gene list.[94] Gene lists of interest were analyzed using DAVID with main focus on the enrichment in GO-terms (Gene Ontology terms) of biological processes (GOTERM_BP_FAT) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.[94]

References

1. Temelkova-Kurktschiev T, Stefanov T. Lifestyle and genetics in obesity and type 2 diabetes. *Exp Clin Endocrinol Diabetes*. 2012;120:1-6
2. Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*. 2005;112:3066-3072
3. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care*. 2001;24:683-689
4. Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, Sowers MR. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: Prevalence and correlates of 2 phenotypes among the us population (nhanes 1999-2004). *Arch Intern Med*. 2008;168:1617-1624
5. Song Y, Manson JE, Meigs JB, Ridker PM, Buring JE, Liu S. Comparison of usefulness of body mass index versus metabolic risk factors in predicting 10-year risk of cardiovascular events in women. *Am J Cardiol*. 2007;100:1654-1658
6. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. *Circulation*. 2009;120:1640-1645
7. Tjonna AE, Lee SJ, Rognmo O, Stolen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slordahl SA, Kemi OJ, Najjar SM, Wisloff U. Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: A pilot study. *Circulation*. 2008;118:346-354
8. Katzmarzyk PT, Leon AS, Wilmore JH, Skinner JS, Rao DC, Rankinen T, Bouchard C. Targeting the metabolic syndrome with exercise: Evidence from the heritage family study. *Med Sci Sports Exerc*. 2003;35:1703-1709
9. Stensvold D, Tjonna AE, Skaug EA, Aspenes S, Stolen T, Wisloff U, Slordahl SA. Strength training versus aerobic interval training to modify risk factors of the metabolic syndrome. *J Appl Physiol*. 2010
10. Kemmler W, Von Stengel S, Engelke K, Kalender WA. Exercise decreases the risk of metabolic syndrome in elderly females. *Med Sci Sports Exerc*. 2009;41:297-305
11. Bateman LA, Slentz CA, Willis LH, Shields AT, Piner LW, Bales CW, Houmard JA, Kraus WE. Comparison of aerobic versus resistance exercise training effects on metabolic syndrome (from the studies of a targeted risk reduction intervention through defined exercise - strride-at/rt). *Am J Cardiol*. 2011;108:838-844
12. Kylin E. Studien. Hypertonie-hyperglykamie-hyperurikamiesyndrome. . *Zentralblatt fur innere Medizon*. 1923;44
13. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a who consultation. *Diabet Med*. 1998;15:539-553
14. Executive summary of the third report of the national cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel iii). *Jama*. 2001;285:2486-2497

15. Ervin R. Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United states, 2003-2006. *National health statistics reports*. 2009;13:1-8
16. Bos MB, de Vries JH, Wolffenbuttel BH, Verhagen H, Hillege JL, Feskens EJ. [the prevalence of the metabolic syndrome in the netherlands: Increased risk of cardiovascular diseases and diabetes mellitus type 2 in one quarter of persons under 60]. *Ned Tijdschr Geneeskd*. 2007;151:2382-2388
17. Grundy SM, Hansen B, Smith SC, Jr., Cleeman JI, Kahn RA. Clinical management of metabolic syndrome: Report of the american heart association/national heart, lung, and blood institute/ american diabetes association conference on scientific issues related to management. *Circulation*. 2004;109:551-556
18. Laaksonen DE, Lakka HM, Niskanen LK, Kaplan GA, Salonen JT, Lakka TA. Metabolic syndrome and development of diabetes mellitus: Application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. *Am J Epidemiol*. 2002;156:1070-1077
19. Ford ES, Li C, Sattar N. Metabolic syndrome and incident diabetes: Current state of the evidence. *Diabetes Care*. 2008;31:1898-1904
20. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in united states adults. *Circulation*. 2004;110:1245-1250
21. Pothiwala P, Jain SK, Yaturu S. Metabolic syndrome and cancer. *Metabolic syndrome and related disorders*. 2009;7:279-288
22. Hu G, Qiao Q, Tuomilehto J, Balkau B, Borch-Johnsen K, Pyorala K. Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in nondiabetic european men and women. *Arch Intern Med*. 2004;164:1066-1076
23. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet*. 2005;365:1415-1428
24. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444:860-867
25. Kalupahana NS, Moustaid-Moussa N, Claycombe KJ. Immunity as a link between obesity and insulin resistance. *Mol Aspects Med*. 2012;33:26-34
26. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA, Hooiveld GJ, Hijmans A, Vroegrijk I, van den Berg S, Romijn J, Rensen PC, Joosten LA, Netea MG, Kanneganti TD. Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci U S A*. 2011;108:15324-15329
27. Elks CM, Francis J. Central adiposity, systemic inflammation, and the metabolic syndrome. *Curr Hypertens Rep*. 2010;12:99-104
28. Ortega FB, Lee DC, Katzmarzyk PT, Ruiz JR, Sui X, Church TS, Blair SN. The intriguing metabolically healthy but obese phenotype: Cardiovascular prognosis and role of fitness. *Eur Heart J*. 2013;34:389-397
29. Koster A, Stenholm S, Alley DE, Kim LJ, Simonsick EM, Kanaya AM, Visser M, Houston DK, Nicklas BJ, Tylavsky FA, Satterfield S, Goodpaster BH, Ferrucci L, Harris TB. Body fat distribution and inflammation among obese older adults with and without metabolic syndrome. *Obesity (Silver Spring, Md*. 2010;18:2354-2361
30. Karelis AD, Faraj M, Bastard JP, St-Pierre DH, Brochu M, Prud'homme D, Rabasa-Lhoret R. The metabolically healthy but obese individual presents a favorable inflammation profile. *J Clin Endocrinol Metab*. 2005;90:4145-4150
31. Bouhours-Nouet N, Dufresne S, de Casson FB, Mathieu E, Douay O, Gatelais F, Rouleau S, Coutant R. High birth weight and early postnatal weight gain protect obese children and adolescents from truncal adiposity and insulin resistance: Metabolically healthy but obese subjects? *Diabetes Care*. 2008;31:1031-1036

32. Brochu M, Tchernof A, Dionne IJ, Sites CK, Eltabbakh GH, Sims EA, Poehlman ET. What are the physical characteristics associated with a normal metabolic profile despite a high level of obesity in postmenopausal women? *J Clin Endocrinol Metab*. 2001;86:1020-1025
33. Farrell SW, Cheng YJ, Blair SN. Prevalence of the metabolic syndrome across cardiorespiratory fitness levels in women. *Obesity research*. 2004;12:824-830
34. Katzmarzyk PT, Church TS, Janssen I, Ross R, Blair SN. Metabolic syndrome, obesity, and mortality: Impact of cardiorespiratory fitness. *Diabetes Care*. 2005;28:391-397
35. Bouchard DR, Langlois MF, Brochu M, Dionne IJ, Baillargeon JP. Metabolically healthy obese women and functional capacity. *Metabolic syndrome and related disorders*. 2011;9:225-229
36. Hayes L, Pearce MS, Firbank MJ, Walker M, Taylor R, Unwin NC. Do obese but metabolically normal women differ in intra-abdominal fat and physical activity levels from those with the expected metabolic abnormalities? A cross-sectional study. *BMC public health*. 2010;10:723
37. Lavoie ME, Rabasa-Lhoret R, Doucet E, Mignault D, Messier L, Bastard JP, Faraj M. Association between physical activity energy expenditure and inflammatory markers in sedentary overweight and obese women. *International journal of obesity (2005)*. 2010;34:1387-1395
38. Umpierre D, Ribeiro PA, Kramer CK, Leitao CB, Zucatti AT, Azevedo MJ, Gross JL, Ribeiro JP, Schaan BD. Physical activity advice only or structured exercise training and association with hba1c levels in type 2 diabetes: A systematic review and meta-analysis. *Jama*. 2011;305:1790-1799
39. Stefanick ML, Mackey S, Sheehan M, Ellsworth N, Haskell WL, Wood PD. Effects of diet and exercise in men and postmenopausal women with low levels of hdl cholesterol and high levels of ldl cholesterol. *N Engl J Med*. 1998;339:12-20
40. Leon AS, Sanchez OA. Response of blood lipids to exercise training alone or combined with dietary intervention. *Med Sci Sports Exerc*. 2001;33:S502-515; discussion S528-509
41. Dickinson HO, Mason JM, Nicolson DJ, Campbell F, Beyer FR, Cook JV, Williams B, Ford GA. Lifestyle interventions to reduce raised blood pressure: A systematic review of randomized controlled trials. *J Hypertens*. 2006;24:215-233
42. Pattyn N, Cornelissen VA, Eshghi SR, Vanhees L. The effect of exercise on the cardiovascular risk factors constituting the metabolic syndrome: A meta-analysis of controlled trials. *Sports Med*. 2013;43:121-133
43. Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD, Bauman A. Physical activity and public health: Updated recommendation for adults from the american college of sports medicine and the american heart association. *Circulation*. 2007;116:1081-1093
44. Lee S, Kuk JL, Katzmarzyk PT, Blair SN, Church TS, Ross R. Cardiorespiratory fitness attenuates metabolic risk independent of abdominal subcutaneous and visceral fat in men. *Diabetes Care*. 2005;28:895-901
45. Lakka TA, Laaksonen DE, Lakka HM, Mannikko N, Niskanen LK, Rauramaa R, Salonen JT. Sedentary lifestyle, poor cardiorespiratory fitness, and the metabolic syndrome. *Med Sci Sports Exerc*. 2003;35:1279-1286
46. Mora S, Cook N, Buring JE, Ridker PM, Lee IM. Physical activity and reduced risk of cardiovascular events: Potential mediating mechanisms. *Circulation*. 2007;116:2110-2118
47. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol*. 2004;561:1-25
48. Green DJ, Walsh JH, Maiorana A, Best MJ, Taylor RR, O'Driscoll JG. Exercise-induced improvement in endothelial dysfunction is not mediated by changes in cv risk factors: Pooled analysis of diverse patient populations. *Am J Physiol Heart Circ Physiol*. 2003;285:H2679-2687

49. Seals DR, Dinenno FA. Collateral damage: Cardiovascular consequences of chronic sympathetic activation with human aging. *Am J Physiol Heart Circ Physiol*. 2004;287:H1895-1905
50. Carter SL, Rennie C, Tarnopolsky MA. Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab*. 2001;280:E898-907
51. Rockl KS, Witczak CA, Goodyear LJ. Signaling mechanisms in skeletal muscle: Acute responses and chronic adaptations to exercise. *IUBMB life*. 2008;60:145-153
52. Thomas DE, Elliott EJ, Naughton GA. Exercise for type 2 diabetes mellitus. *Cochrane Database Syst Rev*. 2006:CD002968
53. Duncan GE, Perri MG, Theriaque DW, Hutson AD, Eckel RH, Stacpoole PW. Exercise training, without weight loss, increases insulin sensitivity and postheparin plasma lipase activity in previously sedentary adults. *Diabetes Care*. 2003;26:557-562
54. Goodyear LJ, Kahn BB. Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med*. 1998;49:235-261
55. Frosig C, Richter EA. Improved insulin sensitivity after exercise: Focus on insulin signaling. *Obesity (Silver Spring, Md*. 2009;17 Suppl 3:S15-20
56. Elbein SC, Kern PA, Rasouli N, Yao-Borengasser A, Sharma NK, Das SK. Global gene expression profiles of subcutaneous adipose and muscle from glucose-tolerant, insulin-sensitive, and insulin-resistant individuals matched for bmi. *Diabetes*. 2011;60:1019-1029
57. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of pgc1 and nrf1. *Proc Natl Acad Sci U S A*. 2003;100:8466-8471
58. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. Pgc-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature genetics*. 2003;34:267-273
59. Stepto NK, Coffey VG, Carey AL, Ponnampalam AP, Canny BJ, Powell D, Hawley JA. Global gene expression in skeletal muscle from well-trained strength and endurance athletes. *Med Sci Sports Exerc*. 2009;41:546-565
60. Teran-Garcia M, Rankinen T, Koza RA, Rao DC, Bouchard C. Endurance training-induced changes in insulin sensitivity and gene expression. *Am J Physiol Endocrinol Metab*. 2005;288:E1168-1178
61. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mrna expression in human skeletal muscle during recovery from endurance exercise. *Faseb J*. 2005;19:1498-1500
62. Blair SN, Morris JN. Healthy hearts--and the universal benefits of being physically active: Physical activity and health. *Annals of epidemiology*. 2009;19:253-256
63. Taylor RS, Brown A, Ebrahim S, Jolliffe J, Noorani H, Rees K, Skidmore B, Stone JA, Thompson DR, Oldridge N. Exercise-based rehabilitation for patients with coronary heart disease: Systematic review and meta-analysis of randomized controlled trials. *Am J Med*. 2004;116:682-692
64. Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Asumi M, Sugawara A, Totsuka K, Shimano H, Ohashi Y, Yamada N, Sone H. Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: A meta-analysis. *Jama*. 2009;301:2024-2035
65. de Groot E, Hovingh GK, Wiegman A, Duriez P, Smit AJ, Fruchart JC, Kastelein JJ. Measurement of arterial wall thickness as a surrogate marker for atherosclerosis. *Circulation*. 2004;109:III33-38
66. Ebrahim S, Papacosta O, Whincup P, Wannamethee G, Walker M, Nicolaides AN, Dhanjil S, Griffin M, Belcaro G, Rumley A, Lowe GD. Carotid plaque, intima media thickness, cardiovascular risk

factors, and prevalent cardiovascular disease in men and women: The british regional heart study. *Stroke*. 1999;30:841-850

67. Wattanakit K, Folsom AR, Selvin E, Weatherley BD, Pankow JS, Brancati FL, Hirsch AT. Risk factors for peripheral arterial disease incidence in persons with diabetes: The atherosclerosis risk in communities (aric) study. *Atherosclerosis*. 2005;180:389-397
68. Wang ZH, Gong HP, Shang YY, Tang MX, Fang NN, Jiang GH, Zhang Y, Zhong M, Zhang W. An integrative view on the carotid artery alterations in metabolic syndrome. *Eur J Clin Invest*. 2012;42:496-502
69. Di Pino A, Alagona C, Piro S, Calanna S, Spadaro L, Palermo F, Urbano F, Purrello F, Rabuazzo AM. Separate impact of metabolic syndrome and altered glucose tolerance on early markers of vascular injuries. *Atherosclerosis*. 2012;223:458-462
70. Iglseder B, Cip P, Malaimare L, Ladurner G, Paulweber B. The metabolic syndrome is a stronger risk factor for early carotid atherosclerosis in women than in men. *Stroke*. 2005;36:1212-1217
71. Dinenno FA, Tanaka H, Monahan KD, Clevenger CM, Eskurza I, DeSouza CA, Seals DR. Regular endurance exercise induces expansive arterial remodelling in the trained limbs of healthy men. *J Physiol*. 2001;534:287-295
72. Green DJ, Swart A, Exterkate A, Naylor LH, Black MA, Cable NT, Thijssen DH. Impact of age, sex and exercise on brachial and popliteal artery remodelling in humans. *Atherosclerosis*. 2010;210:525-530
73. Farpour-Lambert NJ, Aggoun Y, Marchand LM, Martin XE, Herrmann FR, Beghetti M. Physical activity reduces systemic blood pressure and improves early markers of atherosclerosis in pre-pubertal obese children. *J Am Coll Cardiol*. 2009;54:2396-2406
74. Woo KS, Chook P, Yu CW, Sung RY, Qiao M, Leung SS, Lam CW, Metreweli C, Celermajer DS. Effects of diet and exercise on obesity-related vascular dysfunction in children. *Circulation*. 2004;109:1981-1986
75. Laughlin MH, Newcomer SC, Bender SB. Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. *J Appl Physiol*. 2008;104:588-600
76. Whyte JJ, Laughlin MH. The effects of acute and chronic exercise on the vasculature. *Acta Physiol (Oxf)*. 2010;199:441-450
77. Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW, Schuler G. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation*. 2003;107:3152-3158
78. Lammers G, Duijnhoven NT, Hoenderop JG, Horstman AM, Haan AD, Janssen TW, Graaf MJ, Pardoel EM, Verwiel ET, Thijssen DH, Hopman MT. The identification of genetic pathways involved in vascular adaptations after physical deconditioning versus exercise training in humans. *Exp Physiol*. 2012
79. Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R, Hoppeler H. Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol*. 2001;91:173-182
80. Lundby C, Gassmann M, Pilegaard H. Regular endurance training reduces the exercise induced hif-1alpha and hif-2alpha mrna expression in human skeletal muscle in normoxic conditions. *Eur J Appl Physiol*. 2006;96:363-369
81. Padilla J, Simmons GH, Davis JW, Whyte JJ, Zderic TW, Hamilton MT, Bowles DK, Laughlin MH. Impact of exercise training on endothelial transcriptional profiles in healthy swine: A genome-wide microarray analysis. *Am J Physiol Heart Circ Physiol*. 2011;301:H555-564

82. Matsumoto A, Mason SR, Flatscher-Bader T, Ward LC, Marsh SA, Wilce PA, Fassett RG, de Haan JB, Coombes JS. Effects of exercise and antioxidant supplementation on endothelial gene expression. *Int J Cardiol*. 2012;158:59-65

83. Maeda S, Iemitsu M, Miyauchi T, Kuno S, Matsuda M, Tanaka H. Aortic stiffness and aerobic exercise: Mechanistic insight from microarray analyses. *Med Sci Sports Exerc*. 2005;37:1710-1716

84. Hagg U, Johansson ME, Gronros J, Naylor AS, Jonsdottir IH, Bergstrom G, Svensson PA, Gan LM. Gene expression profile and aortic vessel distensibility in voluntarily exercised spontaneously hypertensive rats: Potential role of heat shock proteins. *Physiological genomics*. 2005;22:319-326

85. Hoier B, Nordsborg N, Andersen S, Jensen L, Nybo L, Bangsbo J, Hellsten Y. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *J Physiol*. 2012;590:595-606

86. Gordon PM, Liu D, Sartor MA, IglayReger HB, Pistilli EE, Gutmann L, Nader GA, Hoffman EP. Resistance exercise training influences skeletal muscle immune activation: A microarray analysis. *J Appl Physiol (1985)*. 2012;112:443-453

87. Berg HE, Dudley GA, Haggmark T, Ohlsen H, Tesch PA. Effects of lower limb unloading on skeletal muscle mass and function in humans. *J Appl Physiol (1985)*. 1991;70:1882-1885

88. Dudley GA, Duvoisin MR, Adams GR, Meyer RA, Belew AH, Buchanan P. Adaptations to unilateral lower limb suspension in humans. *Aviat Space Environ Med*. 1992;63:678-683

89. Tack CJ, Ong MK, Lutterman JA, Smits P. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. *Diabetologia*. 1998;41:569-576

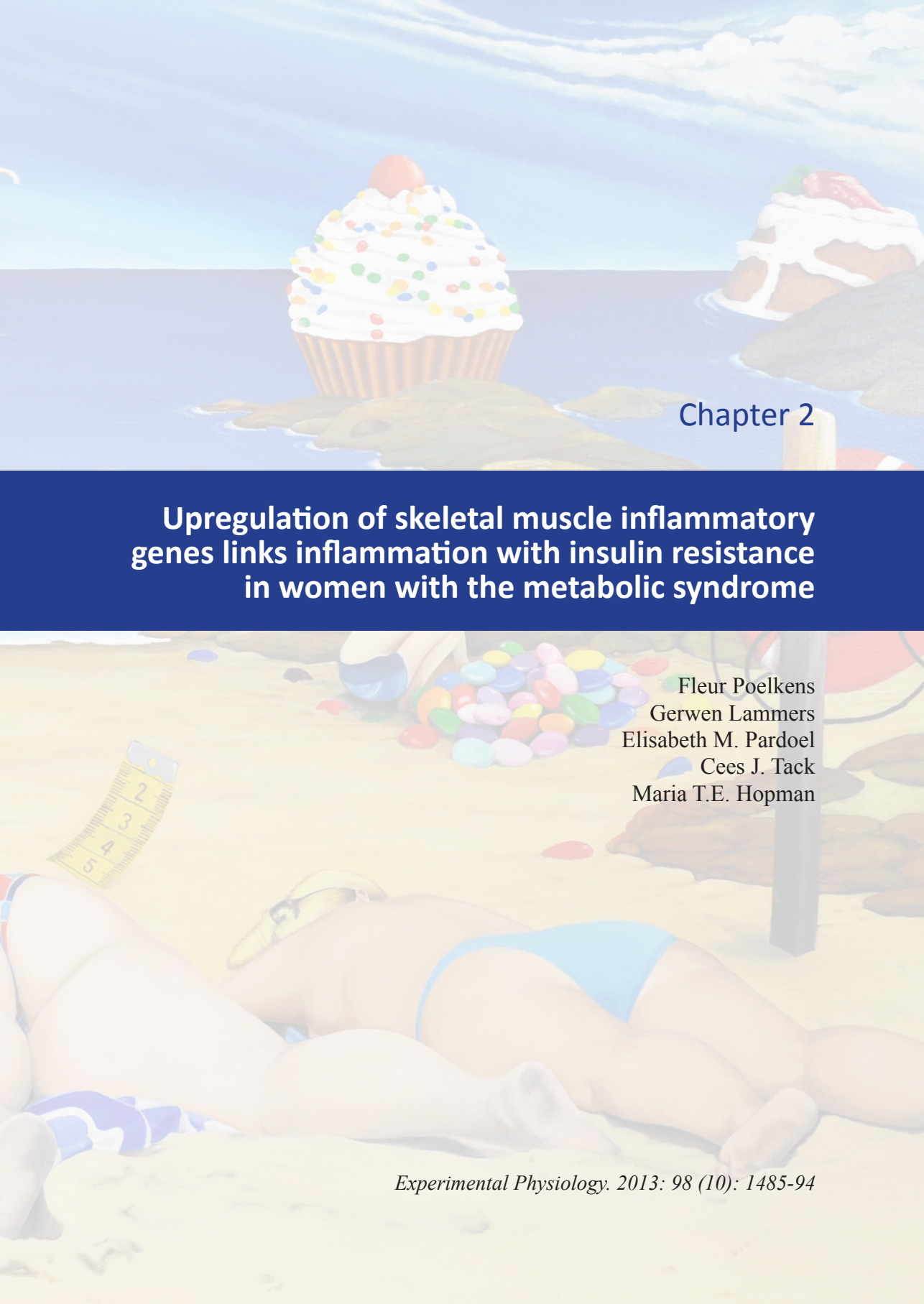
90. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: A method for quantifying insulin secretion and resistance. *Am J Physiol*. 1979;237:E214-223

91. Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R. Intimal plus medial thickness of the arterial wall: A direct measurement with ultrasound imaging. *Circulation*. 1986;74:1399-1406

92. Simon A, Gariepy J, Chironi G, Megnien JL, Levenson J. Intima-media thickness: A new tool for diagnosis and treatment of cardiovascular risk. *J Hypertens*. 2002;20:159-169

93. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)*. 2003;4:249-264

94. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nature protocols*. 2009;4:44-57



Chapter 2

Upregulation of skeletal muscle inflammatory genes links inflammation with insulin resistance in women with the metabolic syndrome

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Experimental Physiology. 2013: 98 (10): 1485-94

Abbreviations

Cd97	CD97 molecule
DXA	Dual-Energy X-ray Absorptiometry
FC	Fold Change
FFA	Free Fatty Acids
GO	Gene Ontology
Hdac9	Histone Deacetylase 9
hs-CRP	High-Sensitivity C-Reactive Protein
IL-1ß	Interleukin 1ß
IL-1Ra	IL-1 Receptor Antagonist
Il6r	Interleukin 6 Receptor
MEF2	Myocyte Enhancing Factor 2
PEG	Polyethylene Glycol
RIN	RNA Integrity Number
RMA	Robust Multiarray Analysis
UBC	Ubiquitin C

Abstract

The metabolic syndrome, a combination of interrelated metabolic risk factors, is associated with insulin resistance and promotes the development of cardiovascular diseases and type 2 diabetes mellitus. There is a close link between inflammation and metabolic disease, but the responsible mechanisms remain elusive. The aim of this study was to identify differentially expressed genes in insulin resistant skeletal muscle tissue of women with the metabolic syndrome compared to healthy controls. Women with the metabolic syndrome (n=19) and healthy controls (n=20) were extensively phenotyped, insulin sensitivity was measured using a hyperinsulinaemic euglycaemic clamp and a skeletal muscle biopsy was obtained. Gene expression levels were compared between the two groups by microarrays. The upregulated genes in skeletal muscle of the women with the metabolic syndrome were primarily enriched for inflammatory response-associated genes. The three most significantly upregulated of this group, interleukin 6 receptor (*Il6r*), histone deacetylase 9 (*Hdac9*), and CD97 molecule (*Cd97*) were significantly correlated with insulin resistance. Taken together, these findings suggest an important role for a number of inflammatory-related genes in the development of skeletal muscle insulin resistance.

Introduction

The metabolic syndrome is a combination of interrelated metabolic risk factors that directly promote the development of cardiovascular diseases [1] and type 2 diabetes mellitus [2]. The pathophysiology of the metabolic syndrome can be characterized by peripheral insulin resistance, which may be caused by excessive central adiposity and/or sedentarism. It has been recognized that a pro-inflammatory state contributes to disease development and progression [3]. In support of such a relationship is the observation that metabolic and immune response pathways are evolutionary integrated and partially overlapping, and it is clear that the immune system plays a role in the development of metabolic diseases [4].

Recent studies demonstrate a strong link between chronic low-grade inflammation and the development of insulin resistance, in which pro-inflammatory cytokines like interleukin 1ß (IL-1ß), IL-6 and the inflammasome play an important role [5-7]. Circulating mononuclear cells also demonstrate an aberrant expression of insulin signaling pathways components in obese subjects and upregulation of pro-inflammatory mediators [8]. These important results were obtained in insulin reactive tissues like fat and circulating cells, while skeletal muscle accounts for the majority (50-75%) of insulin-induced glucose uptake. It has been hypothesized that adipose tissue secretes inflammatory signals that affect target organs like the muscle. However, the underlying molecular mechanisms that link inflammation with skeletal muscle insulin resistance are largely unknown. A closer examination of skeletal muscle inflammatory genes that correlate with insulin resistance may generate new insights.

Gene expression microarray analysis provides the opportunity to assess all known genes in a single experiment, thereby indicating the direction for further research. Only a few previous studies have assessed mRNA gene expression in muscle of insulin resistant and diabetes subjects [9-12] but no study has been performed in women with the metabolic syndrome. Both men and women are at risk for developing the metabolic syndrome, but women with the metabolic syndrome have higher levels of circulating high-sensitivity c-reactive protein (hs-CRP) and IL-1 receptor antagonist (IL-1Ra) than men [13]. This suggests that especially in women chronic low-grade inflammation may play an important role in disease progression. This adds to the raising awareness that cardiovascular disease in women is underdiagnosed because of its atypical presentation, and the chronic low grade inflammation may play a role in the microvascular pathophysiology observed in women.

The aim of this study, therefore, was to identify differentially expressed inflammatory genes in insulin resistant skeletal muscle tissue of women with the metabolic syndrome compared to healthy controls. Identification of these key players

may increase our understanding of the link between obesity induced low-grade skeletal muscle inflammation and insulin resistance in the metabolic syndrome.

Methods

Ethical approval

This study was approved by the Medical Ethical Committee of the Radboud University Nijmegen Medical Centre, and was conducted in accordance with the Declaration of Helsinki (2000). All participants provided a written informed consent.

Subjects

Nineteen women with the metabolic syndrome and twenty healthy lean women participated in this study. Metabolic syndrome was defined as having at least three out of five criteria as defined in the Joint Scientific Statement for Harmonizing the Metabolic Syndrome [14], being 1. waist circumference > 88 cm, 2. triglycerides > 1,7 mmol/l, 3. HDL-cholesterol < 1,3 mmol/l, 4. hypertension > 130/85 mmHg and/or using antihypertensive medication and, 5. fasting glucose > 6,1 mmol/l. Lean healthy women were defined as having a BMI < 25 kg/m^2 and the absence of all metabolic syndrome criteria. Exclusion criteria were a medical history of known diabetes and/or cardiovascular diseases, liver or renal diseases, smoking, and consumption of more than two standardized units (10 grams) of alcohol per day.

Study design

After initial screening, subjects visited the department on two separate days. On day one, after an overnight fast the subjects had a standardized 250 kcal breakfast (79 % carbohydrates, 11.2 % protein, 9.8 % fat) to avoid any dietary effects, at least 2 h before a percutaneous needle biopsy was obtained from the vastus lateralis muscle [15]. This tissue was immediately frozen in liquid nitrogen and subsequently stored at -80 °C until further analysis. At the second visit, which was at least seven days after the first visit, anthropometric measurements including a dual-energy X-ray absorptiometry (DXA) scan, fasting blood sampling and a 2 h, 60mU/m^2/min hyperinsulinaemic euglycaemic clamp were performed [16].

Subject characteristics

Resting blood pressure and heart rate were measured twice after a 10 min supine resting period using a manual sphygmomanometer. Waist circumference was measured midway between the lower rib margin and iliac crest. Hip circumference was measured at the level of widest circumference over greater trochanters. Waist-to-hip ratio was calculated as waist circumference divided by hip circumference.

Table 1. Physical characteristics of the women with the metabolic syndrome (MS, n=19) and the healthy controls (C, n=20).

	MS	C	P value
Age, yrs	52 ± 7	49 ± 11	0.31
Weight, kg	94.7 ± 14.4	63.7 ± 7.8	< 0.001
BMI, kg/m^2	34.5 ± 4.0	22.1 ± 2.0	< 0.001
Waist circumference, cm	108.6 ± 9.8	79.1 ± 7.1	< 0.001
Waist-to-hip ratio	0.92 ± 0.07	0.80 ± 0.06	< 0.001
Systolic pressure, mmHg	138 ± 11	120 ± 13	< 0.001
Diastolic pressure, mmHg	85 ± 6	76 ± 7	< 0.001
Heart rate, bpm	70 ± 10	62 ± 6	0.006
Total fat mass, %	41.3 ± 3.9	28.0 ± 5.4	< 0.001
Trunk fat mass, %	42.3 ± 4.3	25.0 ± 7.5	< 0.001

Values are mean±SD. Bpm=beats per min.

Blood plasma measurements

Fasting venous blood samples were used to determine glucose and lipids via standard laboratory methods. Insulin was measured in duplicate against International Standard 83/500 by an in-house radio-immunoassay (RIA) using a guinea pig anti-human insulin antiserum and radio-iodinated human insulin as a tracer. Bound/free separation was carried out by addition of sheep anti-guinea-pig antiserum and precipitation by means of polyethylene glycol (PEG). Between- and within-run coefficients of variation were 4.6% and 5.8% respectively, at a level of 33mU/L. Adipocytokines, adiponectin and leptin were measured in duplicate by using DuoSet ELISA development system kits (R&D systems, Minneapolis, USA), free fatty acids (FFA) using Cobas Mira Plus (Roche Diagnostics Ltd., Basal, Switzerland), inflammatory markers C-reactive protein (CRP) by Dako high-sensitivity ELISA (Glostrup, Denmark) and interleukin 6 (IL-6) by Luminex assay (Austin, Texas,

USA). A total body DXA scan was performed to determine body fat percentage, fat distribution and lean body mass (QDR 4500 densitometer, Hologic Inc. Waltham, MA).

Hyperinsulinaemic euglycaemic clamp

Peripheral tissue sensitivity to exogenous insulin was measured using a hyperinsulinaemic euglycaemic clamp 16. After an overnight fast, insulin (Actrapid, Novo-Nordisk, Copenhagen, Denmark) was infused intravenously in a dose of 430 pmol m-2 min-1 (=60 mU m-2 min-1) for 120 min in a supine position in a quiet temperature-controlled room (22 – 24 °C) at 8.30 A.M.. Body surface area was calculated according to the formula of Du Bois *et al.* 17. Insulin 50 U/mL was diluted to a concentration of 1 U/m in 50 ml 0.9% NaCl containing 2 ml blood from the subject. Venous plasma glucose concentrations were clamped at 5 mmol/l by a variable 20% glucose infusion rate, adjusted depending on arterialized venous plasma glucose levels measured at 5 min intervals. Venous plasma samples were immediately centrifuged for 10 sec, and glucose was measured in duplicate by the glucose oxidation method (Beckman Glucose Analyzer 2, Beckman Instruments Inc, Fullerton, CA 92634, USA). During the last 30 min, glucose infusion rate and glucose concentration in the arterialized blood had a coefficient of variation of <5%, confirming a proper steady state situation. Whole body glucose disposal (M-value) during the last 30 min of the euglycaemic clamp was calculated.

Gene expression microarrays

Total RNA was isolated and purified from muscle biopsies and the RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). To guarantee good quality microarray analysis and validation, we proceeded with RNA from the six women with the metabolic syndrome and six healthy controls having the largest RNA yield. These numbers were based on previous studies that successfully identified differentially expressed insulin signaling genes in human skeletal muscle tissue [18]. RNA integrity was analyzed on an Agilent Bioanalyser (Santa Clara, CA, USA) and the average RNA integrity number (RIN) for all samples was 7.8. Gene expression profiling was performed using Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix Inc., Santa Clara, CA, USA), according to the manufacturer's instruction. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to generate amplified and biotinylated sense-strands DNA targets from the entire

expressed genome (100 ng of total RNA). The manufacturer's manual was followed for the hybridization, washing, and scanning steps (version 4, P/N 701880 Rev.4). Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 °C for 17 h. After hybridization, the arrays were washed in the Affymetrix GeneChip Fluidics station FS 450. Arrays were scanned using the GeneChip scanner 3000 7G system. For quality control, the average fluorescence intensity of all genes was calculated using the Robust Multiarray Analysis (RMA) Algorithm [19], including a quantile normalization and using a background correction for GC-content. To check overall data quality, the positive (exonic) and negative (intronic) control probe sets were used to calculate the area under the curve which ranged from 0.83 to 0.85, indicating good quality samples. The protocol used was compliant with the MIAME guidelines and data have been submitted to the Gene Expression Omnibus (GEO) repository under no. GSE43760.

Reverse transcription quantitative PCR (RT-qPCR) validation

For reverse transcription quantitative PCR (RT-qPCR) validation of the microarray results, RNA used for the microarrays was transcribed to cDNA using the superscript III first-strand synthesis supermix for qRT-PCR (Invitrogen, Paisley, UK) according to the manufacturers protocol. Ubiquitin C (*Ubc*) was used as a stable reference gene. Intron-spanning primers were designed using NCBI Primer-BLAST and purchased from Biolegio (Malden, The Netherlands). Primer nucleotide sequences (5'>3') were as follows: *Hdac9* forward GGATCAAAGCTCTCCACCCC and reverse TGGGCTCAGAGGCAGTTTTT; *Il6r* forward AGTGTCGGGAGCAAGTTCAG and reverse GGCTGCAAGATTCCACAACC; *Cd97* forward CCCCAGATACTGCTGGTTGG and reverse CAGGTCCCAAGAAGCTCCAG; and *Ubc* forward TAGTTCCGTCGCAGCCGGGA and reverse GCATTGTCAAGTGACGATCACAGCG. Sybr green-based RT-qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). For each primer pair the melt curve, efficiency, and no template controls were assessed to check primer specificity. The comparative CT quantification (ΔΔCt method) was used to compare changes in gene expression.

Statistical analysis

Differences in anthropometric variables between women with the metabolic syndrome and healthy lean women were assessed by a two-tailed unpaired Student's

t-test (Statistical Package for Social Sciences 16.0, SPSS Inc., Chicago, Illinois, USA). Correlations were assessed by a Pearson correlation coefficient.

The Affymetrix CEL-files were imported into Partek® (Genomic Suite Software, version 6.4 Copyright © 2008 Partek Inc., St Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA logarithm with GC background correction. Transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative gene expression levels. To identify differentially expressed genes between the women with the metabolic syndrome and healthy lean women a one-way analysis of variance (ANOVA) was performed. The resulting p-values were corrected for multiple-testing with the Benjamini-Hochberg false discovery rate procedure [20]. The p-value, together with the Fold Change (FC) of every annotated gene, was exported to Microsoft Office Excel.

The gene list of interest (all > 1.3 fold upregulated genes) was analyzed using the Database for Annotation Visualization and Integrated Discovery functional annotation tool (DAVID, http://david.abcc.ncifcrf.gov) with main focus on the enrichment in GO-terms of biological processes (GOTERM_FAT) [21, 22].

To assess differences in the RT-qPCR results, an unpaired Student's t-test was performed on the dCt values of the women with the metabolic syndrome versus healthy lean women. To visualize the fold-changes, the average gene expression levels of the healthy lean women was set at 1 and the average 2^-ddCt value of the women with the metabolic syndrome was calculated.

The level of statistical significance was defined at $\alpha=0.05$ and data are presented as mean ± SD.

Results

Physical characteristics

The physical characteristics of the 19 women with the metabolic syndrome and the 20 healthy controls are presented in Table 1. The groups were age-matched, but as expected the women with the metabolic syndrome had a significantly higher body weight, BMI, waist circumference, waist-to-hip ratio, systolic and diastolic blood pressure, heart rate, and percentage of total and trunk fat mass.

Blood plasma levels

Insulin sensitivity (M-value) was significantly lower in women with the metabolic syndrome (Table 2). Calculation of the M-value per kg fat free mass instead of per total body weight resulted in equal relative values (r^2=0.98). There was a significant negative correlation between the percentage of total fat mass and insulin sensitivity (r^2=0.58, p=0.004). The levels of total cholesterol, triglycerides, LDL-C, apolipoprotein B, leptin and hs-CRP were significantly higher in women with the metabolic syndrome, while HDL-C and adiponectin levels were lower (Table 2).

Table 2. Blood plasma levels of metabolic and inflammatory factors in women with the metabolic syndrome (MS, n=19) and healthy controls (C, n=20).

	MS	C	P value
M-value, mg min^{-1} kg^{-1}	3.82 ± 1.71	8.63 ± 1.71	< 0.001
Total cholesterol, mmol/l	6.25 ± 1.18	4.91 ± 0.77	< 0.001
Triglycerides, mmol/l	1.99 ± 0.80	0.84 ± 0.28	< 0.001
HDL-C, mmol/l	1.20 ± 0.26	1.63 ± 0.33	< 0.001
LDL-C, mmol/l	4.19 ± 1.03	2.90 ± 0.66	< 0.001
Resistin, ng/ml	5.43 ± 1.75	4.80 ± 2.48	0.37
Apolipoprotein B, mg/l	1416 ± 424	758 ± 220	< 0.001
Leptin, ng/ml	65.9 ± 31.3	18.0 ± 9.8	< 0.001
Adiponectin, µg/ml	3.46 ± 1.00	5.07 ± 1.94	0.003
hs-CRP, ng/ml	4.92 ± 7.38	0.55 ± 0.29	0.02
Interleukin-6, pg/ml	< 3.0 (17x) 3.0 (1x) 6.0 (1x)	n.d.(1x) < 3.0 (17x) 4.0 (1x)	
Free fatty acid, mmol/l	0.61 ± 0.23	0.58 ± 0.24	0.70

Values are mean±SD, n.d. not determined

Skeletal muscle gene expression

Skeletal muscle gene expression levels were compared between six women with the metabolic syndrome versus six healthy controls. The physical characteristics and blood plasma levels of this subgroup are provided in supplemental tables 1 and 2, respectively. The two subgroups were representative for the whole group and remained statistically significant different regarding physical characteristics and M-value. Of the 28,870 genes assessed on the microarray, no single gene reached

statistical significant differential expression between groups after stringent correction for multiple testing. Therefore, we focused on biological processes and pathways that were overrepresented in the list of genes with the highest fold-changes. All genes with more than 1.3-fold change were selected for further analysis, resulting in a list of 456 up- and 150 downregulated genes. Apparently, the metabolic syndrome is associated with more transcriptional up- than downregulation. The list of upregulated genes was most significantly enriched for the relatively general gene ontology (GO) term 'defense response' (Table 3, GO:0006952).

Table 3. Enrichment in biological process GO-terms in the list of genes with more than 1.3-fold upregulation in women with the metabolic syndrome versus lean women. Listing is according to significance.

Gene Ontology term (upregulated in MS)	F.E.	P value
GO:0006952~defense response	3.1	1.49E-05
GO:0006954~inflammatory response	3.7	2.72E-04
GO:0045637~regulation of myeloid cell differentiation	8.5	3.38E-04
GO:0045321~leukocyte activation	4.3	4.75E-04
GO:0009611~response to wounding	2.9	4.79E-04
GO:0001775~cell activation	3.8	5.35E-04
GO:0009617~response to bacterium	4.5	7.20E-04
GO:0030595~leukocyte chemotaxis	11.7	8.58E-04
GO:0048511~rhythmic process	5.5	9.37E-04
GO:0060326~cell chemotaxis	11.1	1.12E-03
GO:0050900~leukocyte migration	8.6	1.48E-03
GO:0002237~response to molecule of bacterial origin	6.3	4.25E-03
GO:0006935~chemotaxis	4.4	5.57E-03
GO:0042330~taxis	4.4	5.57E-03
GO:0010033~response to organic substance	2.3	7.77E-03
GO:0006955~immune response	2.3	8.33E-03
GO:0001932~regulation of protein amino acid phosphorylation	4.1	9.09E-03
GO:0032496~response to lipopolysaccharide	6.4	9.40E-03
GO:0048534~hemopoietic or lymphoid organ development	3.3	9.51E-03
GO:0034097~response to cytokine stimulus	6.2	9.51E-03
GO:0030097~hemopoiesis	3.5	1.09E-02
GO:0002274~myeloid leukocyte activation	8.3	1.53E-02
GO:0002520~immune system development	3.1	1.57E-02
GO:0016477~cell migration	3.1	1.57E-02

GO:0045638~negative regulation of myeloid cell differentiation	10.9	1.58E-02
GO:0022610~biological adhesion	2.2	1.58E-02
GO:0007155~cell adhesion	2.2	1.65E-02
GO:0050730~regulation of peptidyl-tyrosine phosphorylation	6.4	1.71E-02
GO:0002763~positive regulation of myeloid leukocyte differentiation	15.1	1.90E-02
GO:0045639~positive regulation of myeloid cell differentiation	9.9	1.99E-02
GO:0046649~lymphocyte activation	3.5	2.04E-02
GO:0050830~defense response to Gram-positive bacterium	13.6	2.62E-02
GO:0006928~cell motion	2.4	2.76E-02
GO:0002366~leukocyte activation during immune response	9.1	2.81E-02
GO:0002263~cell activation during immune response	9.1	2.81E-02
GO:0048870~cell motility	2.8	2.90E-02
GO:0051674~localization of cell	2.8	2.90E-02
GO:0006468~protein amino acid phosphorylation	2.1	2.97E-02
GO:0007626~locomotory behavior	3.0	3.03E-02
GO:0016310~phosphorylation	2.0	3.84E-02
GO:0002761~regulation of myeloid leukocyte differentiation	8.0	4.20E-02
GO:0006968~cellular defense response	6.2	4.24E-02
GO:0006796~phosphate metabolic process	1.8	4.27E-02
GO:0006793~phosphorus metabolic process	1.8	4.27E-02
GO:0042742~defense response to bacterium	4.4	4.81E-02
GO:0031399~regulation of protein modification process	2.8	4.95E-02

F.E. = Fold Enrichment; p-value = Benjamini-Hochberg corrected

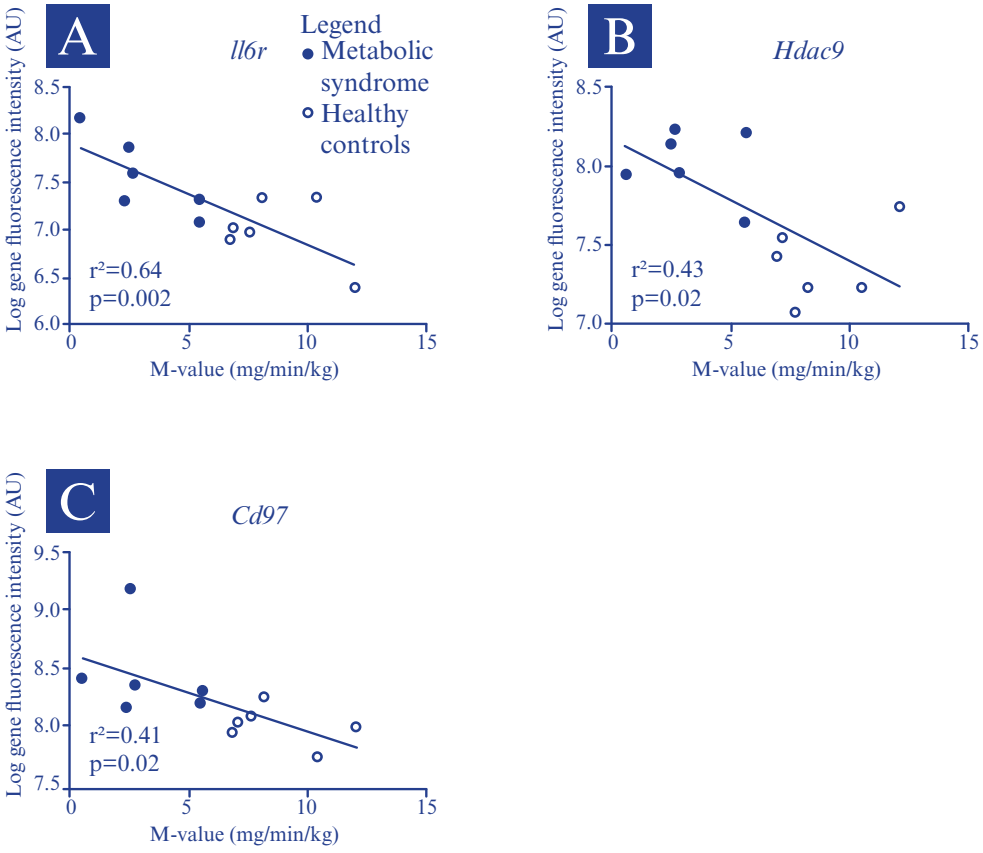
This was better specified by the second term ‘inflammatory response’ (GO:0006954), of which all 22 responsible genes were also present in ‘defense response’ (that contained 13 additional genes). These 22 upregulated genes are listed in Table 4.

Table 4. The 22 upregulated genes responsible for the enriched GO term 'inflammatory response', listed according to significance.

Gene symbol	Gene Name	Fold change	P value
HDAC9	histone deacetylase 9	1.6	< 0.01
IL6R	interleukin 6 receptor	1.5	0.03
CD97	CD97 molecule	1.3	0.03
S100A8	S100 calcium binding protein A8	2.2	0.09
TLR6	toll-like receptor 6	1.5	0.10
LYZ	lysozyme (renal amyloidosis)	1.8	0.10
S100A12	S100 calcium binding protein A12	1.8	0.11
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	1.4	0.14
CCR7	chemokine (C-C motif) receptor 7	1.3	0.15
ALOX5	arachidonate 5-lipoxygenase	1.5	0.16
CXCR2	interleukin 8 receptor, beta	1.7	0.16
PROK2	prokineticin 2	1.5	0.17
S100A9	S100 calcium binding protein A9	1.4	0.18
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	1.4	0.19
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1.6	0.20
CXCR1	interleukin 8 receptor, alpha	1.4	0.23
CR1	complement component (3b/4b) receptor 1 (Knops blood group)	1.3	0.24
CCL5	chemokine (C-C motif) ligand 5	1.3	0.25
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1.3	0.34
AOAH	acyloxyacyl hydrolase (neutrophil)	1.4	0.39
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	1.5	0.41
LBP	lipopolysaccharide binding protein	1.3	0.52

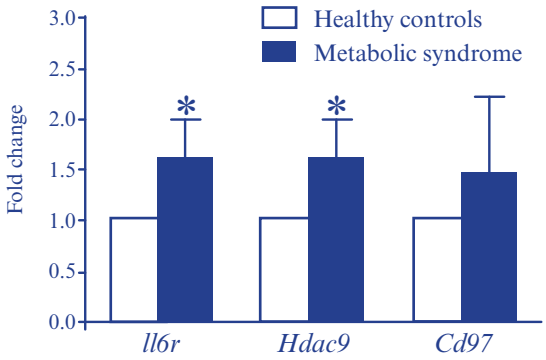
To visualize a possible link of these genes with insulin sensitivity, we correlated the microarray fluorescence intensity of the three most significantly upregulated genes with the M-value (fig. 1A-C), which resulted in a significant correlation for all three genes. RT-qPCR validation of these genes confirmed upregulation of *Hdac9* and *Il6r* (Fig. 2).

Figure 1.



Correlation of insulin sensitivity (M-value) and skeletal muscle gene expression levels of the three most upregulated inflammatory-related genes Il6r (A), Hdac9 (B), and Cd97 (C) in women with the metabolic syndrome (n=6) and healthy controls (n=6).

Figure 2.



RT-qPCR validation of *Il6r, Hdac9* and *Cd97* upregulation in women with the metabolic syndrome (n=6) versus healthy controls (n=6). *p<0.05

Discussion

The main finding of our study is that expression of genes involved in inflammatory pathways is upregulated in skeletal muscle of women with the metabolic syndrome, who are insulin resistant and have chronic low-grade systemic inflammation. This suggests the existence of a close link between obesity and skeletal muscle inflammation and insulin resistance. Correlation of the three most significantly upregulated inflammatory genes, *Hdac9*, *Il6r*, and *Cd97*, with insulin sensitivity suggests an important role for these genes in the development of skeletal muscle insulin resistance.

For this study we selected women that fulfill the criteria of the metabolic syndrome. By definition, these women are obese, which is reflected by a BMI of >30 and an elevated waist circumference. Most important for our study, the women with the metabolic syndrome were more than 2-fold more insulin resistant than the healthy control subjects, as determined by the gold standard hyperinsulinaemic euglycaemic clamp technique. The high negative correlation of total body fat percentage with insulin sensitivity is in line with the idea that adipose tissue plays an important role in the pathophysiology of the metabolic syndrome. Also blood plasma levels had the expected profile with elevated levels of total cholesterol, triglycerides, LDL-C, apolipoprotein B, leptin, and the inflammation marker hs-CRP, and lower HDL-C and adiponectin than controls. Apolipoprotein B has been proposed as an indicator to identify patients with the metabolic syndrome that are at higher cardiovascular risk

[23]. Leptin is an adipose tissue-derived inflammatory mediator that strongly resembles IL-6 [24]. Recently, it has been demonstrated that a high level of hs-CRP, IL-6, or a low level of adiponectin is associated with an increased risk for the metabolic syndrome [25]. Our findings thus confirm the association of elevated blood plasma levels of adipose tissue-derived inflammatory molecules with the metabolic syndrome.

The effect of these altered blood plasma levels on skeletal muscle insulin resistance was further investigated using gene expression microarrays. The metabolic syndrome was mainly associated with the upregulation of genes and these were enriched for GO-terms related to inflammation. The three most significantly upregulated genes responsible for the GO-term 'inflammatory response' were HDAC9, IL6R, and CD97, and their expression levels significantly correlated with insulin resistance. The potential role of these three genes in the development of skeletal muscle insulin resistance is discussed below.

The expression of the interleukin [6] receptor (*Il6r*) gene demonstrated the highest negative correlation with insulin sensitivity in our study. It has been recognized for a long time that elevated blood plasma levels of IL-6 are associated with the metabolic syndrome, but these levels are still below the detection limit of our assay [26]. In mice, it has been demonstrated that treatment with IL-6 causes skeletal muscle insulin resistance, associated with deficient insulin signaling and increased levels of intramuscular fatty acyl-CoA [27]. In contrast to our findings on the mRNA level, a recent study reports a marked downregulation of the IL6 receptor protein in skeletal muscle tissue of obese subjects with and without diabetes 28. This apparent discrepancy between IL-6 receptor mRNA and protein levels has previously been noticed in human exercise physiology studies. Skeletal muscle produces IL-6 during exercise, and it has been demonstrated that exercise training results in upregulation of IL-6 receptor mRNA levels [29]. However, this effect on IL-6 receptor mRNA is probably independent of IL-6 plasma levels, since infusion of IL-6 resulted in upregulation of the IL-6 receptor protein, but not mRNA levels. This is supported by the observation that *Il6* knock-out mice are also able to upregulate IL-6 receptor mRNA after exercise [30]. It will thus be interesting to further investigate the mechanisms that are responsible for the IL-6 receptor mRNA upregulation in women with the metabolic syndrome.

Histone deacetylase 9 (*Hdac9*) has been associated with inflammation and cell-cycle regulation since it can bind to B-cell CLL/lymphoma 6 (BCL6) in the transcriptional regulation of B-cell activation and differentiation [31] *Hdac9* knock-out studies in mice have indicated an important role for this gene in the transcriptional regulation of skeletal muscle fiber type [32, 33]. The slow (type I) fiber type is maintained by the transcription factor myocyte enhancing factor 2 (MEF2) and HDAC9 binds to MEF2, thereby inhibiting its function. Knock-out of this class of histone deacetylases in mice resulted in the loss of *Mef2* repression and consequently the

transition of fast to slow fiber type. We found an upregulation of *Hdac9* in skeletal muscle tissue of insulin resistant women with the metabolic syndrome. It has been demonstrated that skeletal muscle tissue of subjects with type 2 diabetes contains less slow and more fast fibers [34] and this change in fiber type may contribute to increased insulin resistance. In addition, it will be interesting to further study the potential role of histone deacetylase-mediated epigenetic modifications in the development of the metabolic syndrome. We thus identified *Hdac9* due to its association with inflammation, but its role in fiber type determination may provide an alternative explanation and novel insight in the development of insulin resistance in women with the metabolic syndrome.

CD97 molecule (CD97) is a seven plasma membrane spanning glycoprotein, mainly expressed on activated lymphocytes at sites of tissue inflammation [35]. To our knowledge, CD97 has not directly been linked with insulin resistance yet, and also the role of lymphocytes in the development of skeletal muscle insulin signaling remains elusive. It has been demonstrated in adipose tissue in a mouse model that the infiltration of lymphocytes represents an important initial step in tissue inflammation and the subsequent development of insulin resistance [36]. The upregulation of *Cd97* in our study suggests that the infiltration of lymphocytes may also be involved in the development of skeletal muscle insulin resistance in humans.

With the results of the present study it can be hypothesized that central adiposity and associated chronic low-grade inflammation result in chronic elevated levels of circulating inflammatory markers. This probably also includes IL-6, and IL-6 receptor mRNA levels are upregulated, but elucidation of the underlying mechanisms requires further research. At the same time, a transition takes place from slow insulin sensitive to fast, more insulin resistant, skeletal muscle fibers. In addition, lymphocytes may infiltrate the muscle and initiate tissue inflammation. Like in adipose tissue, the infiltration of lymphocytes in the muscle tissue may theoretically also play a role in the development of skeletal muscle insulin resistance.

Limitations

Gene expression analysis was performed in only six subjects from each group, which limited the statistical power of the initial microarray analysis. However, the subgroups were completely representative for the whole group, and additional analysis using gene ontology terms resulted in the identification of inflammatory genes of which the expression level correlated with insulin resistance. This study provides associations, which not necessarily implies a cause and effect relationship. The groups also differed in other parameters like BMI, thereby limiting the possibility to determine the exact contribution of insulin resistance to the observed correlations.

Gene expression microarrays are a powerful tool to assess the expression level of many genes at once, but do not provide information on the cell type distribution in the biopsy. From this study it is not possible to conclude whether one specific cell type (e.g. leukocytes or myocytes) was responsible for the increased expression of inflammatory genes, or that more cells of a specific type (e.g. leukocytes) infiltrated the muscle tissue.

In conclusion, we report that the gene expression in skeletal muscle tissue of women with the metabolic syndrome is mainly enriched for inflammatory response-related genes. Specifically *Il6r*, *Hdac9*, and *Cd97* gene expression negatively correlated with insulin sensitivity. This suggests a role for these three inflammatory genes in the development of skeletal muscle insulin resistance in women with the metabolic syndrome. Further delineation of these pathways may eventually lead to the development of therapeutic interventions to treat insulin resistance.

Acknowledgements

We would like to thank all study participants and the microarray facility of the Department of Genetics of the Radboud University Nijmegen Medical Centre. FP is funded by ZonMW grant number 92003409, GL is funded by the project Gelderland-Overijssel-EFRO no 2009-010034. The authors report no conflict of interest.

References

1. Salminen M, Kuoppamaki M, Vahlberg T, Raiha I, Irjala K, Kivela SL. Metabolic syndrome and vascular risk: A 9-year follow-up among the aged in finland. *Acta Diabetol*. 2011;48:157-165
2. Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*. 2005;112:3066-3072
3. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet*. 2005;365:1415-1428
4. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444:860-867
5. Kalupahana NS, Moustaid-Moussa N, Claycombe KJ. Immunity as a link between obesity and insulin resistance. *Mol Aspects Med*. 2012;33:26-34
6. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest*. 2006;116:1793-1801
7. Stienstra R, Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. *Cell Metab*. 2012;15:10-18
8. Ghanim H, Aljada A, Daoud N, Deopurkar R, Chaudhuri A, Dandona P. Role of inflammatory mediators in the suppression of insulin receptor phosphorylation in circulating mononuclear cells of obese subjects. *Diabetologia*. 2007;50:278-285
9. Elbein SC, Kern PA, Rasouli N, Yao-Borengasser A, Sharma NK, Das SK. Global gene expression profiles of subcutaneous adipose and muscle from glucose-tolerant, insulin-sensitive, and insulin-resistant individuals matched for bmi. *Diabetes*. 2011;60:1019-1029
10. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. Pgc-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* 2003;34:267-273
11. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of pgc1 and nrf1. *Proc Natl Acad Sci U S A*. 2003;100:8466-8471
12. Hittel DS, Kraus WE, Hoffman EP. Skeletal muscle dictates the fibrinolytic state after exercise training in overweight men with characteristics of metabolic syndrome. *J Physiol*. 2003;548:401-410
13. Saltevo J, Vanhala M, Kautiainen H, Kumpusalo E, Laakso M. Gender differences in c-reactive protein, interleukin-1 receptor antagonist and adiponectin levels in the metabolic syndrome: A population-based study. *Diabetic medicine : a journal of the British Diabetic Association*. 2008;25:747-750
14. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. *Circulation*. 2009;120:1640-1645
15. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mrna expression in human skeletal muscle during recovery from endurance exercise. *Faseb J*. 2005;19:1498-1500
16. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: A method for quantifying insulin secretion and resistance. *Am J Physiol*. 1979;237:E214-223

17. Du Bois D, Du Bois EF. A formula to estimate the approximate surface area if height and weight be known. 1916. *Nutrition (Burbank, Los Angeles County, Calif*. 1989;5:303-311; discussion 312-303
18. Rome S, Clement K, Rabasa-Lhoret R, Loizon E, Poitou C, Barsh GS, Riou JP, Laville M, Vidal H. Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. *J Biol Chem*. 2003;278:18063-18068
19. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)*. 2003;4:249-264
20. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behavioural brain research*. 2001;125:279-284
21. Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nat.Protoc.* 2009;4:44-57
22. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*. 2009;37:1-13
23. Sattar N, Williams K, Sniderman AD, D'Agostino R, Jr., Haffner SM. Comparison of the associations of apolipoprotein b and non-high-density lipoprotein cholesterol with other cardiovascular risk factors in patients with the metabolic syndrome in the insulin resistance atherosclerosis study. *Circulation*. 2004;110:2687-2693
24. Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD, Furman TC, Hale JE, Hsiung HM, Schoner BE, Smith DP, Zhang XY, Wery JP, Schevitz RW. Crystal structure of the obese protein leptin-e100. *Nature*. 1997;387:206-209
25. Chen SJ, Yen CH, Huang YC, Lee BJ, Hsia S, Lin PT. Relationships between inflammation, adiponectin, and oxidative stress in metabolic syndrome. *PLoS One*. 2012;7:e45693
26. Pickup JC, Mattock MB, Chusney GD, Burt D. Niddm as a disease of the innate immune system: Association of acute-phase reactants and interleukin-6 with metabolic syndrome x. *Diabetologia*. 1997;40:1286-1292
27. Kim HJ, Higashimori T, Park SY, Choi H, Dong J, Kim YJ, Noh HL, Cho YR, Cline G, Kim YB, Kim JK. Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes*. 2004;53:1060-1067
28. Scheele C, Nielsen S, Kelly M, Broholm C, Nielsen AR, Taudorf S, Pedersen M, Fischer CP, Pedersen BK. Satellite cells derived from obese humans with type 2 diabetes and differentiated into myocytes in vitro exhibit abnormal response to il-6. *PloS one*. 2012;7:e39657
29. Keller C, Steensberg A, Hansen AK, Fischer CP, Plomgaard P, Pedersen BK. Effect of exercise, training, and glycogen availability on il-6 receptor expression in human skeletal muscle. *J Appl Physiol*. 2005;99:2075-2079
30. Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J, Pedersen BK. Interleukin-6 receptor expression in contracting human skeletal muscle: Regulating role of il-6. *Faseb J*. 2005;19:1181-1183
31. Verdin E, Dequiedt F, Kasler HG. Class ii histone deacetylases: Versatile regulators. *Trends Genet*. 2003;19:286-293
32. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: Implications for disease and therapy. *Nat Rev Genet*. 2009;10:32-42
33. Potthoff MJ, Wu H, Arnold MA, Shelton JM, Backs J, McAnally J, Richardson JA, Bassel-Duby R, Olson EN. Histone deacetylase degradation and mef2 activation promote the formation of slow-twitch myofibers. *J Clin Invest*. 2007;117:2459-2467

34. Oberbach A, Bossenz Y, Lehmann S, Niebauer J, Adams V, Paschke R, Schon MR, Bluher M, Punkt K. Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care*. 2006;29:895-900

35. Gray JX, Haino M, Roth MJ, Maguire JE, Jensen PN, Yarme A, Stetler-Stevenson MA, Siebenlist U, Kelly K. Cd97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation. *J Immunol*. 1996;157:5438-5447

36. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, Hauner H, Bluher M, Unger T, Wolf AM, Knippschild U, Hombach V, Marx N. T-lymphocyte infiltration in visceral adipose tissue: A primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol*. 2008;28:1304-1310

Supplemental table 1. Physical characteristics of the women with the metabolic syndrome (MS, n=6) and the healthy controls (C, n=6).

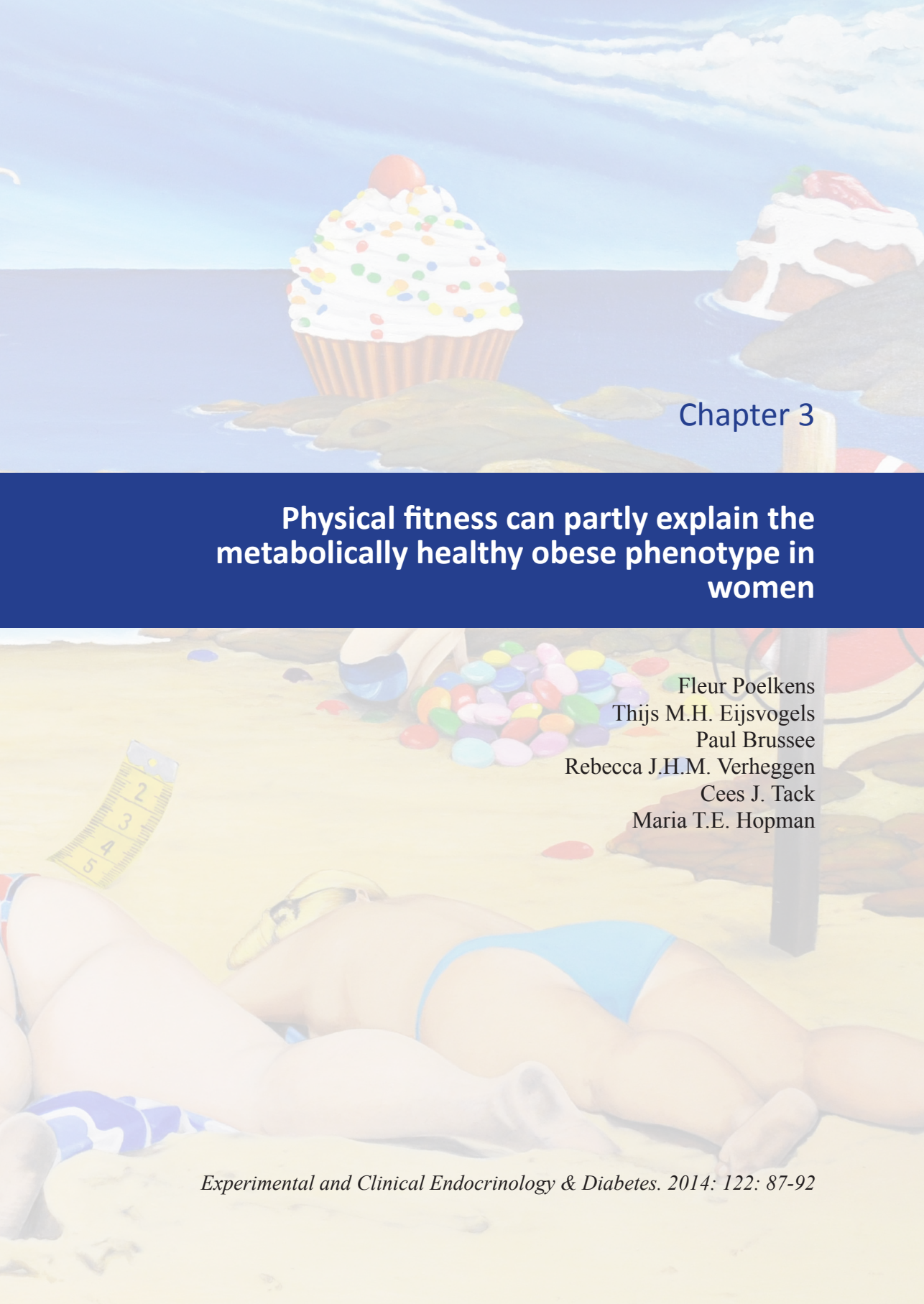
	MS	C	P value
Age, yrs	58 ± 4	50 ± 9	0.06
Weight, kg	103.1 ± 9.3	64.5 ± 6.0	< 0.001
BMI, kg/m^2	36.2 ± 2.1	22.2 ± 1.0	< 0.001
Waist circumference, cm	111.8 ± 11.5	77.6 ± 4.4	< 0.001
Waist-to-hip ratio	0.92 ± 0.10	0.78 ± 0.04	0.01
Systolic pressure, mmHg	142 ± 12	117 ± 8	0.001
Diastolic pressure, mmHg	84 ± 6	75 ± 5	0.02
Heart rate, bpm	69 ± 5	59 ± 5	0.006
Total fat mass, %	40.0 ± 1.4	28.1 ± 4.5	< 0.001
Trunk fat mass, %	40.4 ± 3.8	24.9 ± 6.8	< 0.001

Values are mean±SD. Bpm=beats per min.

Supplemental table 2. Blood plasma levels of metabolic and inflammatory factors in women with the metabolic syndrome (MS, n=6) and healthy controls C, (n=6).

	MS	C	P value
M-value, mg min^{-1} kg^{-1}	3.13 ± 1.96	8.59 ± 2.06	< 0.001
Total cholesterol, mmol/l	6.62 ± 1.41	4.87 ± 0.47	0.03
Triglycerides, mmol/l	2.12 ± 1.06	0.98 ± 0.23	0.05
HDL-C, mmol/l	1.26 ± 0.36	1.53 ± 0.32	0.21
LDL-C, mmol/l	4.50 ± 1.35	2.90 ± 0.23	0.03
Resistin, ng/ml	4.30 ± 1.26	5.52 ± 3.47	0.45
Apolipoprotein B, mg/l	1559 ± 273	721 ± 183	< 0.001
Leptin, ng/ml	71.5 ± 48.6	13.4 ± 4.1	0.03
Adiponectin, µg/ml	3.45 ± 1.29	4.32 ± 0.46	0.17
hs-CRP, ng/ml	4.51 ± 4.25	0.68 ± 0.28	0.08
Free fatty acid, mmol/l	0.58 ± 0.17	0.47 ± 0.16	0.29

Values are mean±SD.



Chapter 3

Physical fitness can partly explain the metabolically healthy obese phenotype in women

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Experimental and Clinical Endocrinology & Diabetes. 2014: 122: 87-92

Abstract

Objective: To investigate whether physical fitness and/or fat distribution and inflammation profile may explain why approximately 30% of the women with obesity are protected against obesity-related disorders. **Method**: Ten metabolically healthy obese women and ten age- and weight-matched women with the metabolic syndrome were enrolled. Physical fitness (VO_{2max}), daily physical activity levels (METs, steps per day), insulin sensitivity (clamp), body fat distribution (DXA scan) and, inflammation markers and adipokines were determined. **Results**: The metabolically healthy obese women had a 17% higher VO_{2max} (25.1 ± 3.9 vs 21.5 ± 3.1 $ml \cdot min^{-1} \cdot kg^{-1}$, p=0.04) and tended to take more steps per day (7388 ± 1440 vs 5927 ± 1301, p=0.06) than women with the metabolic syndrome. Despite equivalent levels of fat mass, metabolically healthy obese women had significantly lower circulating TNF-α levels compared to women with the metabolic syndrome (3.55 ± 3.83 vs 0.43 ± 0.97 ng/ml, p=0.03). No differences were seen in insulin sensitivity, adipokines, and inflammatory markers between both groups. **Conclusion:** Metabolically healthy obese women have a higher cardio-respiratory fitness and lower TNF-α levels, which may partly explain why these women are protected from the detrimental effects of obesity compared to obese women with the metabolic syndrome.

Introduction

Obesity is caused by a complex interplay between genetic predisposition and environmental lifestyle factors such as food overconsumption and physical inactivity[1]. While 70% of the obese people (BMI > 30 kg/m^2) have at least one metabolic and/or cardiovascular risk factor, approximately 30% is metabolically healthy [2]. Obese women with a healthy metabolic profile have a 10 years incidence of cardiovascular disease (CVD) events similar to their lean counterparts [3] while obese women with the metabolic syndrome have an almost 3-fold increased risk for CVD events [4]. With the worldwide growing prevalence of obesity, understanding the physiological mechanisms why some obese women seem to be protected against the detrimental effects of obesity is clearly of utmost importance. In recent years, several hypotheses have been postulated regarding this metabolic healthy obese phenotype. Large epidemiological studies have shown that obese individuals who are in the higher range of physical fitness or physical activity levels, are more likely to have a metabolic healthy phenotype [2, 5, 6]. Recently Ortega *et al.* showed that the metabolically healthy but obese subjects had a better physical fitness than metabolically abnormal subjects[7]. Bouchard *et al.* found similar findings in women with the metabolically healthy obese phenotype who performed a faster and further 6-min walk test compared to obese women with risk factors[8]. Unfortunately, almost all of these studies used self-reported questionnaires or prediction models to determine physical activity levels and physical fitness, respectively. Besides physical fitness, the metabolic activity of fat tissue, including the inflammatory cytokine response [9, 10] and the specific location of fat tissue, have been proposed as possible explanations for the metabolically healthy obese phenotype [11, 12]. Since there seems to be an association between fitness level and subclinical inflammatory profile in obese subjects [13], investigating the role of physical fitness simultaneous with the inflammation profile in metabolically healthy obese women and in obese women with the metabolic syndrome might improve our understanding of the metabolically healthy obese phenotype. In this cross-sectional study we hypothesize that increased physical fitness, using the 'gold standard' maximal oxygen uptake, more than fat distribution or inflammatory profile may explain why these 'metabolically healthy obese' women seem to be protected against obesity-related disorders.

Methods

Subjects

Ten women with the metabolic syndrome and ten age- and weight-matched metabolically healthy obese women were recruited for this study through advertisements in local newspapers (table I). Metabolic syndrome was defined according to the Joint Interim Statement for Harmonizing the Metabolic syndrome [14] as having at least three out of five criteria [15], including waist circumference $\geq$ 88 cm, triglycerides > 1,7 mmol/l, HDL-cholesterol < 1,3 mmol/l, hypertension > 130/85 mmHg and/or using antihypertensive medication and, fasting glucose > 5,6 mmol/l. Metabolically healthy obese was defined as having a BMI > 30 kg/m^2 and a waist circumference above 88 cm, but without any other risk factor, so applying to only one criterion of the metabolic syndrome definition. Accordingly, the Metabolic Syndrome z-score (MS z-score) was calculated for both obesity groups as described previously [16]. Exclusion criteria were a medical history of diabetes, cardiovascular diseases, liver or renal diseases and, performing regular physical activity > 5 hours a week. Before participation, a written informed consent was obtained. This study was approved by the Medical Ethical Committee of the Radboud University Nijmegen Medical Centre, and was conducted in accordance with the Declaration of Helsinki (2000).

Anthropometrics and biochemical parameters

A questionnaire was used to gather information about the duration of obesity, menopausal status, and the use of diet supplements. Height, weight, waist circumference, blood pressure, resting heart rate, plasma lipids, and glucose were measured by standard methods. A total body DXA scan determined body fat percentage, and body fat distribution (QDR 4500 densitometer, Hologic Inc. Waltham, MA). Peripheral tissue sensitivity to exogenous insulin was measured using a hyperinsulinaemic (insulin infusion dose of 430 pmol $\cdot$ m^{-2} $\cdot$ min^{-1}) euglycaemic clamp as previously described [17]. Adiponectin, and leptin were measured in duplicate by using DuoSet ELISA development system kits (R&D systems, Minneapolis, USA), high-sensitive C-reactive protein (hsCRP) via enzyme-immunoassay according to the instructions from the manufacturer (Dako Glastrup, Denmark), and tumor necrosis factor-alpha (TNF-α) in duplicate by using DuoSet ELISA development system kits (R&D systems, Minneapolis, USA).

Physical fitness and daily activity levels

Women performed a maximal exercise test on an leg-cycling ergo meter (Lode, Angio 300, Groningen, the Netherlands) using an incrementing protocol to assess their cardio-respiratory fitness. Workload increased by 10 W per minute, starting at 10 W, until exhaustion. A gas-analyzer was used to measure oxygen consumption continuously (Jaeger Benelux BV, Breda, the Netherlands). Maximal oxygen consumption (VO_{2max}) was analyzed as the mean of all values that were obtained during the last minute of the exercise test. Women had to meet at least two of the three following criteria to fulfill the definition of good quality VO_{2max}-test. One, a heart rate in excess of 90% of age predicted maximum (220 – age); two, identification of a plateau (<150 ml increase) in VO_2 despite further increase in workload; three, a respiratory exchange ratio of ≥ 1.10.

A SenseWear Pro 3 Armband® (Body Media, Inc., Pittsburgh, PA, USA) was worn 24/7 and was used to objectively determine the Metabolic Equivalent of Task (METs) and number of steps taken each day. From each 24 hour interval, data were analyzed from 0700 to 2300 h with a minimum on-body time of 85 %. At least four days had to fulfill these criteria to be used for analysis.

Statistical methods

All data are reported as mean ± SD, while statistical significance was assumed at $P \leq 0.05$. Statistical analyses were performed using SPSS 18.0 (SPSS, Chicago, Illinois). Differences between groups were analysed by an unpaired Student's *t*-test. A binary backward logistic regression (Wald) analysis was used to identify which factor was the strongest predictor to have the metabolically healthy obese phenotype. Parameters that were significantly different between metabolically healthy obese women and metabolic syndrome women in the univariate analysis were selected for this regression model. All Odds Ratios were presented with their 95% confidence interval (CI).

Results

Table I shows characteristics of the metabolically healthy obese women and women with the metabolic syndrome. Both groups differed significantly in criteria of the metabolic syndrome definition, i.e. systolic blood pressure, triglycerides and HDL-cholesterol. No differences between groups were observed for duration of obesity, menopausal status, fasting glucose, insulin, BMI, waist circumference and diastolic blood pressure. Metabolically healthy obese women had a significantly lower MS z-score than women with the metabolic syndrome. The insulin sensitivity (M-value) was comparable between both groups. Metabolically healthy obese women had a significantly lower resting heart rate compared with women with the metabolic syndrome despite the fact that 6 out of 10 women in the metabolic syndrome group used β-blockers.

Physical fitness and physical activity

VO_{2max} was significantly higher in the metabolically healthy obese women compared to women with the metabolic syndrome (fig. IA). No difference was found in METs and minutes per day spend performing activities above an activity level of three METs. However, when assessing daily physical activity levels by number of steps taken each day, metabolically healthy obese women tended to take 25% more steps each day compared to women with the metabolic syndrome (p=0.06)(table I).

Body fat distribution and inflammation

Quantity and distribution of body fat were similar between metabolically healthy obese women and women with the metabolic syndrome, as were total body fat mass, fat mass located on the trunk or on the extremities (table I). Metabolically healthy obese women had higher circulating levels of TNF-α (fig. IB). No differences were measured in resistin, apolipoprotein B, leptin, adiponectin and hsCRP between both groups (table I).

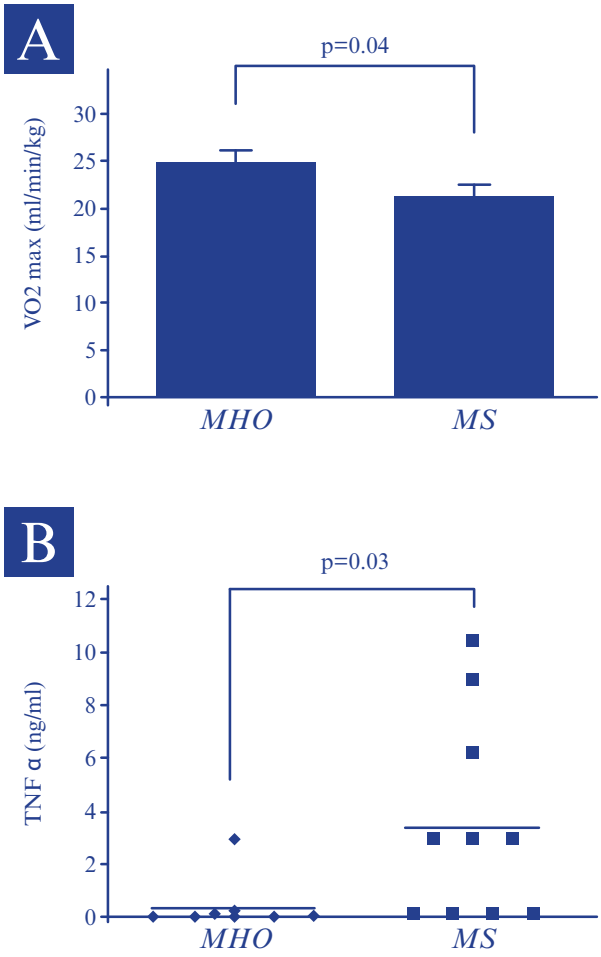
Using cardio-respiratory fitness (VO_{2max}) and TNF-α as co-variate input factors in the binary backward logistic regression (Wald) analysis, cardio-respiratory fitness appeared to be the strongest predictor for having the metabolically healthy obese phenotype (Odds Ratio=1.6, CI 1.0 – 2.4, p-value=0.04).

Table I. Differences between women with the metabolic healthy obese (MHO) phenotype and with the metabolic syndrome (MS).

	MHO	MS	P value
Age (yrs)	50 ± 5	52 ± 7	0.47
Duration of obesity (yrs)	18 ± 11	22 ± 10	0.42
Number of postmenopausal women	3	3	-
Number of MS features	1	4	< 0.00
MS z-score	-1.05 ± 1.22	2.60 ± 1.94	< 0.00
Body composition			
Weight (kg)	91.7 ± 9.3	90.6 ± 9.4	0.81
BMI (kg/m^2)	32.9 ± 3.0	33.7 ± 2.7	0.53
Waist circumference (cm)	103 ± 5	106 ± 5	0.11
Total body fat mass (%)	39.9 ± 3.1	42.1 ± 4.5	0.22
Trunk fat mass (%)	39.9 ± 2.5	42.9 ± 4.8	0.10
Blood pressure			
SBP (mmHg)	124 ± 11	136 ± 13	0.04
DBP (mmHg)	81 ± 5	83 ± 8	0.47
HR_{rest} (bpm)	61 ± 4	69 ± 9	0.03
Blood markers			
Triglycerides (mmol/l)	1.16 ± 0.26	2.08 ± 0.67	< 0.00
HDL-C (mmol/l)	1.52 ± 0.19	1.15 ± 0.16	< 0.00
Glucose (mmol/l)	5.2 ± 0.2	5.3 ± 0.7	0.62
Insulin (mE/L)	13.0 ± 3.9	12.4 ± 3.1	0.59
M-value (µmol/min/kg)	28.0 ± 7.1	26.0 ± 8.0	0.57
Resistin (ng/ml)	6.2 ± 2.3	5.5 ± 1.9	0.45
Apolipoprotein B (mg/L)	1389 ± 335	1398 ± 425	0.96
Leptin (ng/ml)	65.8 ± 27.6	61.1 ± 21.0	0.69
Adiponectin (µg/ml)	4.19 ± 1.13	3.72 ± 1.11	0.37
hsCRP (ng/ml)	2.09 ± 1.80	5.93 ± 9.78	0.25
TNFα (ng/ml)	0.43 ± 0.97	3.55 ± 3.83	0.03
Physical fitness			
VO_{2max} (ml/min/kg)	25.1 ± 3.9	21.5 ± 3.1	0.04
METs, (-)	1.35 ± 0.14	1.29 ± 0.12	0.34
METs > 3 (min)	68 ± 30	50 ± 19	0.23
Number of steps per day	7388 ± 1440	5927 ± 1301	0.06

Values are means ± SD; MHO, metabolically healthy obese; MS, metabolic syndrome; MS z-score, metabolic syndrome z –score; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR_{rest}, resting heart rate; bpm, beats per minute; HDL-C, HDL-cholesterol: hsCRP, high sensitivity C-reactive protein; TNFα, tumor necrosis factor alpha; VO_{2max}, maximal oxygen uptake; METs, Metabolic Equivalent of Task.

Figure I.



A, Significant higher cardio-respiratory fitness (VO_{2max}) in metabolically healthy obese women (MHO) compared to women with the metabolic syndrome (MS). B, Distribution and means of TNFα in metabolically healthy obese women and women with the metabolic syndrome. Error bars represents SEM.

Discussion

In this study we found that metabolically healthy obese women have a higher VO_{2max} compared to age- and weight-matched obese women with the metabolic syndrome. Our regression model reinforced this finding and revealed that VO_{2max} was the strongest predictor for having the metabolically healthy obese phenotype. In addition, the metabolically healthy obese women appeared physically more active as they tended to take more steps per day than the metabolic syndrome women. We did not observe a difference in total body fat mass nor in fat distribution between both groups but we did find lower circulating TNF-α levels in the metabolically healthy obese women, which suggests a lower inflammatory profile of fat tissue.

Previous studies assessing physical fitness used questionnaires, self-reported physical activity scoring systems or predictions of cardio-respiratory fitness and activity levels. These cross-sectional studies reported that with higher levels of cardio-respiratory fitness, the prevalence of the metabolic syndrome is lower [5, 18] and this association between physical fitness and prevalence of metabolic syndrome actually seems to apply for any given level of body weight [19]. Two recent studies, both using a surrogate marker for physical fitness, confirmed the hypothesis that metabolically healthy but obese subjects have a better physical fitness than metabolically abnormal obese subjects [7, 8]. Our results, using the 'gold standard' VO_{2max} for determination of cardio-respiratory fitness, demonstrate that metabolically healthy obese women do have a higher VO_{2max} and, in addition, this parameter appeared to be the strongest predictor of the metabolically healthy obese phenotype. Two other studies found no difference in VO_{2max} between metabolically healthy obese and obese subjects "at-risk". However, these two studies classified their subjects as metabolically healthy obese solely on insulin sensitivity [9, 12], whereas we used the Joint Interim Statement definition of the metabolic syndrome [14] to define our obese women. The important benefit of this approach is the ability to accurately determine the total cardio-metabolic health of the groups. Our findings suggest that VO_{2max} is one of the factors in the phenotype development of subjects with obesity. It appears that the difference in physical fitness between the obesity groups may contribute to the normal blood pressure and cholesterol profile, but does not influence the insulin sensitivity since the M-value did not differ between the obesity groups. As waist circumference did not differ between the groups, the only metabolic syndrome criteria they have in common, waist circumference probably explains the similar insulin sensitivity. This is in agreement with results from the Diabetes Prevention Program Research Group who showed that waist circumference is the strongest predictor of risk for developing diabetes [20]. Although physical fitness can positively influence insulin sensitivity this may only count for a certain threshold of physical fitness level.

Even though our metabolically healthy obese women have a higher physical fitness compared to women with the metabolic syndrome, their fitness level is still below the average value of lean women. Apparently, a small difference in low physical fitness may positively affect blood pressure and cholesterol profile including higher HDL levels, but not insulin sensitivity. The lower resting heart rate in the metabolically healthy obese women, despite the fact that 6 out of 10 metabolic syndrome women used a β-blocker, might be interrelated with the better cardio-respiratory fitness in these women but may also reflect a more favorable autonomic nervous system activity. A lower resting heart rate, suggesting a lower sympathetic nervous system activity, has previously been linked to the metabolically healthy obese phenotype in postmenopausal women [21].

Since chronic subclinical inflammation and physical fitness have opposing relationships with obesity [13], we simultaneously determined physical fitness and the inflammation profile. We found that metabolically healthy obese women had 73% lower circulating TNF-α levels compared to metabolic syndrome women, suggesting that metabolically healthy obese women have a more favorable inflammation profile. Also hsCRP appeared numerically lower in the metabolically healthy obese group, but this difference did not reach statistical significance. One previous study assessed TNF-α levels in metabolically healthy obese subjects compared to obese subjects with the metabolic syndrome. Although this study was performed in older mainly black women they also found lower TNF-α levels in the metabolically healthy obese group [10]. The lower TNF-α levels found in our metabolically healthy obese women cannot be explained by their quantity of fat tissue or degree of insulin resistance since both variables did not differ between groups (table 1). Our results suggest that in obese women TNF-α levels may be influenced by VO_{2max} which corresponds with the fact that exercise training can lower TNF-α levels in diabetic patients [22].

There are a few limitations of this study which need to be considered. First, this study had a cross-sectional design which does not allow to draw causal conclusions. However, exercise intervention studies in obese subjects with multiple cardiovascular risk factors confirm that even without weight loss, improving physical fitness level can reverse these risk factors [23, 24]. Secondly, we selected middle-aged women for this study with different menopausal status. The post-menopausal status is associated with an increased risk of the metabolic syndrome [25] and is related with changes in individual criteria of the metabolic syndrome such as an increase in abdominal adiposity. Although we are aware of this influence we believe this did not affect our results since the distribution of menopausal status and trunk fat mass percentage were equal in both groups. Finally, by using the DXA scan we could determine abdominal trunk fat and peripheral fat located on the extremities, but we were not able to discriminate between subcutaneous and visceral abdominal

fat tissue while specifically visceral abdominal fat can be related to systemic markers of inflammation [11, 26] and consequently with the metabolic phenotype.

Perspectives

The results of the present study show that a higher physical fitness and lower circulating TNF-α levels may partly explain why metabolically healthy obese women are protected against the detrimental effects of obesity, despite corresponding levels of adipose tissue mass, compared to obese women with the metabolic syndrome. Our findings support previous studies which used surrogate markers for physical fitness [7, 8]. In addition, our results show that VO_{2max} appears to be a stronger predictor than TNF-α for having the metabolically healthy obese phenotype. Primary prevention by increasing fitness levels in obese women, might prevent the massive medical and economic burden associated with obesity and is therefore of utmost importance.

Acknowledgement

The work was performed at the department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands. Conception and design of the experiments: FP, CT and MH. Collection, analysis and interpretation of data: FP, TE, PB, RV, CT, MH. Drafting the article or revising it critically for important intellectual content: FP, TE, PB, RV, CT, MH. All authors approved the final version of the manuscript.

References

1. Temelkova-Kurktschiev T, Stefanov T. Lifestyle and genetics in obesity and type 2 diabetes. *Exp Clin Endocrinol Diabetes*. 2012;120:1-6
2. Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, Sowers MR. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: Prevalence and correlates of 2 phenotypes among the us population (nhanes 1999-2004). *Arch Intern Med*. 2008;168:1617-1624
3. Song Y, Manson JE, Meigs JB, Ridker PM, Buring JE, Liu S. Comparison of usefulness of body mass index versus metabolic risk factors in predicting 10-year risk of cardiovascular events in women. *Am J Cardiol*. 2007;100:1654-1658
4. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care*. 2001;24:683-689
5. Farrell SW, Cheng YJ, Blair SN. Prevalence of the metabolic syndrome across cardiorespiratory fitness levels in women. *Obesity research*. 2004;12:824-830
6. Katzmarzyk PT, Church TS, Janssen I, Ross R, Blair SN. Metabolic syndrome, obesity, and mortality: Impact of cardiorespiratory fitness. *Diabetes Care*. 2005;28:391-397
7. Ortega FB, Lee DC, Katzmarzyk PT, Ruiz JR, Sui X, Church TS, Blair SN. The intriguing metabolically healthy but obese phenotype: Cardiovascular prognosis and role of fitness. *Eur Heart J*. 2013;34:389-397
8. Bouchard DR, Langlois MF, Brochu M, Dionne IJ, Baillargeon JP. Metabolically healthy obese women and functional capacity. *Metabolic syndrome and related disorders*. 2011;9:225-229
9. Karelis AD, Faraj M, Bastard JP, St-Pierre DH, Brochu M, Prud'homme D, Rabasa-Lhoret R. The metabolically healthy but obese individual presents a favorable inflammation profile. *J Clin Endocrinol Metab*. 2005;90:4145-4150
10. Koster A, Stenholm S, Alley DE, Kim LJ, Simonsick EM, Kanaya AM, Visser M, Houston DK, Nicklas BJ, Tylavsky FA, Satterfield S, Goodpaster BH, Ferrucci L, Harris TB. Body fat distribution and inflammation among obese older adults with and without metabolic syndrome. *Obesity (Silver Spring, Md*. 2010;18:2354-2361
11. Hayes L, Pearce MS, Firbank MJ, Walker M, Taylor R, Unwin NC. Do obese but metabolically normal women differ in intra-abdominal fat and physical activity levels from those with the expected metabolic abnormalities? A cross-sectional study. *BMC public health*. 2010;10:723
12. Brochu M, Tchernof A, Dionne IJ, Sites CK, Eltabbakh GH, Sims EA, Poehlman ET. What are the physical characteristics associated with a normal metabolic profile despite a high level of obesity in postmenopausal women? *J Clin Endocrinol Metab*. 2001;86:1020-1025
13. Lavoie ME, Rabasa-Lhoret R, Doucet E, Mignault D, Messier L, Bastard JP, Faraj M. Association between physical activity energy expenditure and inflammatory markers in sedentary overweight and obese women. *International journal of obesity (2005)*. 2010;34:1387-1395
14. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. *Circulation*. 2009;120:1640-1645
15. Third report of the national cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel iii) final report. *Circulation*. 2002;106:3143-3421

16. Johnson JL, Slentz CA, Houmard JA, Samsa GP, Duscha BD, Aiken LB, McCartney JS, Tanner CJ, Kraus WE. Exercise training amount and intensity effects on metabolic syndrome (from studies of a targeted risk reduction intervention through defined exercise). *Am J Cardiol*. 2007;100:1759-1766
17. Abbink EJ, De Graaf J, De Haan JH, Heerschap A, Stalenhoef AF, Tack CJ. Effects of pioglitazone in familial combined hyperlipidaemia. *J Intern Med*. 2006;259:107-116
18. Orakzai RH, Orakzai SH, Nasir K, Roguin A, Pimentel I, Carvalho JA, Meneghello R, Blumenthal RS, Santos RD. Association of increased cardiorespiratory fitness with low risk for clustering of metabolic syndrome components in asymptomatic men. *Archives of medical research*. 2006;37:522-528
19. Lee S, Kuk JL, Katzmarzyk PT, Blair SN, Church TS, Ross R. Cardiorespiratory fitness attenuates metabolic risk independent of abdominal subcutaneous and visceral fat in men. *Diabetes Care*. 2005;28:895-901
20. Listed N. Relationship of body size and shape to the development of diabetes in the diabetes prevention program. *Obesity (Silver Spring, Md*. 2006;14:2107-2117
21. Robillard ME, Bellefeuille P, Comtois AS, Aubertin-Leheudre M, Karelis AD. The metabolically healthy but obese postmenopausal woman presents a favourable heart rate variability profile. *Scand Cardiovasc J*. 2011;45:316-320
22. Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, Fallucca S, Alessi E, Letizia C, Jimenez A, Fallucca F, Pugliese G. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr Metab Cardiovasc Dis*. 2010;20:608-617
23. Mourier A, Gautier JF, De Kerviler E, Bigard AX, Villette JM, Garnier JP, Duvallet A, Guezennec CY, Cathelineau G. Mobilization of visceral adipose tissue related to the improvement in insulin sensitivity in response to physical training in niddm. Effects of branched-chain amino acid supplements. *Diabetes Care*. 1997;20:385-391
24. Maiorana A, O'Driscoll G, Goodman C, Taylor R, Green D. Combined aerobic and resistance exercise improves glycemic control and fitness in type 2 diabetes. *Diabetes Res Clin Pract*. 2002;56:115-123
25. Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB. The metabolic syndrome: Prevalence and associated risk factor findings in the us population from the third national health and nutrition examination survey, 1988-1994. *Arch Intern Med*. 2003;163:427-436
26. Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, Keaney JF, Jr., Meigs JB, Lipinska I, Kathiresan S, Murabito JM, O'Donnell CJ, Benjamin EJ, Fox CS. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: The framingham heart study. *Circulation*. 2007;116:1234-1241

Chapter 4

Expression of genes involved in fatty acid transport and insulin signaling is altered by physical inactivity and exercise training in human skeletal muscle

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American Journal of Physiology Endocrinology and Metabolism.
2012: 15: 303(10): E1245-51

Abbreviations

AIS	ASIA Impairment Scale
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AS160 Akt	substrate of 160 kDa
DAVID	database for annotation, visualization and integrated discovery
ECLIA	electrochemiluminescence immunoassay
FA	fatty acid
FABP3	fatty acid binding protein 3
FES	functional electrical stimulation
GLUT4	glucose transporter type 4
GO	gene ontology
HADH	hydroxyacyl-CoA dehydrogenase
HbA_{1c}	glycosylated hemoglobin
HOMA-IR	homeostasis model assessment of insulin resistance
IRS1/2	insulin receptor substrate 1/2
RMA	robust microarray analysis
UBC	ubiquitin C
ULLS	unilateral limb suspension
OXPHOS	oxidative phosphorylation
PKC	protein kinase C
RT-qPCR	reverse transcription quantitative PCR
SCI	spinal cord injury
SLC25A20	solute carrier 25 (carnitine/acylcarnitine translocase), family member A20

Abstract

Physical deconditioning is associated with the development of chronic diseases, including type 2 diabetes and cardiovascular disease. Exercise training effectively counteracts these developments, but the underlying mechanisms are largely unknown. To gain more insight in these mechanisms, muscular gene expression levels were assessed after physical deconditioning and after exercise training of the lower limbs in humans using gene expression microarrays. In order to exclude systemic effects, we used human models for local physical inactivity (three weeks of unilateral limb suspension) and for local exercise training (six weeks of functional electrical stimulation exercise of the extremely deconditioned legs of individuals with a spinal cord injury). The most interesting subset of genes, those downregulated after deconditioning as well as upregulated after exercise training, contained 18 genes related to both the 'insulin action' and 'adipocytokine signaling' pathway. Of these genes, the three with strongest up/downregulation were the muscular fatty acid binding protein 3 (FABP3), the fatty acid oxidizing enzyme hydroxyacyl-CoA dehydrogenase (HADH), and the mitochondrial fatty acid transporter solute carrier 25 family member A20 (SLC25A20). The expression levels of these genes were confirmed using RT-qPCR. The results of the present study indicate an important role for a decreased transport and metabolism of fatty acids which provides a link between physical activity levels and insulin signaling in relation to physical (in) activity.

Introduction

The human genome has evolved based on a physically active lifestyle, but our society has become increasingly sedentary. Physical inactivity has detrimental health consequences, including the development of insulin resistance, and eventually type 2 diabetes and cardiovascular disease[1, 2]. Recently, it has been demonstrated that even a two week reduction in daily activity level can be sufficient to increase insulin resistance in healthy subjects, as does nine days of strict bed rest[3, 4]. On the other end of the spectrum, a single bout of exercise can reduce insulin resistance[5], regular physical activity reduces the risk of developing diabetes[6, 7], and significant exercise effects have been reported in diabetics[8], making exercise a powerful tool in the prevention and management of type 2 diabetes.

Skeletal muscle accounts for the majority of insulin-induced glucose uptake. Binding of insulin to its receptor on the cellular plasma membrane leads, via the activation of intracellular insulin receptor substrate 1 and 2 (IRS1/2), to translocation of the Glut4 glucose transporter to the plasma membrane. Obesity research has demonstrated that elevated levels of free fatty acids in the blood plasma can inhibit insulin signaling directly, or via elevated intramyocellular levels of triglycerides and free fatty acids that attenuate the insulin signaling pathway[9]. In addition, an impaired oxidation of intracellular fatty acids may result in an even larger accumulation of intracellular fatty acids and subsequent inhibition of insulin signaling[10]. On the other hand, exercise can effectively stimulate the insulin signaling cascade[5]. However, it is still unknown how the molecular mechanisms behind the development and prevention of insulin resistance exactly work. Gene expression profiling of skeletal muscle tissue after physical deconditioning and exercise training may provide more insight in the regulation of these mechanisms.

Gene expression microarray analysis is a rapid and comprehensive approach for the first identification of molecular changes in muscle tissue. Using this technique, the expression level of virtually all known genes can be analyzed in a single experiment. Microarrays have been used to study the expression levels of insulin-related genes in human models for deconditioning and exercise training[3, 11], but systemic effects probably play an important role in these bed rest and exercise interventions. In order to exclude systemic effects and to enable us to solely study the muscular effects of the interventions, we used unique human *in vivo* models for local inactivity and local exercise training. The aim of this study was to identify genes related to the insulin signaling pathway that demonstrated an opposite responses to deconditioning versus exercise training. Such genes may be responsible for the detrimental effects of physical inactivity versus the beneficial effects of regular exercise.

Materials and methods

Experimental design

Unilateral lower limb suspension (ULLS) deconditioning:

As a model for local deconditioning, the right leg of six healthy males was suspended and unloaded from all weight bearing for a period of three weeks as described before[12, 13]. To evaluate effectiveness of the ULLS model, calf skin temperature and calf circumference were measured before and after the intervention.

Functional electrical stimulation (FES) exercise training:

As a model for local exercise training, eight subjects with SCI received eight weeks of FES exercise training of their paralyzed legs using a computer-controlled leg cycle ergometer (Ergys 2, Therapeutic Alliances Inc, Fairborn, OH, USA)[14-16]. Self-adhesive 50 x 90 mm surface electrodes (Stimex, Pierenkemper, Wetzlar, Germany) were placed on the hamstring, gluteal, and quadriceps muscles on both legs of each subject. Electrical stimulation was applied using a coordinated sequence of monophasic square wave pulses (450 µs, 30 Hz) permitting cyclic patterns of muscle contraction resulting in leg cycling. The device was programmed to gradually increase the stimulation current amplitude to a maximum of 140 mA, in order to achieve a target pedaling rate of ~50 rpm. Pedal resistance could be altered with 1/8 kp increments, corresponding to ~6.1 W at 50 rpm. Resistance was reduced when pedaling rate dropped below 45 rpm and the stimulation was stopped when pedaling rate dropped below 35 rpm. During the training period, subjects performed 2-3 training sessions per week of 30 min each, resulting in a total of 20 sessions per person. When 30 min exercise was not realized in one run, up to 5 runs were performed until 30 min of cumulated active exercise was achieved. FES-cycling as adopted in our study results in elevations in heart rate for the duration of the exercise bout (up to 30 minutes), but also leads to significant and steady-state elevations in oxygen consumption and improvement in other cardiovascular parameters[14, 17]. This indicates that FES-cycling as adopted in our study can be regarded as an aerobic type of exercise. As a surrogate measure for lower limb fitness level, the total work (in kJ) achieved by a subject during a single bout of FES cycling was assessed during the first and the last training session.

Before and after both interventions, systemic insulin resistance and leg muscular gene expression levels were determined. Muscle biopsies after ULLS deconditioning were obtained before the leg was reactivated. To avoid an acute exercise effect, biopsies after FES exercise were obtained 24-48 h after the last

exercise bout, since the expression level of most metabolic genes returns to baseline within 24 hours following exercise[18].

Subjects

Subject characteristics are provided in Table 1. Seven SCI subjects had a complete lesion, varying between C5 and T11 (ASIA Impairment Scale (AIS) A), while one subject had an incomplete lesion at C5 (AIS B). All lesions were traumatic and existed for at least four years. None of the participants had any known cardiovascular disease, diabetes or cardiovascular risk factors such as hypercholesterolemia and hypertension. The study was approved by the ethics committee of the Radboud University Nijmegen Medical Centre, and conformed to the principles outlined in the declaration of Helsinki. All subjects provided written informed consent prior to testing.

Table 1. Baseline characteristics of healthy subjects before deconditioning (n=6), and subjects with a spinal cord injury (SCI) before exercise training (n=8)

	Healthy subjects	Subjects with SCI
Age, years	21 ± 0	39 ± 3*
Body mass, kg	79 ± 4	72 ± 5
Body mass index, kg/m^2	22 ± 1	22 ± 1
Systolic blood pressure, mmHg	126 ± 3	117 ± 6
Diastolic blood pressure, mmHg	79 ± 2	75 ± 3
Resting heart rate, bpm	64 ± 4	62 ± 2

Values are mean ± SEM. SCI = spinal cord injury; bpm = beats per minute.
*$p<0.05$ for unpaired t-test SCI vs. controls.

Insulin resistance

Blood samples were obtained before and after both interventions. Fasting glucose levels were determined using standard laboratory techniques, and fasting insulin levels were determined with an electrochemiluminescence immunoassay (ECLIA) on the E170 module of a modular analytics EVO analyzer (Roche, Mannheim, Germany) before and after deconditioning (n=5) and exercise training (n=8). Insulin resistance was calculated according to the homeostasis model

assessment of insulin resistance (HOMA-IR) method19, 20, using the equation HOMA-IR = glucose (mmol/l) x insulin (mU/l) / 22.5.

Muscle biopsy and RNA isolation

Muscle biopsies were obtained from the vastus lateralis before and after the interventions using the Bergström technique21. The skin was locally anaesthetized with 1% (w/v) lidocaine, a small incision was made through the skin and fascia, and muscle specimens were immediately frozen in liquid nitrogen and stored at -80°C. Frozen muscle biopsy samples were ground using a mortar and pestle, placed in RNA-Bee, which contains phenol and guanidine thiocyanate (Tel-test, Friendswood, TX, USA), and homogenized using an T25 ultra-turrax dispenser (IKA, Staufen, Germany). RNA was isolated using phenol/chloroform extraction, and purified according to the RNeasy mini kit clean-up protocol including an on-column DNase digestion (Qiagen, Hilden, Germany). The RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity was analyzed on an Agilent Bioanalyzer (Santa Clara, CA, USA). For the deconditioning samples the RIN was between 6.8 and 7.9, and for the exercise samples the RIN was between 8.0 and 8.7, both indicating good RNA quality.

Gene expression microarray analysis

Gene expression analysis was performed before and after deconditioning (for three individuals separately plus one cDNA pool of the other three subjects), and three separate SCI individuals before and after exercise training. From the other SCI subjects we obtained insufficient RNA, which probably relates to the dramatic changes in muscle mass and composition (lots of intramuscular fat) after a SCI, which is further discussed in the limitations section. Total RNA (100 ng) was amplified and labeled according to the Affymetrix GeneChip whole transcript sense target labeling assay, and hybridized on Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA)22. Arrays were scanned, processed, and imported into Genomic Suite software (Version 6.4, Partek, St. Louis, MO, USA). Probe sets were normalized using the robust microarray analysis (RMA) algorithm including background correction for GC content. The mean intensity of probe sets belonging to one gene was calculated, representing the quantitative gene expression levels. Differences in gene expression between groups were calculated and results were exported to Microsoft Excel for further analysis.

Gene lists of interest were analyzed for enriched gene ontology (GO) terms for biological processes at the most specific level available (GOTERM_BP_FAT) using the web-based database for annotation, visualization and integrated discovery (DAVID)23, 24. To summarize extensive lists, functional annotation clustering of the enriched GO terms was performed as indicated. Alternatively, gene lists of interest were analyzed using Pathway studio 7.1 software (Ariadne, Rockville, MD, USA). The microarray data have been submitted to the Gene Expression Omnibus (GEO) repository under number GSE33886. And are accessible for review at:
http://www.ncbi.nlm.nih.gov/geo/query/acc.
cgi?token=dhcpzsgwiskecpi&acc=GSE33886.

Reverse transcription quantitative PCR (RT-qPCR) validation of microarray results

For reverse transcription quantitative PCR (RT-qPCR) validation of the microarray results, RNA used for the microarrays was available for two healthy controls and three individuals with SCI before and after the interventions. Of these samples, 200 ng RNA was transcribed to cDNA using the superscript III first-strand synthesis supermix for qRT-PCR (Invitrogen, Paisley, UK) according to the manufacturers protocol. Based on the microarray results, ubiquitin C (UBC) was selected as a stable reference gene. Intron-spanning primers were designed using NCBI Primer-BLAST and purchased from Biolegio (Malden, The Netherlands). Primer nucleotide sequences (5'>3') were as follows: fatty acid binding protein 3 (FABP3) forward (TGGGACGGGCAAGAGACCACA), FABP3 reverse (TGCCGTGGGTGAGTGTCAGGA), hydroxyacyl-CoA dehydrogenase (HADHB) forward (CACCTGAGCTCCCTGCGGTT), HADHB reverse (TCGGTCTGCAGAGTGGCCCAT), solute carrier 25 (carnitine/acylcarnitine translocase), family member A20 (SLC25A20) forward (TGCTGAGGGAGCTGATCCGGG), SLC25A20 reverse (AACAGGCCGCATTGGCTGGG), UBC forward (TAGTTCCGTCGCAGCCGGGA), and UBC reverse (GCATTGTCAAGTGACGATCACAGCG). Sybr green-based RT-qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). For each primer pair the melt curve, efficiency, and no template controls were assessed to check primer specificity. The comparative CT quantification (ΔΔCt method) corrected for primer efficiency was used to compare changes in gene expression.

Statistical analysis

Subject characteristics, glucose/insulin levels and HOMA-IR are given as mean ± S.E.M. and differences between the groups and between post and pre intervention were assessed using a two-tailed t-test.

To assess differences in gene expression between groups, the corrected intensity of individual genes were used for an analysis of variance (ANOVA) on 1) After deconditioning versus before deconditioning, and 2) After exercise training versus before exercise training, using Partek Genomic Suite. The focus of this study was on biological processes and pathways instead of individual genes, and therefore unadjusted p-values ($p<0.05$) were used. Enriched GO terms were considered significant if $p<0.05$ after Benjamini-Hochberg correction for multiple testing.

To validate the microarray data, a Pearson correlation coefficient was calculated on the fold-changes of FABP3, HADHB, and SLC25A20 as determined with RT-qPCR versus the fold-changes as determined with gene expression microarrays.

Results

Effectiveness of the interventions

Deconditioning by ULLS resulted in a significant decrease of 2.3±0.1 °C in calf skin temperature and a significant decrease of 1.2±0.4 cm in calf circumference of the suspended leg (pre 28.1±0.2, post 25.8±0.2 °C, $P<0.001$ and pre 37.7±0.9, post 36.5±0.7 cm, $P=0.028$, respectively). This indicates that ULLS deconditioning was effective.

The exercise training period was successfully completed by all eight subjects with SCI. Work during FES exercise increased significantly after training (median (25%-75%): pre: 0 kJ (0-2.2), post: 11.0 kJ (5.5-19.7), $P=0.008$). This indicates that FES exercise training was effective in improving condition of the paralyzed muscles in SCI individuals.

Systemic insulin resistance, as determined by the HOMA-IR, was not affected by ULLS deconditioning, nor by FES exercise training (Table 2).

Table 2. Fasting glucose and insulin levels, and deduced insulin resistance (HOMA-IR) before and after deconditioning of healthy subjects (n=5) and exercise training of subjects with a spinal cord injury (n=8)

	Deconditioning		Exercise training	
	Before	After	Before	After
Glucose (mmol/L)	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.2
Insulin (mU/L)	4.9 ± 1.0	6.6 ± 0.6	12.1 ± 4.6	9.7 ± 2.9
Insulin resistance (HOMA-IR)	1.0 ± 0.2	1.4 ± 0.1	2.7 ± 1.1	2.1 ± 0.7

Values are mean±SEM. HOMA-IR = homeostasis model assessment of insulin resistance

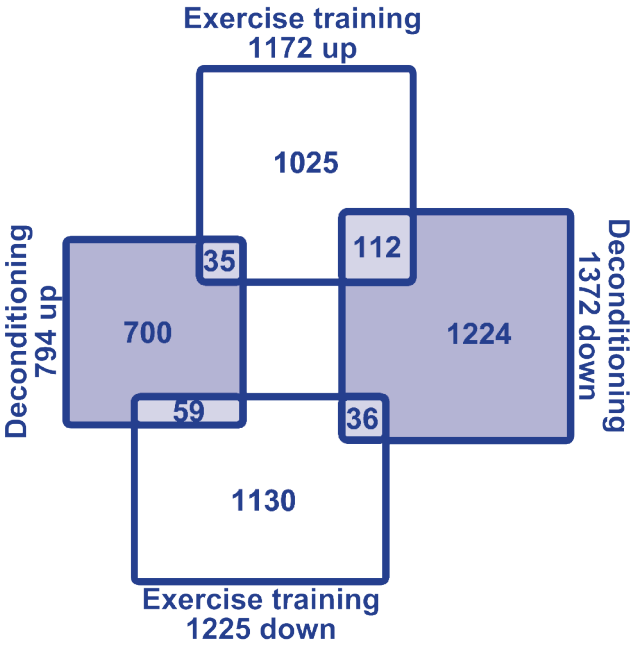
Gene expression microarray analysis

Gene expression levels were analyzed for a subgroup of the participants. We identified 2,166 genes with an altered expression level after deconditioning (1,372 down- and 794 upregulated), and 2,397 genes after exercise training (1,172 up- and 1,225 downregulated) (Fig. 1). Further analysis revealed an overlap in 112 genes that were upregulated after exercise training and downregulated after deconditioning and 59 genes that showed the opposite response. In addition, we found upregulation after both interventions in 35 genes and downregulation in 36 genes.

Effect of the interventions on biological processes

To gain insight in the biological implication of the observed changes in gene expression, lists of up- and downregulated genes were analyzed for overrepresented biological process gene ontology (GO) terms and summarized using functional annotation clustering (Table 3). The list of genes upregulated after deconditioning was not enriched for any GO term, while the list of genes downregulated after deconditioning was significantly enriched for 15 clusters of GO terms that are mainly related to cellular respiration. The list of upregulated genes after exercise training was enriched for the GO term cluster ‘generation of precursor metabolites and energy’. The list of genes downregulated after exercise training was enriched for GO terms related to RNA splicing and chromosome organization.

Figure 1



Schematic representation of the microarray results. Number of genes up- and downregulated after deconditioning and exercise training, and overlap between these gene lists ($p<0.05$).

Table 3. The most significant gene ontology (GO) terms representing the overrepresented clusters in the lists of genes up- and downregulated after deconditioning and exercise training

DOWNREGULATED AFTER DECONDITIONING		
Gene ontology term	**F.E.**	**P value**
GO:0006091 generation of precursor metabolites and energy	5.2	7.2E-34
GO:0006119 oxidative phosphorylation	7.2	8.2E-19
GO:0006631 fatty acid metabolic process	3.7	6.7E-10
GO:0006732 coenzyme metabolic process	3.8	5.7E-08
GO:0006090 pyruvate metabolic process	5.9	1.0E-04
GO:0006006 glucose metabolic process	3.0	4.3E-04
GO:0010565 regulation of cellular ketone metabolic process	4.3	2.5E-03
GO:0046486 glycerolipid metabolic process	2.7	2.9E-03
GO:0015718 monocarboxylic acid transport	4.6	3.2E-03
GO:0055085 transmembrane transport	1.7	8.1E-03
GO:0015908 fatty acid transport	5.4	2.6E-02
GO:0003012 muscle system process	2.4	2.8E-02
GO:0046474 glycerophospholipid biosynthetic process	3.3	3.9E-02

UPREGULATED AFTER EXERCISE TRAINING		
Gene ontology term	**F.E.**	**p-value**
GO:0006091 generation of precursor metabolites and energy	2.4	3.0E-03

DOWNREGULATED AFTER EXERCISE TRAINING		
Gene ontology term	**F.E.**	**p-value**
GO:0008380 RNA splicing	3.8	8.6E-14
GO:0051028 mRNA transport	3.9	2.2E-03
GO:0051276 chromosome organization	1.9	2.3E-02

F.E. = Fold enrichment

Biological processes and pathways affected by both interventions

Analysis of the overlapping gene lists, being the genes that responded to deconditioning as well as exercise training, resulted in significantly enriched GO terms only for the 112 genes that were downregulated after deconditioning as well as upregulated after exercise training (Table 4). These 112 genes were further analyzed for overrepresented "Ariadne signaling pathways". This resulted in two significantly enriched pathways, namely "insulin action" (P=5.5E-07, 18 of 905 members present) and "adipocytokine signaling" (P=1.0E-03, 12 of 780 members present). The 12 genes of the second pathway completely overlapped with the 18 genes of the first, and are all listed in table 5.

Table 4. Overrepresented gene ontology terms in the overlap between the lists of 'downregulated after deconditioning' and 'upregulated after exercise training'

Gene ontology term	F.E.	P value
GO:0006091 generation of precursor metabolites and energy	9.3	1.7E-08
GO:0055114 oxidation reduction	5.4	9.7E-07
GO:0045333 cellular respiration	14.1	3.6E-04
GO:0022900 electron transport chain	12.0	8.0E-04
GO:0006119 oxidative phosphorylation	12.2	2.8E-03
GO:0015980 energy derivation by oxidation of organic compounds	9.5	3.0E-03
GO:0006631 fatty acid metabolic process	6.9	1.6E-02
GO:0006732 coenzyme metabolic process	7.8	2.4E-02
GO:0006006 glucose metabolic process	7.8	2.4E-02
GO:0016054 organic acid catabolic process	9.3	3.9E-02
GO:0046395 carboxylic acid catabolic process	9.3	3.9E-02
GO:0015908 fatty acid transport	24.5	4.2E-02

F.E. = Fold enrichment

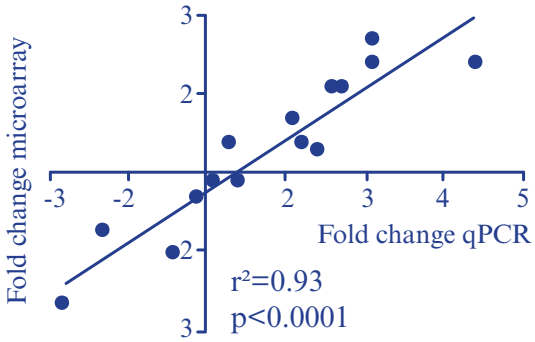
Table 5. Fold-change of all 18 genes downregulated after deconditioning and upregulated after exercise training that belong to the "insulin action" and "adipocytokine signaling" pathways

Gene symbol	Name	Deconditioning	Exercise training
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	-1.89	2.47
HADHB	hydroxyacyl-CoA dehydrogenase/3-keto-acyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	-1.25	1.87
SLC25A20	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	-1.56	1.44
LDHD	lactate dehydrogenase D	-1.59	1.28
PDK2	pyruvate dehydrogenase kinase, isozyme 2	-1.42	1.38
DLST	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	-1.40	1.34
PET112L	PET112-like (yeast)	-1.36	1.32
MRPS2	mitochondrial ribosomal protein S2	-1.46	1.18
MRPS18B	mitochondrial ribosomal protein S18B	-1.42	1.21
PC	pyruvate carboxylase	-1.25	1.35
MRPL33	mitochondrial ribosomal protein L33	-1.25	1.32
MRPL12	mitochondrial ribosomal protein L12	-1.33	1.22
ENO3	enolase 3 (beta, muscle)	-1.37	1.16
PECI (ECI2)	enoyl-CoA delta isomerase 2	-1.28	1.24
GPI	glucose-6-phosphate isomerase	-1.23	1.26
HADHA	hydroxyacyl-CoA dehydrogenase/3-keto-acyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	-1.18	1.30
EIF3K	eukaryotic translation initiation factor 3, subunit K	-1.27	1.13
NR2F2	nuclear receptor subfamily 2, group F, member 2	-1.06	1.18

Reverse transcription quantitative PCR (RT-qPCR) validation

Of the 18 genes associated with insulin and adopocytokine signaling, RT-qPCR analysis was performed to assess the expression levels of the three genes that demonstrated the strongest up- and downregulation after exercise training and deconditioning, respectively. The fold-changes of FABP3, HADHB, and SLC25A20 as determined with RT-qPCR demonstrated a significant correlation with the fold-changes determined with the gene expression microarrays, thereby confirming the microarray findings (Fig. 2).

Figure 2.



RT-qPCR validation of the microarray results. Correlation of the expressional changes of FABP3, HADHB, and SLC20A25 after deconditioning (n=2) and exercise training (n=3), as determined with RT-qPCR versus gene expression microarrays. The origin of this graph represents a -1 or 1-fold difference (=unchanged expression).

Discussion

In order to study only the local effects of physical inactivity and activity and minimize possible systemic effects, we used unique human models for inactivity and exercise training. The sudden ULLS inactivation of the active legs of healthy subjects is in our opinion best mirrored by the sudden FES activation of the extremely inactive legs of subjects with a SCI[25, 26]. The models for local deconditioning and exercise training were successful, since three weeks of ULLS deconditioning decreased calf skin temperature and leg circumference, and FES exercise training increased the total work performed by the participants.

Due to the local nature of our interventions, we did not find a statistically significant effect on systemic insulin resistance. Recent studies on more systemic types of deconditioning did report an increase in insulin resistance, for example after a two week reduction in daily steps[4], and after nine days of bed rest[3]. Subjects with SCI have an increased risk for developing insulin resistance and subsequent type 2 diabetes[27], but our local FES exercise training did not alter fasting glucose and insulin levels. This is in line with a comparable intervention study, that required the administration of one dose of glucose (oral glucose tolerance test) to visualize differences in insulin sensitivity[28, 29]. In addition, they reported an increase in the skeletal muscle protein levels of the glucose transporters GLUT1 and GLUT4 after intervention. However, we did not confirm this on the gene expression level (1.02 and 1.06-fold upregulated, respectively). Our study could have been strengthened by assessment of local insulin resistance by 2-deoxyglucose uptake or oral glucose tolerance tests with concomitant muscle biopsies and subsequent analysis for phosphorylation status of insulin-signaling proteins.

The focus of this study was to identify insulin signaling-related genes that changed after both interventions. Of the 112 genes that were downregulated after deconditioning as well as upregulated after exercise training, pathway analysis identified 18 genes to be involved in insulin action and adipocytokine signaling. Expression levels of the three genes with the most contrasting up/downregulation (FABP3, HADHB, and SLC25A20) were confirmed using RT-qPCR validation.

Previous reports on these three genes describe their possible role in insulin and adipocytokine signaling. FABP3 (also known as H-FABP) facilitates the cytoplasmic transport of fatty acids between intracellular membranes[30]. It has previously been demonstrated in a rat model that hind limb unloading results in FABP3 downregulation[31], while exercise training of humans results in the upregulation of FABP3 expression[32]. Trained athletes also have higher FABP3 expression levels than untrained subjects[33]. In addition, FABP3 is one of the genes demonstrating a positive correlation with both maximal oxygen uptake and proportion of skeletal muscle type I fibers in men, suggesting a role for FABP3 in muscular aerobic metabolism[34]. Gene and protein expression of muscular FABP3 has been correlated to body weight in a diet-induced obesity mouse model, and increased expression in cultured skeletal muscle cells resulted in phosphorylation of the protein Akt substrate of 160 kDa (AS160) and glucose uptake via activation of AMP-activated protein kinase (AMPK) and insulin-dependent Akt activation[35]. FABP3 thus provides a first link between physical activity and insulin resistance.

The fatty acid beta oxidizing enzyme HADH functions in the mitochondrial matrix and is commonly used as a marker for skeletal muscle oxidative capacity. The protein consists of an alpha and beta subunit, and in our study both were downregulated after deconditioning and upregulated after exercise training. This is

in line with previous studies that reported a decreased HADH activity after hind limb unloading in a rat model[36], and HADH activation following exercise training programs in both animal and human models[37-39]. The role of HADH in skeletal muscle insulin signaling remains more elusive.

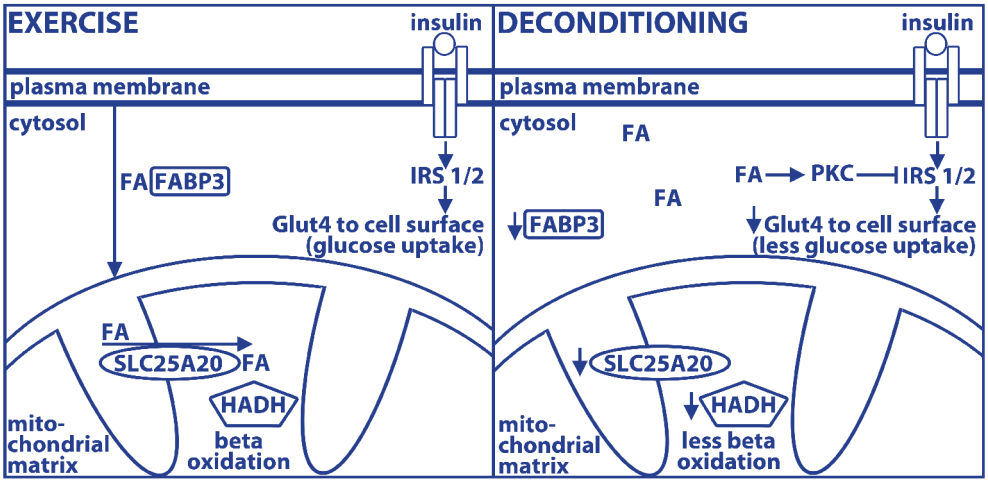
The mitochondrial fatty acid transporter SLC25A20 (also known as CACT) is required to translocate fatty acids across the inner mitochondrial membrane into the mitochondrial matrix, where fatty acid beta oxidation takes place[40]. Despite this crucial role in the generation of energy in muscle, little is known about the effects of deconditioning and exercise on SLC25A20 expression levels. Regarding insulin resistance, a significant decrease in muscular SLC25A20 mRNA and protein levels has been demonstrated in insulin resistant subjects and in their isolated mitochondria the activity of the enzyme was lower compared to healthy subjects[41].

Thus, our results indicate that one of the opposite effects of physical deconditioning and exercise training is an altered transport (intracellular FABP3 and SLC25A20 over the inner mitochondrial membrane) and metabolism (beta oxidation by HADH) of fatty acids. This is in line with theories that inactivity-induced accumulation of intramuscular fatty acids may lead to insulin resistance, while exercise induces the beta oxidation of these fatty acids[9, 10]. Integration of our findings with these existing models results in a proposed working mechanism of physical activity on insulin resistance as depicted in figure 3. In this model, exercise training upregulates FABP3 that transports fatty acids between the cellular plasma membrane and the mitochondrial membranes. SLC25A20 is also upregulated and transports the fatty acids over the inner mitochondrial membrane. Inside the mitochondria, upregulated HADH is involved in the beta oxidation of fatty acids. Physical deconditioning, on the other hand, results in downregulation of these genes, which leads to the accumulation of intracellular fatty acids. This activates protein kinase C (PKC), which inhibits insulin receptor substrate 1/2 (IRS 1/2), resulting in less translocation of Glut4 to the cell membrane and thus a reduced insulin-induced glucose uptake. However, additional experiments are required to prove the validity of this preliminary model.

Apart from the genes that were regulated by both interventions, we separately analyzed the lists of genes that were up- and downregulated after the individual interventions. Deconditioning resulted in the downregulation of biological processes involved in cellular respiration including oxidative phosphorylation. This is in line with a previous study that reported a marked downregulation of the oxidative phosphorylation (OXPHOS) pathway after bed rest deconditioning3. The most significantly downregulated GO term after deconditioning: ‘generation of precursor metabolites and energy’, was identical to the single term upregulated after exercise training, confirming the opposite effects of physical deconditioning and exercise training on the gene expression level. A surprising observation was

the downregulation of RNA splicing and related gene ontology terms after exercise training in SCI individuals. These terms were not significantly upregulated after deconditioning of our healthy controls. This suggests that altered RNA splicing may be a long-term consequence of spinal cord injury, which is not induced by short-term deconditioning, but can be counteracted by exercise training. This is supported by a recent study in a mouse model for spinal muscular atrophy, which proposes that changes in RNA splicing are mainly a secondary effect of the paralysis and may be a response to cell stress[42]. Interestingly, changes in alternative splicing have been linked with the development of insulin resistance[43] and may thus provide an alternative explanation for SCI-induced insulin resistance. Exploring possible other (patho-) physiological consequences of this altered alternative splicing machinery requires further investigation[44].

Figure 3.



Preliminary representation of the proposed link between physical activity and inactivity and insulin signaling in skeletal muscle cells. Exercise training (left panel) results in the upregulation of FABP3 that transports fatty acids (FAs) between the cellular plasma membrane and the membranes of the mitochondria. At the inner mitochondrial membrane, SLC25A20 is upregulated and transports FAs over the membrane. Inside the mitochondria, HADH is upregulated and involved in the beta oxidation of FAs. Physical deconditioning (right panel), on the contrary, downregulates these key players. This results in a reduced FA transport and the subsequent accumulation of intracellular FAs. This activates protein kinase C (PKC), which inhibits insulin receptor substrate 1/2 (IRS 1/2), resulting in less translocation of Glut4 to the cell membrane and a reduced insulin-induced glucose uptake. Partially based on previously published models[9, 10].

Limitations

Gene expression microarrays are a comprehensive approach for the fast identification of interesting genes, but also have their limitations. Changes in gene expression are only physiologically relevant under the general assumption of a good correlation between mRNA and protein levels. However, this is not always the case, and validation on the protein level would therefore be a logical follow-up[45]. In addition, protein phosphorylation is a key feature in insulin signal transduction, but gene expression levels cannot provide information about protein modifications. Unfortunately, additional experiments on the protein level are hampered in this study by the limited availability of muscle tissue in the subjects with SCI. Indeed, it has been demonstrated that muscle fiber type[46], the amount of muscle and percentage of intramuscular fat changes dramatically after a SCI[47, 48]. Therefore, we used most of the obtained tissue to isolate sufficient RNA to perform good quality microarrays. However, the poor muscle quality in SCI also limited the total number of microarrays that could be performed. This drawback has to be acknowledged before SCI can serve as a unique and very informative model for physical inactivity.

Conclusions

Analysis of gene expression levels after local deconditioning versus exercise training resulted in the identification of 18 genes that are mainly involved in the transport and metabolism of intramuscular fatty acids. These genes, including FABP3, HADH, and SLC25A20 provide a link between physical activity levels and insulin signaling.

Acknowledgements

Duby Ballak is acknowledged for his internship work on this topic. We would like to thank Joris Veltman, Suzanne Keijzers-Vloet, and Eugène Verwiel of the Microarray Facility Nijmegen of the department of human genetics (Radboud University Nijmegen Medical Centre) for their assistance. GL was funded by the project Gelderland-Overijssel-EFRO no 2009-010034, NTLvD and DHJT were supported by the Netherlands Heart Foundation (grants NHS 2005B113 and NHS 2009T064, respectively), and JGH was supported by the EURYI award.

References

1. Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP. Reduced physical activity and risk of chronic disease: The biology behind the consequences. *Eur J Appl Physiol*. 2008;102:381-390
2. Szostak J, Laurant P. The forgotten face of regular physical exercise: A 'natural' anti-atherogenic activity. *Clin Sci (Lond)*. 2011;121:91-106
3. Alibegovic AC, Sonne MP, Hojbjerre L, Bork-Jensen J, Jacobsen S, Nilsson E, Faerch K, Hiscock N, Mortensen B, Friedrichsen M, Stallknecht B, Dela F, Vaag A. Insulin resistance induced by physical inactivity is associated with multiple transcriptional changes in skeletal muscle in young men. *Am J Physiol Endocrinol Metab*. 2010;299:E752-763
4. Krogh-Madsen R, Thyfault JP, Broholm C, Mortensen OH, Olsen RH, Mounier R, Plomgaard P, van Hall G, Booth FW, Pedersen BK. A 2-wk reduction of ambulatory activity attenuates peripheral insulin sensitivity. *J Appl Physiol (1985)*. 2010;108:1034-1040
5. Frosig C, Richter EA. Improved insulin sensitivity after exercise: Focus on insulin signaling. *Obesity (Silver Spring, Md*. 2009;17 Suppl 3:S15-20
6. Helmrich SP, Ragland DR, Leung RW, Paffenbarger RS, Jr. Physical activity and reduced occurrence of non-insulin-dependent diabetes mellitus. *N Engl J Med*. 1991;325:147-152
7. Manson JE, Rimm EB, Stampfer MJ, Colditz GA, Willett WC, Krolewski AS, Rosner B, Hennekens CH, Speizer FE. Physical activity and incidence of non-insulin-dependent diabetes mellitus in women. *Lancet*. 1991;338:774-778
8. Boule NG, Haddad E, Kenny GP, Wells GA, Sigal RJ. Effects of exercise on glycemic control and body mass in type 2 diabetes mellitus: A meta-analysis of controlled clinical trials. *Jama*. 2001;286:1218-1227
9. Boden G. Obesity and free fatty acids. *Endocrinol Metab Clin North Am*. 2008;37:635-646, viii-ix
10. Venables MC, Jeukendrup AE. Physical inactivity and obesity: Links with insulin resistance and type 2 diabetes mellitus. *Diabetes/metabolism research and reviews*. 2009;25 Suppl 1:S18-23
11. Teran-Garcia M, Rankinen T, Koza RA, Rao DC, Bouchard C. Endurance training-induced changes in insulin sensitivity and gene expression. *Am J Physiol Endocrinol Metab*. 2005;288:E1168-1178
12. Berg HE, Dudley GA, Haggmark T, Ohlsen H, Tesch PA. Effects of lower limb unloading on skeletal muscle mass and function in humans. *J Appl Physiol (1985)*. 1991;70:1882-1885
13. Bleeker MW, De Groot PC, Poelkens F, Rongen GA, Smits P, Hopman MT. Vascular adaptation to 4 wk of deconditioning by unilateral lower limb suspension. *Am J Physiol Heart Circ Physiol*. 2005;288:H1747-1755
14. Gerrits HL, de Haan A, Sargeant AJ, van Langen H, Hopman MT. Peripheral vascular changes after electrically stimulated cycle training in people with spinal cord injury. *Arch Phys Med Rehabil*. 2001;82:832-839.
15. Petrofsky JS, Heaton HH, 3rd, Phillips CA. Leg exerciser for training of paralysed muscle by closed-loop control. *Medical & biological engineering & computing*. 1984;22:298-303
16. Van Duijnhoven NT, Janssen TW, Green DJ, Minson CT, Hopman MT, Thijssen DH. Effect of functional electrostimulation on impaired skin vasodilator responses to local heating in spinal cord injury. *J Appl Physiol (1985)*. 2009;106:1065-1071
17. Davis GM, Hamzaid NA, Fornusek C. Cardiorespiratory, metabolic, and biomechanical responses during functional electrical stimulation leg exercise: Health and fitness benefits. *Artif Organs*. 2008;32:625-629

18. Yang Y, Creer A, Jemiolo B, Trappe S. Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. *J Appl Physiol (1985)*. 2005;98:1745-1752
19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419
20. Turner RC, Holman RR. Insulin rather than glucose homoeostasis in the pathophysiology of diabetes. *Lancet*. 1976;1:1272-1274
21. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest*. 1975;35:609-616
22. Lammers G, Gilissen C, Nillesen ST, Uijtdewilligen PJ, Wismans RG, Veltman JA, Daamen WF, van Kuppevelt TH. High density gene expression microarrays and gene ontology analysis for identifying processes in implanted tissue engineering constructs. *Biomaterials*. 2010;31:8299-8312
23. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*. 2009;37:1-13
24. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nature protocols*. 2009;4:44-57
25. de Groot PC, Bleeker MW, Hopman MT. Magnitude and time course of arterial vascular adaptations to inactivity in humans. *Exerc Sport Sci Rev*. 2006;34:65-71
26. Thijssen DH, Green DJ, Hopman MT. Blood vessel remodeling and physical inactivity in humans. *J Appl Physiol (1985)*. 2011;111:1836-1845
27. Bauman WA, Spungen AM. Disorders of carbohydrate and lipid metabolism in veterans with paraplegia or quadriplegia: A model of premature aging. *Metabolism*. 1994;43:749-756
28. Chilibeck PD, Bell G, Jeon J, Weiss CB, Murdoch G, MacLean I, Ryan E, Burnham R. Functional electrical stimulation exercise increases glut-1 and glut-4 in paralyzed skeletal muscle. *Metabolism*. 1999;48:1409-1413
29. Jeon JY, Weiss CB, Steadward RD, Ryan E, Burnham RS, Bell G, Chilibeck P, Wheeler GD. Improved glucose tolerance and insulin sensitivity after electrical stimulation-assisted cycling in people with spinal cord injury. *Spinal Cord*. 2002;40:110-117
30. Glatz JF, Schaap FG, Binas B, Bonen A, van der Vusse GJ, Luiken JJ. Cytoplasmic fatty acid-binding protein facilitates fatty acid utilization by skeletal muscle. *Acta Physiol Scand*. 2003;178:367-371
31. Fluck M, Schmutz S, Wittwer M, Hoppeler H, Desplanches D. Transcriptional reprogramming during reloading of atrophied rat soleus muscle. *Am J Physiol Regul Integr Comp Physiol*. 2005;289:R4-14
32. Schmutz S, Dapp C, Wittwer M, Vogt M, Hoppeler H, Fluck M. Endurance training modulates the muscular transcriptome response to acute exercise. *Pflugers Arch*. 2006;451:678-687
33. Schmitt B, Fluck M, Decombaz J, Kreis R, Boesch C, Wittwer M, Graber F, Vogt M, Howald H, Hoppeler H. Transcriptional adaptations of lipid metabolism in tibialis anterior muscle of endurance-trained athletes. *Physiological genomics*. 2003;15:148-157
34. Parikh H, Nilsson E, Ling C, Poulsen P, Almgren P, Nittby H, Eriksson KF, Vaag A, Groop LC. Molecular correlates for maximal oxygen uptake and type 1 fibers. *Am J Physiol Endocrinol Metab*. 2008;294:E1152-1159
35. Kusudo T, Kontani Y, Kataoka N, Ando F, Shimokata H, Yamashita H. Fatty acid-binding protein 3 stimulates glucose uptake by facilitating as160 phosphorylation in mouse muscle cells. *Genes to cells : devoted to molecular & cellular mechanisms*. 2011;16:681-691
36. Simard C, Lacaille M, Vallieres J. Enzymatic adaptations to suspension hypokinesia in skeletal muscle of young and old rats. *Mech Ageing Dev*. 1985;33:1-9

37. Criswell D, Powers S, Dodd S, Lawler J, Edwards W, Renshler K, Grinton S. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Med Sci Sports Exerc*. 1993;25:1135-1140

38. Nakai N, Sato Y, Oshida Y, Fujitsuka N, Yoshimura A, Shimomura Y. Insulin activation of pyruvate dehydrogenase complex is enhanced by exercise training. *Metabolism*. 1999;48:865-869

39. Simoneau JA, Lortie G, Boulay MR, Marcotte M, Thibault MC, Bouchard C. Effects of two high-intensity intermittent training programs interspaced by detraining on human skeletal muscle and performance. *Eur J Appl Physiol Occup Physiol*. 1987;56:516-521

40. Indiveri C, Iacobazzi V, Tonazzi A, Giangregorio N, Infantino V, Convertini P, Console L, Palmieri F. The mitochondrial carnitine/acylcarnitine carrier: Function, structure and physiopathology. *Mol Aspects Med*. 2011;32:223-233

41. Peluso G, Petillo O, Margarucci S, Mingrone G, Greco AV, Indiveri C, Palmieri F, Melone MA, Reda E, Calvani M. Decreased mitochondrial carnitine translocase in skeletal muscles impairs utilization of fatty acids in insulin-resistant patients. *Frontiers in bioscience : a journal and virtual library*. 2002;7:a109-116

42. Baumer D, Lee S, Nicholson G, Davies JL, Parkinson NJ, Murray LM, Gillingwater TH, Ansorge O, Davies KE, Talbot K. Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. *PLoS Genet*. 2009;5:e1000773

43. Savkur RS, Philips AV, Cooper TA. Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. *Nature genetics*. 2001;29:40-47

44. Witten JT, Ule J. Understanding splicing regulation through rna splicing maps. *Trends Genet*. 2011;27:89-97

45. Nie L, Wu G, Culley DE, Scholten JC, Zhang W. Integrative analysis of transcriptomic and proteomic data: Challenges, solutions and applications. *Critical reviews in biotechnology*. 2007;27:63-75

46. Burnham R, Martin T, Stein R, Bell G, MacLean I, Steadward R. Skeletal muscle fibre type transformation following spinal cord injury. *Spinal Cord*. 1997;35:86-91

47. Elder CP, Apple DF, Bickel CS, Meyer RA, Dudley GA. Intramuscular fat and glucose tolerance after spinal cord injury--a cross-sectional study. *Spinal Cord*. 2004;42:711-716

48. Gorgey AS, Dudley GA. Skeletal muscle atrophy and increased intramuscular fat after incomplete spinal cord injury. *Spinal Cord*. 2007;45:304-309

Chapter 5a

Letter to the Editor
Does the HOMA-IR accurately determine insulin sensitivity after an exercise training program in subjects with the metabolic syndrome?

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Circulation. 2009: 119: e225

Abstract original article

Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome. A pilot study.

Tjønna AE, Lee SJ, Rognmo Ø, Stølen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slørdahl SA, Kemi OJ, Najjar S, Wisløff U.

Background Individuals with the metabolic syndrome are 3 times more likely to die of heart disease than healthy counterparts. Exercise training reduces several of the symptoms of the syndrome, but the exercise intensity that yields the maximal beneficial adaptations is in dispute. We compared moderate and high exercise intensity with regard to variables associated with cardiovascular function and prognosis in patients with the metabolic syndrome. **Methods and Results** Thirty-two metabolic syndrome patients (age, 52.3±3.7 years; maximal oxygen uptake [$V\dot{}O2max$], 34 mL · kg^{-1} · min^{-1}) were randomized to equal volumes of either moderate continuous moderate exercise (CME; 70% of highest measured heart rate [Hfmax]) or aerobic interval training (AIT; 90% of Hfmax) 3 times a week for 16 weeks or to a control group. $V\dot{}O2max$ increased more after AIT than CME (35% versus 16%; $P<0.01$) and was associated with removal of more risk factors that constitute the metabolic syndrome (number of factors: AIT, 5.9 before versus 4.0 after; $P<0.01$; CME, 5.7 before versus 5.0 after; group difference, $P<0.05$). AIT was superior to CME in enhancing endothelial function (9% versus 5%; $P<0.001$), insulin signaling in fat and skeletal muscle, skeletal muscle biogenesis, and excitation-contraction coupling and in reducing blood glucose and lipogenesis in adipose tissue. The 2 exercise programs were equally effective at lowering mean arterial blood pressure and reducing body weight (-2.3 and -3.6 kg in AIT and CME, respectively) and fat. **Conclusions** Exercise intensity was an important factor for improving aerobic capacity and reversing the risk factors of the metabolic syndrome. These findings may have important implications for exercise training in rehabilitation programs and future studies.

To the editor

We read with great interest the article by Tjønna et al[1]1 that addressed the efficacy of different modes of exercise training to reverse features of the metabolic syndrome. The metabolic syndrome, considered an ailment of the 20th century, consists of a clustering of risk factors, with insulin resistance still viewed as the common pathophysiological pathway.[2] We were intrigued by the fact that in patients with the metabolic syndrome, aerobic interval training substantially improved insulin sensitivity (homeostatic model assessment score increasing from 62.2±8.0% to 77.2±4.9%), whereas continuous moderate exercise seemed to decrease insulin sensitivity (64.4±5.7% versus 50.2±4.9% before and after training, respectively). It should be noted that the homeostatic model assessment score as a derivate index for insulin sensitivity has clear limitations.[3] The gold standard for measuring insulin sensitivity is the euglycemic hyperinsulinemic clamp, with the frequently sampled intravenous glucose tolerance test as the second best. Houmard et al[4] and Johnson et al[5] studied the effects of different aerobic training volumes and intensity on insulin sensitivity in 154 overweight/obese individuals and 171 individuals with the metabolic syndrome, respectively. Both studies demonstrated a clear improvement in insulin sensitivity shown by frequently sampled intravenous glucose tolerance test after continuous moderate exercise training. These studies[4, 5] provide clear evidence that training volume is more important than exercise intensity for improvement of insulin sensitivity. Although we are still excited about the efficacy of the type of aerobic interval training used by Tjønna et al[1] on the maximal oxygen uptake (exercise capacity) compared with continuous exercise, we think there is currently no conclusive evidence for a more favorable effect of aerobic interval training on insulin sensitivity in comparison to aerobic continuous exercise. Clearly, additional studies comparing the effect of different modes of exercise on insulin sensitivity measured by reliable methods are needed.

References

1. Tjonna AE, Lee SJ, Rognmo O, Stolen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slordahl SA, Kemi OJ, Najjar SM, Wisloff U. Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: A pilot study. *Circulation*. 2008;118:346-354
2. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet*. 2005;365:1415-1428
3. Wallace TM, Levy JC, Matthews DR. Use and abuse of homa modeling. *Diabetes Care*. 2004;27:1487-1495
4. Houmard JA, Tanner CJ, Slentz CA, Duscha BD, McCartney JS, Kraus WE. Effect of the volume and intensity of exercise training on insulin sensitivity. *J Appl Physiol*. 2004;96:101-106
5. Johnson JL, Slentz CA, Houmard JA, Samsa GP, Duscha BD, Aiken LB, McCartney JS, Tanner CJ, Kraus WE. Exercise training amount and intensity effects on metabolic syndrome (from studies of a targeted risk reduction intervention through defined exercise). *Am J Cardiol*. 2007;100:1759-1766

Chapter 5b

Effects of exercise training on insulin sensitivity and global gene expression profile of skeletal muscle in women with and without the metabolic syndrome

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Maria T.E. Hopman

Submitted

Abstract

Aims. Exercise training can effectively improve insulin sensitivity via several metabolic adaptations in skeletal muscle tissue but the responsible mechanism remains elusive. The aim of this study was to determine changes in insulin sensitivity with concomitant changes in the global expression profile of genes after exercise training in women with and without the metabolic syndrome. **Methods** Before and after six months of endurance exercise training, whole body insulin sensitivity (euglycaemic clamp) was determined and skeletal muscle biopsies were taken in 18 women with the metabolic syndrome and in 11 lean control women. Differentially expressed genes (Affymetrix GeneChip Human Gene 1.0 ST arrays) after exercise training were identified and subsequently pathways analyses was performed. **Results** Eleven women with the metabolic syndrome and ten control women completed the training program. The exercise training increased insulin sensitivity by 45% and 22% in women with the metabolic syndrome and in control women, respectively, independent of changes in body weight and/or composition ($p<0.05$). Pathway analyses of genes with a >1.3-fold change in women with the metabolic syndrome revealed four enriched pathways, in which insulin-like growth factor 2 (IGF2) showed the most significant upregulation. In lean women, pathway analysis revealed clustering of genes related to the immune system and inflammatory response. The most significantly upregulated gene in these pathways was CD2 molecule (CD2). **Conclusion** Exercise training improves insulin sensitivity in both groups of women, independent of changes in body composition. Different molecular mechanisms seem involved in the exercise-induced improvements in insulin sensitivity in women with and without the metabolic syndrome, which need further delineation.

Introduction

The metabolic syndrome is a complex of interrelated risk factors resulting in an increased risk for the development of diabetes[1] and cardiovascular diseases[2]. The pathophysiology of the metabolic syndrome can be characterized by peripheral insulin resistance, which may be caused by excessive central adiposity and/or physical inactivity. Exercise training can effectively counteract insulin resistance via several metabolic adaptations including changes in skeletal muscle tissue such as a fiber type transformation[3], an increase in the number of mitochondria[4, 5] and an increase in glucose transporter 4 (GLUT4) protein levels[6]. Several intracellular signaling molecules have been identified as key factors to mediate exercise-induced changes in skeletal muscle insulin sensitivity. These include AMP-activated protein kinase (AMPK), calcineurin, peroxisome-proliferator-activated Receptor-γ Coactivator 1α (PGC-1α), atypical protein kinase C (aPKC) and p38 mitogen activated protein kinase (p38 MAPK)[7]. Although the beneficial effects of exercise on insulin resistance are well established, the exact molecular mechanisms by which exercise promotes these beneficial effects are not fully understood.

Gene expression microarrays provide the opportunity to analyse the expression level of virtually all known genes in a single experiment, and are therefore a rapid and comprehensive approach for the first identification of exercise-induced molecular changes. Previous studies have assessed mRNA gene expression after exercise training in insulin sensitive subjects[8] and after a few weeks of exercise training in young healthy men[9]. No previous microarray studies however, have been performed in insulin resistant women with the metabolic syndrome who are prone to develop diabetes in the nearby future[1]. Especially these women will benefit from exercise training and may show large improvement in insulin resistance.

Therefore, the aim of this study was to determine exercise-induced improvement in insulin sensitivity and concomitant changes in the global expression of genes after six month, three times a week of endurance exercise training in insulin resistant skeletal muscle tissue of women with the metabolic syndrome and in lean control women. Identification of key players may increase our understanding of the molecular mechanisms by which long-term endurance exercise induces improvement in insulin sensitivity.

Methods

Participants

Eighteen women with the metabolic syndrome and eleven lean healthy control women participated in this study. Metabolic syndrome was defined as having at least three out of five criteria as defined in the Joint Scientific Statement for Harmonizing the Metabolic Syndrome[10], including waist circumference ≥88 cm, triglycerides >1.7 mmol/l, HDL-cholesterol <1.3 mmol/l, hypertension >130/85 mmHg (systolic/diastolic blood pressure) and/or use of antihypertensive medication, and fasting glucose >6.1 mmol/l. Lean women were defined as having a body mass index (BMI) < 25 kg/m^2 and the absence of all metabolic syndrome criteria. We excluded women with a medical history of known diabetes and/or cardiovascular diseases, liver or renal diseases, smoking, more than two alcohol consumptions (10 g) a day, and performing regular physical activity >2 times a week. Before participation, a written informed consent was obtained. This study was approved by the Medical Ethical Committee of the Radboud University Nijmegen Medical Centre, and was conducted in accordance with the Declaration of Helsinki (2000).

Exercise intervention

Women trained for six months, three times a week under the supervision of a professional trainer with preferably one day between subsequent exercise bouts. Training consisted of cycling exercise on a ergometer (Lode, Groningen, the Netherlands) starting with a 10 minute warming-up, followed by 30 minutes of exercise at 65% of the individual heart rate reserve (HRR) and finished off with a cooling-down of 5 minutes. As their exercise tolerance improved, the intensity of the training was increased by 5% HRR to a maximum of 85% of the HRR. Exercise intensity was documented through the use of heart rate monitors (Polar). Women had to attend at least 90% of the training sessions during this six month period to be eligible for inclusion of the statistical analysis.

Cardio-respiratory fitness test

Women performed a maximal exercise test on an electrically braked leg-cycling ergo meter (Lode, Angio 300, Groningen, the Netherlands) using an incremental protocol to assess their cardio-respiratory fitness. Workload increased

Table 1. Physiological characteristics before and after the exercise intervention.

	Women with the metabolic syndrome			Healthy control women		
	Pre	Post	P value	Pre	Post	P value
Age, years	53 ± 7			49 ± 10		
Body composition						
Weight, kg	96.4 ± 11.3	94.8 ± 9.6	0.136	67.0 ± 6.6	66.9 ± 6.2	0.909
Body mass index, kg/m^2	34.5 ± 3.2	34.0 ± 2.9	0.150	22.8 ± 1.7	22.7 ± 1.5	0.901
Waist, cm	109 ± 9	105 ± 8	0.119	80 ± 6	79 ± 5	0.072
Waist-to-hip ratio	0.91 ± 0.08	0.90 ± 0.07	0.757	0.80 ± 0.05	0.78 ± 0.04	0.115
Body fat, %	40.6 ± 4.4	40.3 ± 4.2	0.505	28.8 ± 4.7	28.1 ± 4.2	0.433
Trunk fat, %	41.2 ± 4.6	41.4 ± 3.9	0.841	26.0 ± 6.9	25.3 ± 5.9	0.445
Blood variables						
Fasting glucose, mmol/l	5.5 ± 0.6	5.7 ± 0.8	0.356	4.5 ± 0.3	4.7 ± 0.4	0.138
Insulin, mE/l	17.4 ± 10.4	15.4 ± 5.2	0.428	8.9 ± 3.0	10.0 ± 3.8	0.076
M-value, mg/min/kg	4.0 ± 1.8	5.8 ± 0.9	<0.001	8.1 ± 1.9	9.8 ± 2.0	0.001
HDL-cholesterol, mmol/l	1.21 ± 0.30	1.28 ± 0.29	0.106	1.59 ± 0.29	1.58 ± 0.19	0.831
Triglycerides, mmol/l	1.98 ± 0.84	1.83 ± 0.66	0.338	0.87 ± 0.27	0.93 ± 0.22	0.316
Free fatty acids, mmol/l	0.58 ± 0.15	0.45 ± 0.18	0.008	0.52 ± 0.16	0.43 ± 0.23	0.229
Blood pressure, mmHg						
Systolic blood pressure	138 ± 11	132 ± 11	0.102	120 ± 9	114 ± 9	0.089
Diastolic blood pressure	84 ± 5	80 ± 7	0.195	76 ± 5	73 ± 6	0.021
Resting heart rate, bpm	68 ± 5	59 ± 7	0.006	60 ± 7	58 ± 5	0.260
Exercise parameters						
VO_2 max, ml/min/kg	22.8 ± 4.5	25.3 ± 3.8	0.003	32.0 ± 4.7	35.6 ± 5.5	0.002
Power, Watt	158 ± 28	185 ± 23	<0.001	180 ± 21	205 ± 27	0.011

by 10 W per minute, starting at 10 W, until exhaustion. A gas-analyzer was used to measure oxygen consumption continuously (Jaeger Benelux BV, Breda, the Netherlands). Maximal oxygen consumption (VO_{2max}) was analyzed as the mean of the last minute of the exercise bout. During the test, heart rate was measured continuously. Two minutes after cessation of the test, blood lactate level (Roche Diagnostics GmbH, Mannheim, Germany) was measured.

Clinical examinations and insulin sensitivity

Before and after 6 months of endurance exercise training height, weight, waist circumference, waist-to-hip ratio, blood pressure, resting heart rate, plasma lipids, free fatty acids, and glucose were measured by standard methods. A total body DXA scan determined body fat percentage and body fat distribution (QDR 4500 densitometer, Hologic Inc. Waltham, MA).

Peripheral tissue sensitivity to exogenous insulin was measured using a hyperinsulinaemic euglycaemic clamp as previously described [11, 12]. After an overnight fast (10-h), with the patient supine in a quiet, temperature controlled room (22 – 24 ^{0}C), insulin (Actrapid, Novo-Nordisk, Copenhagen, Denmark) was infused intravenously in a dose of 430 pmol · m^{-2} · min^{-1} (60 mU · m^{-2} · min^{-1}) for 120 minutes. Insulin 50 U · ml^{-1} was diluted in 48 ml NaCl 0.9% with the addition of 2 ml blood from the subject to a concentration of 1 U·ml^{-1}. Venous plasma glucose concentrations were clamped at 5.0 mmol·l^{-1} by a variable glucose 20% infusion rate, adjusted depending on venous plasma glucose level measured at 5-minute intervals. Venous plasma glucose was measured in duplicate, in samples that were immediately centrifuged during 10 seconds, by the glucose oxidation method (Beckman Glucose Analyzer 2, Beckman Instruments Inc, Fullerton, CA 92634, USA). Insulin was measured in duplicate against International Standard 83/500 by an in-house radio-immunoassay (RIA) using an anti-human insulin antiserum raised in guinea pig and radio-iodinated human insulin as a tracer. Bound/free separation was carried out by addition of sheep anti-guinea-pig antiserum and precipitation by means of polyethylene glycol (PEG). Between- and within-run coefficients of variation were 4.6% and 5.8% respectively, at a level of 33mU·L^{-1}. Whole body glucose disposal during the last 30-min of the euglycaemic clamp was calculated as the M-value.

RNA gene expression

Muscle biopsies from the vastus lateralis muscle were taken after a standardized 250 kcal breakfast (79 % carbohydrates, 11.2 % protein, 9.8 % fat).

From muscle biopsies of 6 women of each group (being a representative sample from the total) total RNA was isolated and purified. The characteristics of the array 'cohort' did not differ from the whole group (Table S2). RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The 260/280 ratio indicated 'pure RNA' quality. RNA integrity was analyzed on an Agilent Bioanalyser (Santa Clara, CA, USA) and the average RNA integrity number for all muscle samples was 7.8. RNA gene expression profiling was performed using Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix Inc., Santa Clara, CA, USA), according to the manufacturer's instruction. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to generate amplified and biotinylated sense-strands DNA targets from the entire expressed genome (100 ng of total RNA). The manufacturer's manual was followed for the hybridization, washing, and scanning steps (version 4, P/N 701880 Rev.4). Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 ^{0}C for 17 h. After hybridization, the arrays were washed in the Affymetrix GeneChip Fluidics station FS 450. Arrays were scanned using the GeneChip scanner 3000 7G system. The average fluorescence intensity of all genes was calculated using the Robust Multiarray Analysis (RMA) Algorithm[13], including a quantile normalization and using a background correction for GC-content. To check overall data quality, the positive (exonic) and negative (intronic) control probe sets were used to calculate the area under the curve which ranged from 0.83 to 0.85, indicating good quality samples. The protocol used was compliant with the MIAMI guidelines, and data have been submitted to the Gene Expression Omnibus (GEO) repository under no. GSE43760.

Statistical analysis

Effects of the exercise intervention on physiological characteristics in women with the metabolic syndrome and in lean control women were assessed by a paired Student's *t* tests (Statistical Package for Social Sciences 18.0, SPSS Inc., Chicago, Illinois, USA). Correlations were assessed by a Pearson correlation coefficient. The level of statistical significance was defined at α=0.05 and data are presented as mean ± SD, unless stated otherwise.

The Affymetrix CEL-files were imported into Partek® (Genomic Suite Software version 6.4, Partek Inc., St Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA logarithm with GC background correction. The distribution of the intensity values on the individual arrays was visualized in a signal histogram and one outlier in the group of women with the metabolic syndrome was detected. This outlier was removed from further data

analysis. Transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative gene expression levels. To identify differentially expressed genes after exercise training, the corrected intensity of individual genes were used for an one-way analysis of variance (ANOVA). The resulting p-values were corrected for multiple-testing with the Benjamini-Hochberg false discovery rate procedure[14]. The p-value together with the fold change of every annotated gene, were exported to Microsoft Office Excel. The gene list of interest (>1.3-fold change) was analysed using the Database for Annotation Visualization and Integrated Discovery functional annotation tool (DAVID, http:david.abcc.ncifcrf.gov)) with main focus on the enrichment in GO-terms (Gene Ontology terms) of biological processes (GOTERM_BP_FAT)[15].

Results

Effectiveness of the training intervention

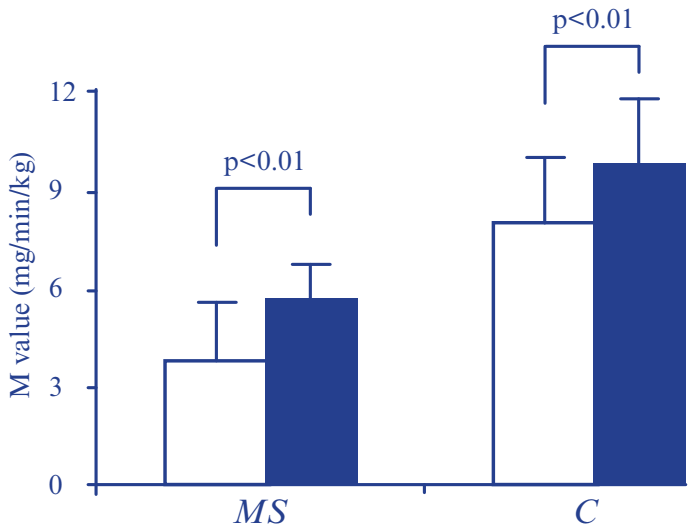
Eleven women with the metabolic syndrome and ten healthy control women successfully completed the exercise intervention, with a training compliance of 92%. Cardio-respiratory fitness, determined as maximal oxygen uptake, improved significantly by 11% in both exercise groups (Table 1). Seven women with the metabolic syndrome and one healthy control woman dropped out of the study; five due to medical reasons unrelated to the exercise intervention, one woman dropped out as a consequence of social/family circumstances, and two women (one control woman) dropped out due to lack of motivation. There were no significant differences at baseline between women who successfully completed the study and those who dropped out of the study (Table S1).

Exercise-induced changes in physiological characteristics

Six months of endurance exercise training significantly improved insulin sensitivity (M-value) by 45% in women with the metabolic syndrome and by 22% in lean control women (Figure 1). After 6 months of exercise cycling training, no significant changes were observed in the variables of the metabolic syndrome; waist circumference, diastolic or systolic blood pressure, glucose, HDL-cholesterol and triglycerides for the whole group (Table 1). Nonetheless, three out of eleven women with the metabolic syndrome (27%) did not meet the criteria for the metabolic syndrome anymore after the exercise intervention (Table 1). The cycling training

did not alter body composition including body weight, total body fat, body mass index, waist circumference, or waist-to-hip ratio. In women with the metabolic syndrome, resting heart rate and free fatty acid plasma levels decreased significantly (Table 1). In lean control women diastolic blood pressure decreased after the training intervention (Table 1).

Figure 1.



Insulin sensitivity (M-value) before (white bars) en after (blue bars) six months of endurance exercise training in women with the metabolic syndrome (MS) and in lean control women (C).

Exercise-induced changes in skeletal muscle gene expression

Skeletal muscle gene expression levels were compared before and after the exercise intervention in five women with the metabolic syndrome and in six lean control women. The physiological characteristics of these subgroups were representative for the whole group and showed similar statistical significant results after the exercise intervention (Table S2). In both groups, of the 28,870 genes assessed on the microarray, no single gene reached statistical significance after stringent correction for multiple testing. Therefore, we focused on biological processes and pathways that were overrepresented in the list of genes with the highest fold change.

All genes with >1.3-fold change were selected for further analysis, resulting in a list of 139 upregulated and 193 downregulated genes in women with the metabolic syndrome. In these women, the list of upregulated genes was significantly enriched for 4 gene ontology (GO) terms as depicted in Table 2. These 4 GO terms consisted of 24 unique genes of which insulin-like growth factor 2 (IGF2) showed the highest significance (Table 3).

Table 2. Enrichment of biological process gene ontology (GO) terms in the list of genes with > 1.3-fold upregulation after 6 months of exercise training in women with the metabolic syndrome and in lean control women. F.E. = Fold Enrichment.

Gene Ontology term	F.E.	Bonferroni
Women with the metabolic syndrome		
GO:0010033 response to organic substance	5.4	0.0001
GO:0051789 response to protein stimulus	15.8	0.0046
GO:0007610 behavior	5.7	0.0167
GO:0009611 response to wounding	5.0	0.0469
Lean control women		
GO:0006952 defense response	4.8	<0.001
GO:0006955 immune response	3.7	0.001
GO:0002274 myeloid leukocyte activation	19.6	0.002
GO:0001775 cell activation	5.8	0.003
GO:0045321 leukocyte activation	6.4	0.003
GO:0050900 leukocyte migration	15.8	0.006
GO:0006954 inflammatory response	4.8	0.049

In lean control women, the gene list of interest (>1.3-fold change) consisted of 261 upregulated and 58 downregulated genes. Apparently, exercise training induces more transcriptional upregulation than downregulation in lean control women. The list of upregulated genes were significantly enriched for GO terms related to the immune system (Table 2). These seven immune system related GO terms consisted of 38 unique genes as listed in Table 4 of which CD2 molecule (CD2) showed the most significant change. In both groups of women, the downregulated genes were not significantly enriched for GO-terms. To visualize a possible link of the identified genes with insulin sensitivity, we correlated the microarray fluorescence intensity with the change in M-value, which did not result in significant correlations.

Table 3. The 24 upregulated genes responsible for the significantly enriched GO terms after exercise training in women with the metabolic syndrome, listed according to significance.

Gene symbol	Gene name	P value	Fold change
IGF2	insulin-like growth factor 2 (somatomedin A)	0.01	1.4
HSPD1	heat shock 60kDa protein 1 (chaperonin)	0.03	1.3
COL1A1	collagen, type I, alpha 1	0.05	1.3
CXCL10	chemokine (C-X-C motif) ligand 10	0.06	1.4
EGR1	early growth response 1	0.09	2.9
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	0.21	1.7
OSMR	oncostatin M receptor	0.21	1.3
CTGF	connective tissue growth factor	0.22	1.4
JUNB	jun B proto-oncogene	0.22	1.5
NR4A2	nuclear receptor subfamily 4, group A, member 2	0.26	1.7
THBD	thrombomodulin	0.26	1.4
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.26	1.5
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0.32	2.4
CXCL2	chemokine (C-X-C motif) ligand 2	0.35	1.3
SERPINE1	serpin peptidase inhibitor, clade E, member 1	0.36	1.4
CCL2	chemokine (C-C motif) ligand 2	0.40	1.7
CYR61	cysteine-rich, angiogenic inducer, 61	0.42	1.7
HSPA6	heat shock 70kDa protein 7 (HSP70B)	0.43	1.6
EGR2	early growth response 2	0.47	1.3
TNC	tenascin C	0.49	1.5
IL18	interleukin 18 (interferon-gamma-inducing factor)	0.53	1.3
HBEGF	heparin-binding EGF-like growth factor	0.58	1.5
THBS1	thrombospondin 1	0.58	1.6
NR4A3	nuclear receptor subfamily 4, group A, member 3	0.67	1.4

Table 4. The 38 upregulated genes responsible for the significantly enriched GO terms after exercise training in lean control women, listed according to significance.

Gene symbol	Gene name	P value	Fold change
CD2	CD2 molecule	<0.01	1.3
ALOX5	arachidonate 5-lipoxygenase	0.01	1.4
PTPRC	protein tyrosine phosphatase, receptor type, C	0.01	1.8
TLR6	toll-like receptor 6	0.01	1.3
CD48	CD48 molecule	0.02	1.3
LCP1	lymphocyte cytosolic protein 1 (L-plastin)	0.02	1.7
SELPLG	selectin P ligand	0.02	1.3
ITGA6	integrin, alpha 6	0.02	1.3
DOCK2	dedicator of cytokinesis 2	0.02	1.4
IL7R	interleukin 7 receptor	0.02	1.7
ITK	IL2-inducible T-cell kinase	0.02	1.3
LCP2	lymphocyte cytosolic protein 2	0.03	1.3
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	0.03	2.1
CD96	CD96 molecule	0.03	1.3
CXCR1	interleukin 8 receptor, alpha	0.03	1.5
CCL5	chemokine (C-C motif) ligand 5	0.04	1.4
IL2RG	interleukin 2 receptor, gamma	0.04	1.4
S100A8	S100 calcium binding protein A8	0.04	1.8
LYZ	lysozyme (renal amyloidosis)	0.04	1.5
MYO1F	myosin IF	0.05	1.3
KLRK1	killer cell lectin-like receptor subfamily C, member 4	0.05	1.4
PXDN	peroxidasin homolog (Drosophila)	0.05	1.4
CORO1A	coronin, actin binding protein, 1A	0.06	1.4
CSF3R	colony stimulating factor 3 receptor (granulocyte)	0.06	1.4
MNDA	myeloid cell nuclear differentiation antigen	0.07	1.5
AQP9	aquaporin 9	0.07	1.8
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	0.08	1.7
FYB	FYN binding protein (FYB-120/130)	0.08	1.5
NCF2	neutrophil cytosolic factor 2	0.08	1.4
CXCR2	interleukin 8 receptor, beta	0.08	1.5
PPBP	pro-platelet basic protein (ligand 7)	0.10	1.6
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	0.11	1.3
S100A9	S100 calcium binding protein A9	0.11	1.3
IGSF6	immunoglobulin superfamily, member 6	0.13	1.3

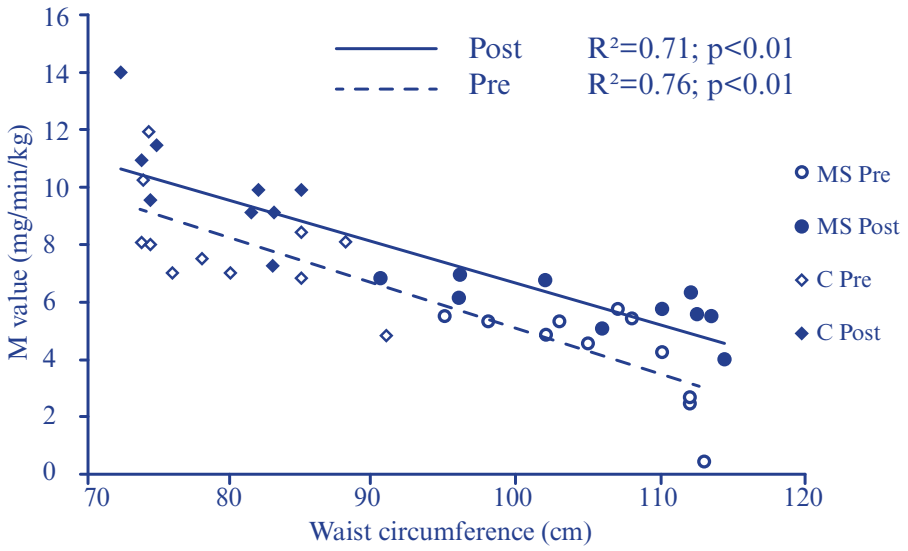
SERPINA1	serpin peptidase inhibitor, clade A, member 1	0.17	1.4
COL3A1	collagen, type III, alpha 1	0.20	1.3
S100A12	S100 calcium binding protein A12	0.22	1.3
C7	complement component 7	0.45	1.4

Discussion

The main finding of this study is that six months of endurance exercise training significantly improves insulin sensitivity in both women with the metabolic syndrome as well as in lean women, independent of changes in body weight and/or composition. Pathway analysis revealed four enriched pathways in women with the metabolic syndrome, in which insulin-like growth factor 2 (IGF2) showed the most significant upregulation. In lean women, expression of genes related to the immune system and inflammatory response is upregulated, with CD2 molecule (CD2) showing the most significant upregulation. These results indicate that different molecular mechanisms seem involved in exercise-induced improvements in insulin sensitivity in women with and without the metabolic syndrome.

Six months of endurance exercise training significantly improved physical fitness in both women with the metabolic syndrome and in lean control women, indicating the successful performance of the exercise training program. But more important, the exercise training program greatly improved insulin sensitivity in both groups. These results are in agreement with previous studies in diabetic patients[16, 17], obese women with impaired glucose tolerance[18] and in lean women[18]. Although exercise training significantly improved insulin sensitivity in both groups, the beneficial effects were relatively larger in insulin resistant subjects with the metabolic syndrome compared to the more insulin sensitive lean subjects. The extent of improvement in insulin resistance after the training program is large compared to the effect of glucose lowering medication, such as metformin[19], emphasizing the beneficial effects of exercise. As noted by previous studies, waist circumference has a strong inverse correlation with whole-body insulin sensitivity[20]. Our results however, show that exercise training can improve insulin sensitivity, even independent of changes in body weight and/or composition including waist circumference (as depicted in Figure 2) and trunk fat mass. This was also observed by Duncan et al. who, in middle-aged sedentary adults, noted an 40% increase in insulin sensitivity independent of any change in BMI or waist circumference after a moderate-intensity walking intervention[21].

Figure 2.



Correlation of waist circumference with M-value before (dotted line) and after (blue line) 6 months of endurance exercise training in women with the metabolic syndrome and in control women.

The effects of endurance exercise training on skeletal muscle insulin sensitivity were further investigated using gene expression microarrays. After stringent correction for multiple testing, no differentially expressed genes were seen after the 6 months of exercise training, despite the large observed improvements in insulin sensitivity. Focusing on genes with >1.3-fold change revealed in women with the metabolic syndrome four GO-terms, consisting of 24 unique genes. The most significant upregulated gene was insulin-like growth factor 2 (IGF2). IGF2 is a key factor in human growth and development and shares structural similarity to insulin. Only few studies have examined associations between gene expression of IGF2 in obesity and type 2 diabetes. These studies show that subjects who are prenatally exposed to the Dutch famine have lower methylation of IGF2 in blood and have an increased prevalence of obesity, insulin resistance and type 2 diabetes in later life[22, 23]. Chen *et al*., confirmed the lower methylation of IGF2 in muscle tissue of patients with type 2 diabetes[24]. Our results indicate that exercise training can upregulate IGF2 expression in skeletal muscle tissue of insulin resistant women. It will thus be interesting to further investigate the possible role of IGF2 in exercise-induced improvements is insulin resistance.

In lean control women, those genes with >1.3-fold change after the exercise training were overrepresented in GO-terms related to the immune system and

inflammatory response. The most significant upregulated gene in lean women was CD2, which is an adhesion molecule found on the surface of T cells and natural killer (NK) cells. Exercise training is known to induce changes in the immune system and function[25]. Acute exercise induces a stress response with a rapid and profound neutrophilia, an increase in circulating NK cells, inflammatory cytokines and monocytosis[26]. Long-term exercise training however, specifically in subjects with chronic low-grade inflammation such as the metabolic syndrome, is known to have anti-inflammatory and insulin-sensitizing effects[27]. Our results of an upregulation of genes related to the immune system and inflammatory response in lean women need further investigation. Despite similar exercise duration and intensity compared to women with the metabolic syndrome, lean women may have experienced post-exercise immune dysfunction due to the continuous, prolonged and high intensity exercise training. This may have induced elevated circulating stress hormones and alterations in the pro/anti-inflammatory cytokine balance.

Limitations

Unfortunately ~40 % of the subjects with the metabolic syndrome were not able to complete the 6-month training period. Only one of these women stopped due to a lack of motivation despite repeated encouragement. The other 6 women with the metabolic syndrome could not complete the training program due to medical reasons, none of which were related to the participation in our study.

Gene expression microarrays are a powerful tool to assess the expression level of many genes at once, but do not provide information on the cell type distribution in the biopsy. From this study it is not possible to conclude whether one specific cell type (e.g. leukocytes or myocytes) was responsible for the increased expression of immune and inflammatory genes, or that more cells of a specific type (e.g. natural killer cells) infiltrated the muscle tissue.

Conclusion

In conclusion, six months of endurance exercise training improves insulin sensitivity in women with and without the metabolic syndrome, independent of changes in body weight and/or composition. Different molecular mechanisms seem involved in these exercise-induced improvements in insulin sensitivity. We identified upregulation of IGF2 expression in women with the metabolic syndrome and found that expression of genes in lean women were overrepresented for immune system and inflammatory response genes. Further delineation of these genes and exercise

intervention studies with multiple time-course assessments of gene expression and protein levels should be the next step.

Acknowledgements

FP is funded by ZonMW grant number 92003409, GL is funded by the project Gelderland-Overijssel-EFRO no 2009-010034.

References

1. Ford ES, Li C, Sattar N. Metabolic syndrome and incident diabetes: Current state of the evidence. *Diabetes Care*. 2008;31:1898-1904
2. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in united states adults. *Circulation*. 2004;110:1245-1250
3. Pette D, Staron RS. Transitions of muscle fiber phenotypic profiles. *Histochem Cell Biol*. 2001;115:359-372
4. Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci*. 2006;61:534-540
5. Hoppeler H, Howald H, Conley K, Lindstedt SL, Claassen H, Vock P, Weibel ER. Endurance training in humans: Aerobic capacity and structure of skeletal muscle. *J Appl Physiol*. 1985;59:320-327
6. Christ-Roberts CY, Pratipanawatr T, Pratipanawatr W, Berria R, Belfort R, Kashyap S, Mandarino LJ. Exercise training increases glycogen synthase activity and glut4 expression but not insulin signaling in overweight nondiabetic and type 2 diabetic subjects. *Metabolism*. 2004;53:1233-1242
7. Frosig C, Richter EA. Improved insulin sensitivity after exercise: Focus on insulin signaling. *Obesity (Silver Spring, Md*. 2009;17 Suppl 3:S15-20
8. Teran-Garcia M, Rankinen T, Koza RA, Rao DC, Bouchard C. Endurance training-induced changes in insulin sensitivity and gene expression. *Am J Physiol Endocrinol Metab*. 2005;288:E1168-1178
9. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mrna expression in human skeletal muscle during recovery from endurance exercise. *Faseb J*. 2005;19:1498-1500
10. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. *Circulation*. 2009;120:1640-1645
11. Tack CJ, Ong MK, Lutterman JA, Smits P. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. *Diabetologia*. 1998;41:569-576
12. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: A method for quantifying insulin secretion and resistance. *Am J Physiol*. 1979;237:E214-223
13. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)*. 2003;4:249-264
14. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behavioural brain research*. 2001;125:279-284
15. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nature protocols*. 2009;4:44-57
16. Glans F, Eriksson KF, Segerstrom A, Thorsson O, Wollmer P, Groop L. Evaluation of the effects of exercise on insulin sensitivity in arabian and swedish women with type 2 diabetes. *Diabetes Res Clin Pract*. 2009;85:69-74
17. Segerstrom AB, Glans F, Eriksson KF, Holmback AM, Groop L, Thorsson O, Wollmer P. Impact of exercise intensity and duration on insulin sensitivity in women with t2d. *European journal of internal medicine*. 2010;21:404-408

18. Straczkowski M, Kowalska I, Dzienis-Straczkowska S, Stepien A, Skibinska E, Szelachowska M, Kinalska I. Changes in tumor necrosis factor-alpha system and insulin sensitivity during an exercise training program in obese women with normal and impaired glucose tolerance. *European journal of endocrinology / European Federation of Endocrine Societies*. 2001;145:273-280
19. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346:393-403
20. Wahrenberg H, Hertel K, Leijonhufvud BM, Persson LG, Toft E, Arner P. Use of waist circumference to predict insulin resistance: Retrospective study. *Bmj*. 2005;330:1363-1364
21. Duncan GE, Perri MG, Theriaque DW, Hutson AD, Eckel RH, Stacpoole PW. Exercise training, without weight loss, increases insulin sensitivity and postheparin plasma lipase activity in previously sedentary adults. *Diabetes Care*. 2003;26:557-562
22. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A*. 2008;105:17046-17049
23. Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, Bleker OP. Glucose tolerance in adults after prenatal exposure to famine. *Lancet*. 1998;351:173-177
24. Chen M, Macpherson A, Owens J, Wittert G, Heilbronn LK. Obesity alone or with type 2 diabetes is associated with tissue specific alterations in DNA methylation and gene expression of ppargc1a and igf2. *Journal of Diabetes Research and Clinical Metabolism*. 2012;1
25. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, Simon P. Position statement. Part one: Immune function and exercise. *Exercise immunology review*. 2011;17:6-63
26. Gleeson M, Bishop NC. The t cell and nk cell immune response to exercise. *Annals of transplantation : quarterly of the Polish Transplantation Society*. 2005;10:43-48
27. Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, Fallucca S, Alessi E, Letizia C, Jimenez A, Fallucca F, Pugliese G. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr Metab Cardiovasc Dis*. 2010;20:608-617

Supplement 1. No difference in physical characteristics of women with the metabolic syndrome who successfully completed the study and those who dropped out of the study.

	Metabolic syndrome	
	Successful women (n=11)	Drop outs (n=6)
Age, yrs	53 ± 7	50 ± 7
Weight, kg	96.4 ± 11.3	92.4 ± 18.4
BMI, kg/m^2	34.5 ± 3.2	34.6 ± 5.2
Waist circumference, cm	109 ± 9	109 ± 11
Systolic pressure, mmHg	138 ± 11	138 ± 12
Diastolic pressure, mmHg	84 ± 5	85 ± 8
Heart Rate $_{rest}$, bpm	68 ± 5	73 ± 15
Glucose, mmol/l	5.5 ± 0.6	5.4 ±0.8
Triglycerides, mmol/l	1.98 ± 0.84	1.99 ± 0.79
HDL-C, mmol/l	1.21 ± 0.30	1.18 ±0.21

Values are mean ± SD.

Supplement 2. Physical characteristics and M-value of all subjects versus the subjects in the array cohorts.

	Metabolic syndrome		Controls	
	All subjects (n=11)	Array cohort (n=5)	All subjects (n=10)	Array cohort (n=6)
Age, yrs	53 ± 7	58 ± 5	49 ± 10	50 ± 9
Weight, kg	96.4 ± 11.3	105.8 ± 7.1	67.0 ± 6.6	64.5 ± 6.0
BMI, kg/m^2	34.5 ± 3.2	36.7 ± 2.0	22.8 ± 1.7	22.2 ± 1.0
Waist circumference, cm	109 ± 9	115 ± 9	80 ± 6	78 ± 4
Waist-to-Hip Ratio, -	0.91 ± 0.08	0.95 ± 0.08	0.80 ± 0.05	0.78 ± 0.04
Systolic pressure, mmHg	138 ± 11	143 ± 13	120 ± 9	117 ± 8
Diastolic pressure, mmHg	84 ± 5	83 ± 5	76 ± 5	75 ± 5
Heart Rate $_{rest}$, bpm	68 ± 5	68 ± 4	60 ± 7	59 ± 5
Glucose, mmol/l	5.5 ± 0.6	6.0 ± 0.2	4.5 ± 0.3	4.5 ± 0.2
Triglycerides, mmol/l	1.98 ± 0.84	1.83 ± 0.89	0.87 ± 0.27	0.98 ± 0.23
HDL-C, mmol/l	1.21 ± 0.30	1.28 ± 0.40	1.59 ± 0.29	1.53 ± 0.32
M-value, mg/min/kg	4.0 ± 1.8	3.1 ± 2.0	8.1 ± 1.9	8.6 ± 2.1

Values are mean ± SD.

Chapter 6

Identification of genetic pathways involved in vascular adaptations after exercise training in women with the metabolic syndrome

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Abstract

Women with the metabolic syndrome are prone to develop atherosclerosis and subsequently cardiovascular diseases (CVD). Exercise training reduces the CVD risk via vascular remodeling including adaptations of arterial wall thickness. However, whether this happens similarly in central/peripheral arteries and the molecular mechanisms underlying these adaptations are currently unknown. Therefore, the aim of this study was to assess the effect of 6-month exercise training on vascular wall thickness and diameter (using ultrasound) of central (i.e. carotid artery (CA)) and peripheral arteries (i.e. brachial (BA), common femoral (CFA) and superficial femoral artery (SFA)), and concomitant changes in gene expression levels (microarray; muscle biopsy) in eighteen women with the metabolic syndrome. Eleven women completed the training program. Exercise training induced peripheral vascular adaptations including an increase in BA and SFA diameter, and a decrease in CFA intima-media wall thickness/lumen ratio (all $P<0.05$). No adaptations were observed in the central CA diameter or wall thickness. Regarding gene expression levels, groups of genes related to vascular remodeling (GO term: vasculature development, blood vessel development, blood vessel morphogenesis, and angiogenesis) were identified, including enhanced gene expression of delta like protein 4 (DLL4) and collagen type 1 alpha 1 (COL1A1). Taken together, exercise training in women with the metabolic syndrome leads to vascular remodeling in peripheral, and not central, arteries through upregulation of vascular-remodeling pathways, with a dominance for DLL4 and COL1A1.

Introduction

Approximately 25% of the adult population in Western countries fulfills the criteria of the metabolic syndrome[1] which represents a cluster of cardiovascular risk factors.[2] Subjects with the metabolic syndrome are prone to develop atherosclerosis[3], evident through gradual thickening of the arterial wall[4] which finally results in the development of cardiovascular disease (CVD).[5] Exercise training is a powerful tool to decrease CVD risk by improving traditional cardiovascular risk factors[6], but also by inducing arterial adaptations and hence reversing the atherosclerotic process.[7] Assessment of intima-media thickness (IMT) is a validated surrogate marker for atherosclerosis[8] and an increased carotid IMT has been associated with increased risk for both cardio/cerebro-vascular[9] and peripheral vascular events.[10] Exercise training is associated with a smaller arterial wall thickness.[11] However, relatively little is known whether this occurs in both central and/or peripheral arteries. A recent study found a decrease in carotid and femoral artery wall thickness after exercise training in healthy young subjects.[12] However, longitudinal[13] and cross-sectional[14] data in subjects with cardiovascular risk/disease suggest that remodeling of the arterial wall primarily takes place in peripheral, but not in the central arteries (i.e. carotid artery). Whether exercise training in women with the metabolic syndrome leads to systemic or localized adaptations in wall thickness is currently unknown.

The molecular mechanisms associated with exercise-induced vascular remodeling, such as changes in wall thickness and diameter, are gradually elucidated.[15] Gene expression microarrays are a powerful tool to comprehensively study these molecular mechanisms. So far, microarrays have been extensively used when studying the impact of exercise bouts and short-term exercise training programs, predominantly in healthy men and focused on the muscular pathway.[16, 17] Only one previous study in men linked microarrays with vascular adaptations after physical inactivity and exercise training.[18] They identified groups of genes including the vascular endothelial growth factor pathway, transforming growth factor β1 and extracellular matrix proteins which showed associations with vascular adaptations.

The aim of this study was to assess the effects of 6 months exercise training on vascular diameter and wall thickness of central (i.e. carotis artery (CA)) and peripheral arteries (i.e. brachial (BA), common femoral (CFA) and superficial femoral artery (SFA)), and concomitant changes in gene expression levels in women with the metabolic syndrome. We hypothesized to find changes in peripheral but not in central arterial structure, accompanied by changes in gene expression level of vascular-related genes such as growth factors and extra-cellular matrix components. These results will enable us to identify key genes that play a role in vascular adaptations after exercise training in women with multiple cardiovascular risk factors i.e. the metabolic syndrome.

Methods

Subjects

Eighteen women with the metabolic syndrome participated in this study. Metabolic syndrome was defined as having at least three out of five criteria including waist circumference >88 cm, triglycerides >1.7 mmol/l, HDL-cholesterol <1.3 mmol/l, hypertension >130/85 mmHg (systolic/diastolic blood pressure) and/or use of antihypertensive medication, and fasting glucose >5.5 mmol/l.2 We excluded subjects with a medical history of known diabetes and/or cardiovascular diseases, liver or renal diseases, smoking, more than two alcohol consumptions a day, and performing regular physical activity >2 times a week. Before participation, a written informed consent was obtained. This study was approved by the Medical Ethical Committee of the Radboud University Nijmegen Medical Centre, and was conducted in accordance with the Declaration of Helsinki (2000).

Table 1. Subject characteristics of women with the metabolic syndrome (n=11) before and after training.

	Pre	Post	p-value
Age, yrs	53 ± 7		
Weight, kg	96.4 ± 11.3	94.8 ± 9.6	0.14
BMI, kg/m^2	34.5 ± 3.2	34.0 ± 2.9	0.15
WHR, -	0.91 ± 0.08	0.90 ± 0.07	0.76
Waist circumference, cm	109 ± 9	105 ± 8	0.12
Systolic pressure, mmHg	138 ± 11	132 ± 11	0.10
Diastolic pressure, mmHg	84 ± 5	80 ± 7	0.19
HR_{rest}, bpm	68 ± 5	59 ± 7	<0.01
Glucose, mmol/l	5.5 ± 0.6	5.7 ± 0.8	0.36
Triglycerides, mmol/l	1.98 ± 0.84	1.83 ± 0.73	0.34
HDL-C, mmol/l	1.21 ± 0.30	1.28 ± 0.29	0.11
LDL-C, mmol/l	4.25 ± 1.03	4.25 ± 0.58	0.99
Incremental exercise test			
VO_{2max}, ml/min/kg	22.7 ± 4.7	25.3 ± 3.8	<0.01
Workload max, W	158 ± 28	185 ± 23	<0.01

WHR, waist-to-hip ratio; HR, heart rate, VO2 peak, peak oxygen uptake. Values are means ± SD. One woman with the metabolic syndrome did not perform a maximal incremental exercise test after the six months of endurance training due to acute raised lower back pain one day prior testing.

Study design

Before and after six months of endurance cycling training, subjects visited our laboratory on three separate days. On day one, a percutaneous needle muscle biopsy (Bergström technique) was taken from the vastus lateralis muscle under local anesthesia (2% lidocaine) and the obtained muscle tissue was immediately frozen in liquid nitrogen and subsequently stored at -80 0C until analysis. On the second day, resting diameter and blood flow of the common carotid artery (CA), brachial artery (BA), common femoral artery (CFA), and superficial femoral artery (SFA) were measured. In addition, CA and CFA intima-media thickness (IMT) was measured as a measure of wall thickness. On day three, peak oxygen consumption was measured during an incremental cycling test.

Exercise Intervention

Subjects trained for 6 months, three times a week under the supervision of a researcher with preferably one day between subsequent exercise bouts. A cycling ergometer (Lode, Groningen) was used for cycling exercise. Each session started with a 10 minute warming-up followed by 30 minutes of cycling exercise at 65% of the individual Heart Rate Reserve (HRR). As their exercise tolerance improved, the intensity of the training was increased by 5% HRR to a maximum of 85% of the HRR. Exercise intensity was documented through the use of heart rate monitors (Polar). Subjects had to attend at least 90% of the training sessions during this six month period to be eligible for inclusion of the statistical analysis.

Experimental Procedures

Cardio-respiratory Exercise Test

Subjects performed a maximal exercise test on an electrically braked leg-cycling ergo meter (Lode, Angio 300, Groningen, the Netherlands) using an incrementing protocol to assess their cardio-respiratory fitness. Workload increased

by 10 W per minute, starting at 10 W, until exhaustion. A gas-analyzer was used to measure oxygen consumption continuously (Jaeger Benelux BV, Breda, the Netherlands). Peak oxygen consumption (VO2max) was analyzed as the mean of the last minute of the exercise bout. During the test, heart rate was measured continuously. Two minutes after cessation of the test, blood lactate level (Roche Diagnostics GmbH, Mannheim, Germany) was measured.

Vascular characteristics

Vascular measurements were performed after a 4-hour fast and 12 hours of abstinence from caffeine, chocolate, alcohol, kiwi and vitamin supplements in a temperature-controlled room by an experienced sonographer.[19] After a 20-minute supine resting period, baseline diameter of the CA, BA, CFA and SFA and IMT of the CA and CFA were measured using high resolution echo ultrasonography (Picus, Pie Medical Benelux, Maastricht, the Netherlands or T3000, Terason, Burlington, MA, USA). Baseline blood flow (i.e. red blood cell velocity) was measured by the Doppler technique (WAKI e, Atys, France or Terason). Blood flow data was collected using the lowest possible insonation angle (<600). The same procedure was used before and after training. The CA was measured 2 cm proximal to the bulbus, the BA was measured 3 cm proximal of the olecranon process, the CFA was measured 2 cm proximal of the bifurcation and the SFA was measured 2 to 3 cm distal from the bifurcation. IMT was defined as the distance from lumen-intima interface to media-adventitia interface and was normalized for lumen diameter size (IMT/lumen).[20] For baseline assessment using the Picus, artery diameter and IMT were measured in 4 series of 6 consecutive cardiac cycles and stored for later off-line, automated analysis. This off-line analysis was performed with an echo tracking system (ART-Laboratory, Esaote Europe BV, Eindhoven, the Netherlands) as previously described elsewhere.[21] For baseline assessment using the Terason, artery diameter, IMT and blood flow were measured continuously for 1 minute. Post-test analysis was performed using custom-designed, edge-detection, and wall tracking software, which is independent of investigator bias.[22] The echo Doppler signal was real-time encoded and stored as a digital file. Subsequent software analysis of this data was performed at 30 Hz using an icon-based graphical programming language and toolkit (LabVIEW 6.02, National instruments, Austin, TX, USA). For baseline assessment of blood flow using the Waki, flow profiles were analyzed from the Doppler signal by a custom-designed program that detects the envelope of the velocity waveform (MatLab, MathWorks Inc, USA). Mean blood flow was calculated as (p.(Dmean/2)2) . ((Vmean/2)).60, where Vmean (cm/s) is mean blood cell velocity.

mRNA Gene Expression

Muscle biopsies from the vastus lateralis muscle were taken after a standardized 250 kcal breakfast (79% carbohydrates, 11.2% protein, 9.8% fat). From muscle biopsies of 6 women (being a representative sample from the total) with the metabolic syndrome total RNA was isolated and purified. The characteristics of the array 'cohort' did not differ from the whole group (Supplement 2). RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The 260/280 ratio indicated 'pure RNA' quality. RNA integrity was analyzed on an Agilent Bioanalyser (Santa Clara, CA, USA) and the average RNA integrity number for all muscle samples was 7.8. RNA gene expression profiling was performed using Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix Inc., Santa Clara, CA, USA), according to the manufacturer's instruction. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to generate amplified and biotinylated sense-strands DNA targets from the entire expressed genome (100 ng of total RNA). The manufacturer's manual was followed for the hybridization, washing, and scanning steps (version 4, P/N 701880 Rev.4). Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 0C for 17 h. After hybridization, the arrays were washed in the Affymetrix GeneChip Fluidics station FS 450. Arrays were scanned using the GeneChip scanner 3000 7G system. The average fluorescence intensity of all genes was calculated using the Robust Multiarray Analysis (RMA) Algorithm[23], including a quantile normalization and using a background correction for GC-content. To check overall data quality, the positive (exonic) and negative (intronic) control probe sets were used to calculate the area under the curve which ranged from 0.83 to 0.85, indicating good quality samples. The Affymetrix CEL-files were imported into Partek (Genomic Suite Software version 6.4, Partek Inc., St Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA logarithm with GC background correction. The distribution of the intensity values on the individual arrays was visualized in a signal histogram and one outlier was detected. This outlier was removed from further data analysis. Transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative gene expression levels. The microarray data have been submitted to the Gene Expression Omnibus (GEO) repository under number GSE46697.

The Database for Annotation Visualization and Integrated Discovery functional annotation tool (DAVID, http:david.abcc.ncifcrf.gov)) was used as an integrated biological knowledgebase and analytical tool to systematically extract biological meaning from large gene list.[24] Gene lists of interest (top 500 positive and top 500 negative fold change) were analyzed using DAVID with main focus on the

enrichment in GO-terms (Gene Ontology terms) of biological processes (GOTERM_BP_FAT) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.[24]

RT-qPCR Validation

Microarray results were validated by reverse transcription quantitative PCR (RT-qPCR). From the 5 microarray subjects, 150 ng total RNA was transcribed to cDNA using the superscript III first-strand synthesis supermix for qRT-PCR (invitrogen, paisley, UK) according to the manufacturer's protocol. Based on the microarray results, ubiquitin c (UBC) was selected as a stable reference gene with relatively high expression levels. Intron-spanning primers were designed using NCBI Primer-BLAST and purchased from Biolegio (Malden, the Netherlands). Primer nucleotide sequences (5' > 3') were as follows: DLL4 forward TGTCATTGCCACGGAGGTAT , DLL4 reverse TGAGCAGGGATGTCCAGGTA ; COL1A1 forward ACATGTTCAGCTTTGTGGACC, COL1A1 reverse TGATTGGTGGGATGTCTTCGT; COL3A1 forward GTTGCACGAAACACACTGGG, COL3A1 reverse ACAGCCTTGCGTGTTCGATA; UBC forward TAGTTCCGTCGCAGCCGGGA, UBC reverse GCATTGTCAAGTGACGATCACAGCG. RT-qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). The PCR reaction volume was 12.5 µl, containing 400 nM of each primer, 2.5 µl cDNA (5 ng/µl) and 6.[25] µl iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. The comparative CT quantification (ΔΔCt method) corrected for primer efficiency was used to compare changes in gene expression.

Statistical Analysis

Differences in anthropometric and vascular variables in women with the metabolic syndrome before and after six months of cycling training were assessed by paired student's t-tests (Statistical Package for Social Sciences 20.0, SPSS Inc., Chicago, Illinois, USA). The level of statistical significance was defined at α=0.05 and data are presented as mean ± SD, unless stated otherwise.

To identify differentially expressed genes after exercise training, the corrected intensity of individual genes were used for an one-way analysis of variance (ANOVA). The resulting p-values were corrected for multiple-testing with the Benjamini-Hochberg false discovery rate procedure.[25] With this stringent correction for multiple-testing all statistical significances disappeared. Therefore, we used plain fold changes to assess enrichment in GO-terms of biological processes since we were interested in the physiological meaning of our array results.

Results

Effectiveness of the training intervention

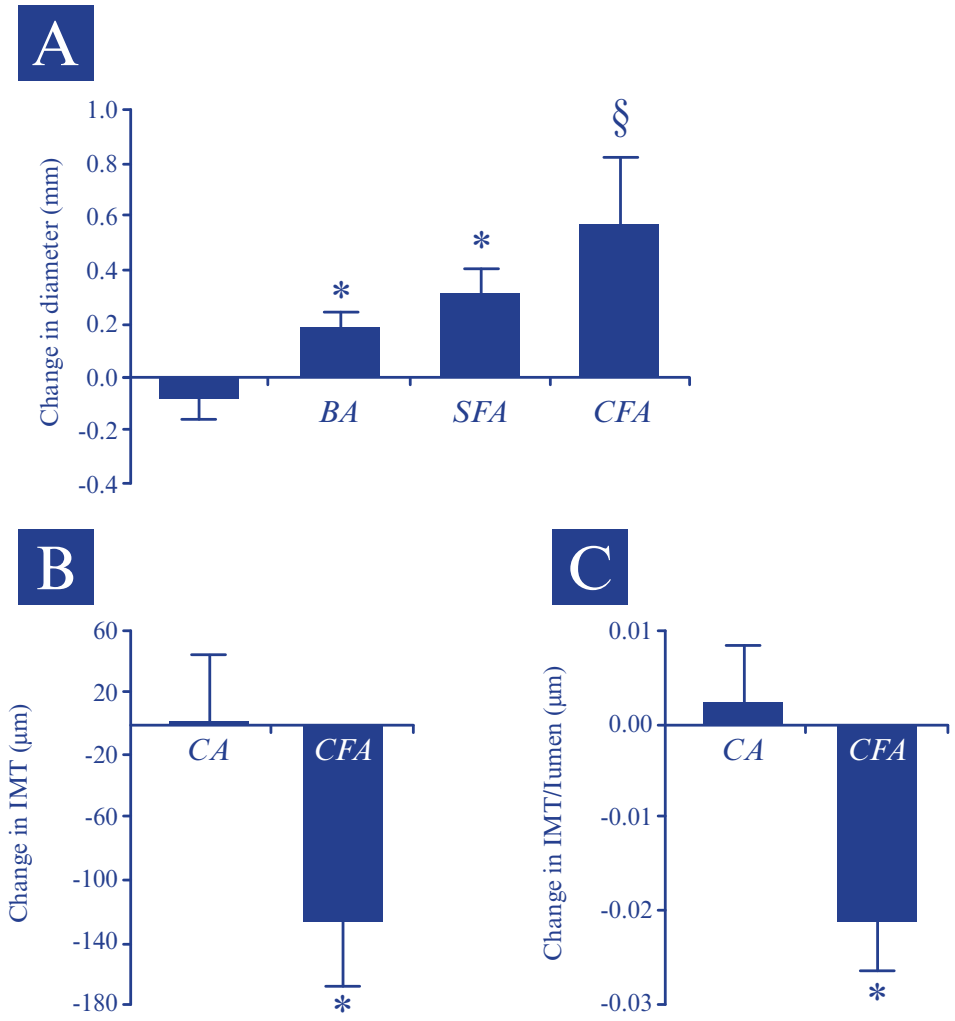
Eleven women successfully completed the training intervention with a training compliance of 92%. Cardio-respiratory fitness, determined as peak oxygen uptake, improved significantly by 11% and peak workload increased significantly by 17% after six months of cycling training (Table 1). Seven women dropped out of the study: five due to medical reasons unrelated to the exercise intervention, one dropped out as a consequence of social/family circumstances, and one woman dropped out due to lack of motivation. There were no significant differences at baseline between women who successfully completed the study and those who dropped out of the study (Supplement 1).

After 6 months of exercise cycling training, no significant changes were observed in the variables of the metabolic syndrome; waist circumference, diastolic or systolic blood pressure, glucose, HDL-cholesterol and triglycerides for the whole group (Table 1). Nonetheless, three out of eleven women (27%) did not meet the criteria for the metabolic syndrome anymore (Table 1). The cycling training did not alter body weight, body mass index or waist-to-hip ratio. Resting heart rate decreased significantly from 68 ± 5 beats per minute to 59 ± 7 beats per minute (Table 1).

Vascular effects of training

After 6 months of endurance exercise training, resting baseline diameters of the BA and SFA were significantly increased (Table 2 and Figure 1). The diameter of the CFA tended to increase ($p=0.07$) and no significant change was observed in the CA diameter. No changes were observed in resting blood flow of the arteries. CFA IMT and the IMT/lumen significantly decreased after exercise training (Table 2 and Figure 1, respectively). No changes were observed in the IMT and IMT/lumen ratio of the CA.

Figure 1.



Change in diameter (A), Intima-media thickness (IMT) (B), and IMT/lumen ratio (C) of the carotid artery (CA), brachial artery (BA), superficial femoral artery (SFA), and common femoral artery (CFA) after 6 months of exercise training. Error bars represent SEM. *p<0.05 and §p=0.07 pre vs post.

Table 2. Diameters and IMT(/lumen) in women with the metabolic syndrome before and after training.

	Pre	Post	p-value
Carotid artery			
Diameter, mm	7.0 ± 0.7	6.9 ± 0.7	0.38
Blood flow$_{rest}$, ml/min	610 ± 161	572 ± 139	0.52
IMT, µm	726 ± 168	727 ± 194	0.99
IMT/lumen	0.10 ± 0.02	0.11 ± 0.03	0.71
Brachial artery			
Diameter, mm	3.8 ± 0.6	4.0 ± 0.6	0.01
Blood flow$_{rest}$, ml/min	42 ± 27	58 ± 47	0.25
Common femoral artery			
Diameter, mm	8.5 ± 1.2	9.1 ± 1.2	0.07
Blood flow$_{rest}$, ml/min	515 ± 251	500 ± 187	0.80
IMT, µm	798 ± 206	670 ± 164	0.02
IMT/lumen	0.10 ± 0.03	0.07 ± 0.01	<0.01
Superficial femoral artery			
Diameter, mm	6.4 ± 0.7	6.7 ± 0.6	0.03
Blood flow$_{rest}$, ml/min	117 ± 59	146 ± 110	0.43

IMT, intima-media thickness. Values are means ± SD. Due to technical constraints the baseline diameter and IMT of the carotid artery was not analyzed in one woman.

Changes in gene expression; the identification of biological processes

In order to gain more insight into the biological meaning of the observed changes in gene expression, a list of top 500 up- and top 500 down-regulated genes was analyzed for overrepresented biological process gene ontology (GO) terms and summarized using functional annotation clustering (Table 3). The list of top 500 up-regulated genes after exercise training was significantly enriched for 27 clusters of GO terms. The most significant enriched GO term for the up-regulated genes was 'response to organic substance'. More strikingly, four GO terms in the top ten of most significant enriched GO terms were related to the vasculature: 'vasculature development', 'blood vessel development', 'blood vessel morphogenesis', and 'angiogenesis'. These four vascular related GO terms included a total of 17 unique genes (Table 4), which demonstrated a response to exercise training in women with the metabolic syndrome.

Table 3. Enrichment in biological process GO-terms (FAT level) of the top 500 positive fold change in women with the metabolic syndrome before and after training. Listing is according to significance. F.E. = Fold Enrichment.

Term	F.E.	Benjamini
GO:0010033 response to organic substance	3.6	4.46E-06
GO:0001944 vasculature development	5.9	1.67E-05
GO:0001568 blood vessel development	6.0	1.78E-05
GO:0051789 response to protein stimulus	8.9	1.69E-04
GO:0048514 blood vessel morphogenesis	5.8	3.12E-04
GO:0007167 enzyme linked receptor protein signaling pathway	4.3	5.51E-04
GO:0032570 response to progesterone stimulus	26.0	6.10E-04
GO:0001525 angiogenesis	6.4	1.46E-03
GO:0007610 behavior	3.5	1.50E-03
GO:0007169 transmembrane receptor protein tyrosine kinase sign. pathway	5.0	1.59E-03
GO:0009611 response to wounding	3.3	1.74E-03
GO:0008284 positive regulation of cell proliferation	3.6	3.07E-03
GO:0022610 biological adhesion	2.6	1.79E-02
GO:0006986 response to unfolded protein	8.5	1.80E-02
GO:0007155 cell adhesion	2.6	1.90E-02
GO:0042127 regulation of cell proliferation	2.4	2.24E-02
GO:0048870 cell motility	3.7	2.29E-02
GO:0051674 localization of cell	3.7	2.29E-02
GO:0009719 response to endogenous stimulus	3.2	2.31E-02
GO:0032496 response to lipopolysaccharide	7.9	2.34E-02
GO:0030199 collagen fibril organization	15.0	2.53E-02
GO:0016477 cell migration	3.8	2.59E-02
GO:0001501 skeletal system development	3.5	2.59E-02
GO:0006928 cell motion	2.9	2.68E-02
GO:0002237 response to molecule of bacterial origin	7.1	3.18E-02
GO:0030335 positive regulation of cell migration	6.8	3.67E-02
GO:0030334 regulation of cell migration	4.6	4.69E-02

Two genes called delta like protein 4 (DLL4) and collagen 1 type 1 (COL1A1) were significantly changed when using the less stringent ANOVA test. Using the list of top 500 up-regulated genes after exercise training revealed one significantly changed KEGG pathway called 'hsa04512; extra-cellular matrix(ECM)-receptor interaction' (8.0-fold enrichement; Benjamini 7.14E-05). The list of top 500 down-regulated genes after exercise was enriched for only the GO term 'sensory perception of taste'.

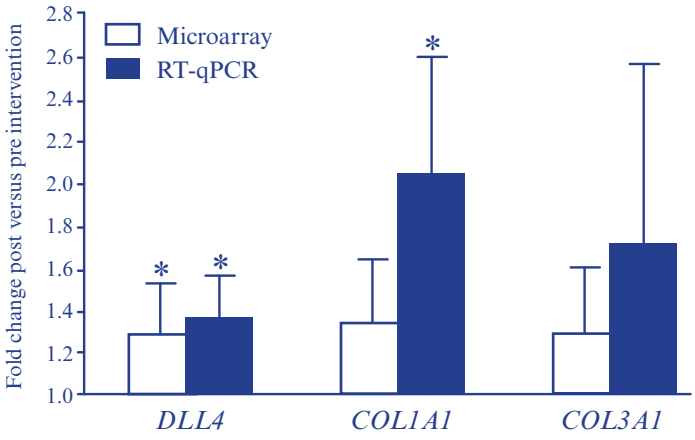
Table 4. All 17 unique genes that included the four vascular GO-terms 'vasculature development', 'blood vessel development', 'blood vessel morphogenesis', and 'angiogenesis'. F.C. = Fold Change.

Gene symbol	Gene Name	F.C.	p-value
DLL4	delta-like 4	1.27	0.04
COL1A1	collagen, type I, alpha 1	1.31	0.05
COL3A1	collagen, type III, alpha 1	1.26	0.10
ANXA2	annexin A2	1.30	0.10
Bak1	BCL2-antagonist/killer 1	1.22	0.21
BGN	biglycan	1.29	0.21
CTGF	connective tissue growth factor	1.43	0.22
NRP2	neuropilin 2	1.20	0.22
junb	jun B proto-oncogene	1.55	0.22
cyr61	cysteine-rich angiogenic inducer 61	1.74	0.42
IL18	interleukin 18	1.33	0.48
FGF9	fibroblast growth factor 9	1.24	0.49
Itgav	Integrin alpha V	1.23	0.53
Klf5	Kruppel-like factor 5	1.28	0.54
TGFB2	transforming growth factor beta 2	1.26	0.58
PLAU	plasminogen activator urokinase	1.21	0.61
Thbs1	thrombospondin 1	1.58	0.64

Reverse transcription quantitative PCR (RT-qPCR) validation

Of the 17 identified vascular-related genes, RT-qPCR analysis was performed on the three genes that demonstrated the lowest p-value after exercise training. Figure 2 demonstrates the validated significant expression of DLL4 and COL1A1.

Figure 2.



Real time-qPCR validation of the delta like protein 4 (DLL4), collagen type 1 alpha 1 (COL1A1) and collagen type 3 alpha 1 (COL3A1). * $p<0.05$

Discussion

This study provides a number of findings. First, we found that 6 months of leg exercise training in women with the metabolic syndrome leads to a larger diameter and a decrease in wall thickness in peripheral arteries, but not in the central area (i.e. CA). Second, microarray analysis demonstrated that exercise training in women with the metabolic syndrome induced upregulated expression of genes involved in vascular remodeling (GO-term: vasculature development, blood vessel development, blood vessel morphogenesis, and angiogenesis), including enhanced gene expression of delta like protein 4 (DLL4) and collagen 1 type 1 (COL1A1). Taken together, this study demonstrates that exercise training in women with the metabolic syndrome leads to vascular remodeling in peripheral, but not central, arteries through upregulation of vascular remodeling-pathways, with a dominance for DLL4 and COL1A1.

After 6 months of exercise training, physical fitness level of the women with the metabolic syndrome is improved by 11%, which is comparable to previous endurance training programs in men and women with the metabolic syndrome of similar duration.[26, 27] This indicates the successful performance of exercise training in our group. On the vascular level, the training induced structural changes in peripheral conduit arteries supplying both the active leg regions (i.e. CFA and SFA) as well as

the inactive arm regions (i.e. BA). As indicated in previous studies, exercise-induced arterial remodeling in diameter is believed to depend on local, shear-dependent processes, while adaptations in wall thickness more likely depends on systemic, rather than local stimuli during repeated bouts of exercise.[28-30]. Interestingly, we also found the brachial artery diameter to adapt despite leg cycling exercise. This is probably the result of thermoregulatory responses during exercise that can lead to significant elevations in brachial artery blood flow.[31, 32] Our findings of increased peripheral artery diameter are in accordance with previous aerobic exercise intervention studies in healthy women[33]. Our study extends these observations with the finding of a decrease in peripheral artery wall thickness (i.e. IMT CFA). In marked contrast to the remodeling in peripheral arteries, no significant changes were observed in the central CA diameter and wall thickness, suggesting that exercise training has a more pronounced effect on remodeling of the arterial wall of peripheral conduit arteries than on the central artery. One potential explanation relates the potential impact of cardiovascular risk factors on the ability of peripheral and central conduit arteries to adapt. Indeed, studies in healthy subjects suggest the presence of remodelling in response to training in peripheral and central arteries.12 However, the effects of exercise training on remodeling of peripheral and central arteries in those with increased cardiovascular risk may be different.13 Although the exact mechanisms for this disparity in arterial remodelling between vessels is unknown, we found that in women with the metabolic syndrome, who have multiple CVD risk factors, exercise training induces systemic peripheral vascular changes in conduit arteries, but not in a central artery.

The beneficial adaptations in peripheral arterial structure after exercise training depend on local and systemic stimuli on the endothelial cells lining the inner vascular wall. Gene expression microarray analysis was performed as a fast and comprehensive method to assess changes in gene expression levels that are associated with the observed vascular adaptations. Those genes with the highest fold-change after exercise training were analyzed for overrepresented biological process gene ontology (GO) terms. We identified four significant enriched GO-terms related to vascular remodeling including ‘vasculature development’, ‘blood vessel development’, ‘blood vessel morphogenesis’ and ‘angiogenesis’. These GO-terms included a total of 17 unique genes. RT-qPCR validation of the expression of genes with the most prominent changes confirmed the microarray data. The most significant changed gene is DLL4, an endothelial cell-specific Notch ligand which once activated by vascular endothelial growth factor (VEGF) can induce Notch signaling. Notch signaling is known to play a critical role in physiological arterial remodeling.[34] Recently, it has been suggested that DLL4 expression acts as a switch from the proliferative phase of angiogenesis to the maturation and stabilization of

endothelial cells.[35, 36] We demonstrated a possible role of DLL4 in exercise-induced adaptations in arterial wall structure in humans. Well-controlled regulation of arterial remodeling is important for preventing excessive vascular growth. The other significant up-regulated gene after 6 months of exercise is COL1A1. COL1A1 is an extra-cellular matrix component, which contributes to the stiffness of an artery.[37] Initially degradation of the extra-cellular matrix surrounding the artery is necessary to allow outward arterial remodeling. When a new stable arterial structure has been established strengthening of the extracellular matrix occurs.[38] KEGG pathways analysis supported this notion by our identification of the extracellular matrix (ECM)-receptor interaction pathway.

Clinical relevance

Our findings have important clinical relevance. First, the smaller femoral artery wall thickness is of clinical importance, as there is growing evidence that peripheral artery wall thickness (e.g. FA) has clinical and prognostic value.[39, 40] Moreover, the observation that 6 months of exercise training is effective to reverse the metabolic syndrome in 3 women is of clinical importance. This effect was independent of a generalized effect of exercise training on the individual risk factors of the metabolic syndrome. This observation is in agreement with various other studies, which demonstrate that prolonged exercise training has no or a modest impact on traditional cardiovascular risk factors such as weight, blood pressure and cholesterol.[41-43] Therefore, our findings reinforce the multi-target effects and benefits of exercise training, with the important clinical message that emphasis on a single outcome measure after exercise training can be misleading.

Study limitations

Unfortunately ~40 % of the subjects with the metabolic syndrome were not able to complete the 6-month training period. Only one of these women stopped due to a lack of motivation despite repeated encouragement. The other 6 women with the metabolic syndrome could not complete the training program due to medical reasons, none of which were related to the participation in our study.

Unfortunately, direct sampling of the arterial wall is limited in human in vivo studies. Muscle biopsies are a good and valid alternative, since it is the primary organ affected by exercise training, is highly vascularized en surrounds the peripheral vasculature. There is growing evidence, which points to a strong relation between muscle capillary development and large arterial structural adaptations.[44-46]

Furthermore, it has been demonstrated that exercise-induced muscle-derived factors (i.e. IL-6 and VEGF) can induce elevated blood plasma levels[47, 48] and can even induce gene expression in another organ via blood plasma.[49] This supports our assumption that changes in muscular gene expression reflect genes involved in arterial adaptations.

In conclusion, this study showed that 6 months of exercise training in women with the metabolic syndrome induces arterial remodeling of peripheral conduit arteries including an increase in diameter and decrease in arterial wall thickness. Interestingly, such remodeling was not present in central arteries, suggesting remodeling in response to exercise training occurs more rapidly in peripheral arteries. Gene expression microarray analysis identified four molecular pathways related to vascular remodeling including enhanced gene expression of DLL4 and COL1A1. Both DLL4 and COL1A1 are involved in stabilizing the arterial wall and are for the first time identified as possible factors in exercise-induced vascular remodeling in humans.

Funding

This work was supported by ZonMW [92003409 to F.P.]; the project Gelderland-Overijssel-EFRO [no 2009-010034 to G.L.]; and by the Netherlands Heart Foundation [E Dekker stipend, 2009T064 to D.H.J.T.]

Acknowledgements

We thank the Microarray facility for their assistance with the microarray experiment. We would like to thank Bregina Kersten for her excellent echo Doppler measurements and analysis. We would like to acknowledge Jos Evers, Anne Arts, Paul Brussee, Duby Ballak and Margo Dona for their enthusiastic assistance during the training period.

Author contributions

Author contributions: F.P., G.L., and E.P. performed experiments; F.P., G.L., I.M., M.H. analyzed and interpreted data ; F.P., G.L., D.T., C.T., and M.H. drafted manuscript; F.P., G.L., I.M., E.P., C.T., D.T., and M.H. approved final version of manuscript; F.P., D.T., C.T. and M.H. conception and design research.

References

1. Ford ES, Li C, Zhao G. Prevalence and correlates of metabolic syndrome based on a harmonious definition among adults in the us. *Journal of diabetes*. 2010;2:180-193
2. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC, Jr. Harmonizing the metabolic syndrome: A joint interim statement of the international diabetes federation task force on epidemiology and prevention; national heart, lung, and blood institute; american heart association; world heart federation; international atherosclerosis society; and international association for the study of obesity. *Circulation*. 2009;120:1640-1645
3. Kawamoto R, Tomita H, Ohtsuka N, Inoue A, Kamitani A. Metabolic syndrome, diabetes and subclinical atherosclerosis as assessed by carotid intima-media thickness. *Journal of atherosclerosis and thrombosis*. 2007;14:78-85
4. de Groot E, van Leuven SI, Duivenvoorden R, Meuwese MC, Akdim F, Bots ML, Kastelein JJ. Measurement of carotid intima-media thickness to assess progression and regression of atherosclerosis. *Nature clinical practice. Cardiovascular medicine*. 2008;5:280-288
5. Alexander CM, Landsman PB, Teutsch SM, Haffner SM. Ncep-defined metabolic syndrome, diabetes, and prevalence of coronary heart disease among nhanes iii participants age 50 years and older. *Diabetes*. 2003;52:1210-1214
6. Mora S, Cook N, Buring JE, Ridker PM, Lee IM. Physical activity and reduced risk of cardiovascular events: Potential mediating mechanisms. *Circulation*. 2007;116:2110-2118
7. Joyner MJ, Green DJ. Exercise protects the cardiovascular system: Effects beyond traditional risk factors. *J Physiol*. 2009;587:5551-5558
8. de Groot E, Hovingh GK, Wiegman A, Duriez P, Smit AJ, Fruchart JC, Kastelein JJ. Measurement of arterial wall thickness as a surrogate marker for atherosclerosis. *Circulation*. 2004;109:III33-38
9. Ebrahim S, Papacosta O, Whincup P, Wannamethee G, Walker M, Nicolaides AN, Dhanjil S, Griffin M, Belcaro G, Rumley A, Lowe GD. Carotid plaque, intima media thickness, cardiovascular risk factors, and prevalent cardiovascular disease in men and women: The british regional heart study. *Stroke*. 1999;30:841-850
10. Wattanakit K, Folsom AR, Selvin E, Weatherley BD, Pankow JS, Brancati FL, Hirsch AT. Risk factors for peripheral arterial disease incidence in persons with diabetes: The atherosclerosis risk in communities (aric) study. *Atherosclerosis*. 2005;180:389-397
11. Thijssen DH, Cable NT, Green DJ. Impact of exercise training on arterial wall thickness in humans. *Clin Sci (Lond)*. 2012;122:311-322
12. Thijssen DH, Dawson EA, van den Munckhof IC, Birk GK, Timothy Cable N, Green DJ. Local and systemic effects of leg cycling training on arterial wall thickness in healthy humans. *Atherosclerosis*. 2013;229:282-286
13. Maiorana AJ, Naylor LH, Exterkate A, Swart A, Thijssen DH, Lam K, O'Driscoll G, Green DJ. The impact of exercise training on conduit artery wall thickness and remodeling in chronic heart failure patients. *Hypertension*. 2011;57:56-62
14. Moreau KL, Donato AJ, Seals DR, Dinenno FA, Blackett SD, Hoetzer GL, Desouza CA, Tanaka H. Arterial intima-media thickness: Site-specific associations with hrt and habitual exercise. *Am J Physiol Heart Circ Physiol*. 2002;283:H1409-1417
15. Whyte JJ, Laughlin MH. The effects of acute and chronic exercise on the vasculature. *Acta Physiol (Oxf)*. 2010;199:441-450

16. Hoier B, Nordsborg N, Andersen S, Jensen L, Nybo L, Bangsbo J, Hellsten Y. Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *J Physiol*. 2012;590:595-606
17. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mrna expression in human skeletal muscle during recovery from endurance exercise. *Faseb J*. 2005;19:1498-1500
18. Lammers G, Duijnhoven NT, Hoenderop JG, Horstman AM, Haan AD, Janssen TW, Graaf MJ, Pardoel EM, Verwiel ET, Thijssen DH, Hopman MT. The identification of genetic pathways involved in vascular adaptations after physical deconditioning versus exercise training in humans. *Exp Physiol*. 2012
19. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME, Green DJ. Assessment of flow-mediated dilation in humans: A methodological and physiological guideline. *Am J Physiol Heart Circ Physiol*. 2011;300:H2-12
20. Simon A, Gariepy J, Chironi G, Megnien JL, Levenson J. Intima-media thickness: A new tool for diagnosis and treatment of cardiovascular risk. *J Hypertens*. 2002;20:159-169
21. Brands PJ, Hoeks AP, Willigers J, Willekes C, Reneman RS. An integrated system for the non-invasive assessment of vessel wall and hemodynamic properties of large arteries by means of ultrasound. *Eur J Ultrasound*. 1999;9:257-266
22. Woodman RJ, Playford DA, Watts GF, Cheetham C, Reed C, Taylor RR, Puddey IB, Beilin LJ, Burke V, Mori TA, Green D. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *J Appl Physiol*. 2001;91:929-937
23. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)*. 2003;4:249-264
24. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nature protocols*. 2009;4:44-57
25. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behavioural brain research*. 2001;125:279-284
26. Tjonna AE, Lee SJ, Rognmo O, Stolen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slordahl SA, Kemi OJ, Najjar SM, Wisloff U. Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: A pilot study. *Circulation*. 2008;118:346-354
27. Stensvold D, Tjonna AE, Skaug EA, Aspenes S, Stolen T, Wisloff U, Slordahl SA. Strength training versus aerobic interval training to modify risk factors of the metabolic syndrome. *J Appl Physiol*. 2010
28. Rowley NJ, Dawson EA, Birk GK, Cable NT, George K, Whyte G, Thijssen DH, Green DJ. Exercise and arterial adaptation in humans: Uncoupling localized and systemic effects. *J Appl Physiol (1985)*. 2011;110:1190-1195
29. Rowley NJ, Dawson EA, Hopman MT, George KP, Whyte GP, Thijssen DH, Green DJ. Conduit diameter and wall remodeling in elite athletes and spinal cord injury. *Med Sci Sports Exerc*. 2012;44:844-849
30. Thijssen DH, Dawson EA, van den Munckhof IC, Tinken TM, den Drijver E, Hopkins N, Cable NT, Green DJ. Exercise-mediated changes in conduit artery wall thickness in humans: Role of shear stress. *Am J Physiol Heart Circ Physiol*. 2011;301:H241-246
31. Simmons GH, Padilla J, Young CN, Wong BJ, Lang JA, Davis MJ, Laughlin MH, Fadel PJ. Increased brachial artery retrograde shear rate at exercise onset is abolished during prolonged cycling: Role of thermoregulatory vasodilation. *J Appl Physiol (1985)*. 2011;110:389-397

32. Green D, Cheetham C, Mavaddat L, Watts K, Best M, Taylor R, O'Driscoll G. Effect of lower limb exercise on forearm vascular function: Contribution of nitric oxide. *Am J Physiol Heart Circ Physiol*. 2002;283:H899-907
33. Sabatier MJ, Schwark EH, Lewis R, Sloan G, Cannon J, McCully K. Femoral artery remodeling after aerobic exercise training without weight loss in women. *Dyn Med*. 2008;7:13
34. Hofmann JJ, Iruela-Arispe ML. Notch signaling in blood vessels: Who is talking to whom about what? *Circ Res*. 2007;100:1556-1568
35. Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. Regulation of notch1 and dll4 by vascular endothelial growth factor in arterial endothelial cells: Implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*. 2003;23:14-25
36. Corada M, Nyqvist D, Orsenigo F, Caprini A, Giampietro C, Taketo MM, Iruela-Arispe ML, Adams RH, Dejana E. The wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating dll4/notch signaling. *Dev Cell*. 2010;18:938-949
37. Schmidt A, Lorkowski S, Seidler D, Breithardt G, Buddecke E. Tgf-beta1 generates a specific multicomponent extracellular matrix in human coronary smc. *Eur J Clin Invest*. 2006;36:473-482
38. Fleenor BS, Marshall KD, Durrant JR, Lesniewski LA, Seals DR. Arterial stiffening with ageing is associated with transforming growth factor-beta1-related changes in adventitial collagen: Reversal by aerobic exercise. *J Physiol*. 2010;588:3971-3982
39. Paul TK, Chen W, Srinivasan SR, Rice J, Toprak A, He J, Berenson GS. Framingham risk score is associated with femoral artery intima-media thickness in asymptomatic young adults (the bogalusa heart study). *Atherosclerosis*. 2010;213:627-631
40. Rietzschel ER, De Buyzere ML, Duprez DA, Clement DL. Interchangeability of carotid and femoral intima-media thickness in risk stratification. *Int Angiol*. 2001;20:38-46
41. Cornelissen VA, Fagard RH. Effect of resistance training on resting blood pressure: A meta-analysis of randomized controlled trials. *J Hypertens*. 2005;23:251-259
42. Kodama S, Tanaka S, Saito K, Shu M, Sone Y, Onitake F, Suzuki E, Shimano H, Yamamoto S, Kondo K, Ohashi Y, Yamada N, Sone H. Effect of aerobic exercise training on serum levels of high-density lipoprotein cholesterol: A meta-analysis. *Arch Intern Med*. 2007;167:999-1008
43. Weinheimer EM, Sands LP, Campbell WW. A systematic review of the separate and combined effects of energy restriction and exercise on fat-free mass in middle-aged and older adults: Implications for sarcopenic obesity. *Nutrition reviews*. 2010;68:375-388
44. Robbins JL, Jones WS, Duscha BD, Allen JD, Kraus WE, Regensteiner JG, Hiatt WR, Annex BH. Relationship between leg muscle capillary density and peak hyperemic blood flow with endurance capacity in peripheral artery disease. *J Appl Physiol*. 2011;111:81-86
45. Rizzoni D, Porteri E, Duse S, De Ciuceis C, Rosei CA, La Boria E, Semeraro F, Costagliola C, Sebastiani A, Danzi P, Tiberio GA, Giulini SM, Docchio F, Sansoni G, Sarkar A, Rosei EA. Relationship between media-to-lumen ratio of subcutaneous small arteries and wall-to-lumen ratio of retinal arterioles evaluated noninvasively by scanning laser doppler flowmetry. *J Hypertens*. 2012;30:1169-1175
46. White FC, Bloor CM, McKirnan MD, Carroll SM. Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart. *J Appl Physiol*. 1998;85:1160-1168
47. Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK. Il-6 and tnf-alpha expression in, and release from, contracting human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2002;283:E1272-1278
48. Hiscock N, Fischer CP, Pilegaard H, Pedersen BK. Vascular endothelial growth factor mrna expression and arteriovenous balance in response to prolonged, submaximal exercise in humans. *Am J Physiol Heart Circ Physiol*. 2003;285:H1759-1763

49. Pedersen L, Pilegaard H, Hansen J, Brandt C, Adser H, Hidalgo J, Olesen J, Pedersen BK, Hojman P. Exercise-induced liver chemokine cxcl-1 expression is linked to muscle-derived interleukin-6 expression. *J Physiol*. 2011;589:1409-1420

Supplement 1. No difference in physical characteristics of women with the metabolic syndrome who successfully completed the study and those who dropped out of the study.

	Metabolic syndrome	
	Successful women (n=11)	**Drop outs (n=6)**
Age, yrs	53 ± 7	50 ± 7
Weight, kg	96.4 ± 11.3	92.4 ± 18.4
BMI, kg/m^2	34.5 ± 3.2	34.6 ± 5.2
Waist circumference, cm	109 ± 9	109 ± 11
Systolic pressure, mmHg	138 ± 11	138 ± 12
Diastolic pressure, mmHg	84 ± 5	85 ± 8
Heart Rate$_{rest}$, bpm	68 ± 5	73 ± 15
Glucose, mmol/l	5.5 ± 0.6	5.4 ±0.8
Triglycerides, mmol/l	1.98 ± 0.84	1.99 ± 0.79
HDL-C, mmol/l	1.21 ± 0.30	1.18 ±0.21

Supplement 2. Physical and vascular characteristics of all subjects versus the subjects in the array cohort.

	All subjects (n=11)	Array subject cohort (n=6)
Age, yrs	53 ± 7	58 ± 5
Weight, kg	96.4 ± 11.3	105.8 ± 7.1
BMI, kg/m^2	34.5 ± 3.2	36.7 ± 2.0
Waist circumference, cm	109 ± 9	115 ± 9
Waist-to-Hip Ratio, -	0.91 ± 0.08	0.95 ± 0.08
Systolic pressure, mmHg	138 ± 11	143 ± 13
Diastolic pressure, mmHg	84 ± 5	83 ± 5
Heart Rate $_{rest}$, bpm	68 ± 5	68 ± 4
Glucose, mmol/l	5.5 ± 0.6	6.0 ± 0.2
Triglycerides, mmol/l	1.98 ± 0.84	1.83 ± 0.89
HDL-C, mmol/l	1.21 ± 0.30	1.28 ± 0.40
Vascular characteristics		
Common carotid artery		
Diameter, mm	7.0 ± 0.7	7.3 ± 0.4
Blood flow$_{rest}$, ml/min	610 ± 161	631 ± 198
IMT, µm	726 ± 168	783 ± 163
IMT/lumen	0.10 ± 0.02	0.11 ± 0.02
Brachial artery		
Diameter, mm	3.8 ± 0.6	4.0 ± 0.6
Blood flow$_{rest}$, ml/min	42 ± 27	49 ± 10
Common femoral artery		
Diameter, mm	8.7 ± 1.2	9.2 ± 0.8
Blood flow$_{rest}$, ml/min	515 ± 251	565 ± 314
IMT, µm	798 ± 206	855 ± 247
IMT/lumen	0.09 ± 0.03	0.09 ± 0.03
Superficial femoral artery		
Diameter, mm	6.4 ± 0.7	6.4 ± 0.4
Blood flow$_{rest}$, ml/min	117 ± 59	140 ± 71

Bpm=beats per minute; IMT= intima-media thickness

Chapter 7

General discussion and future perspectives

With the ever expanding pandemic of obesity, millions of people are at increased risk of developing type 2 diabetes and cardiovascular diseases (CVD)[1, 2], which is a serious threat to human health. Insulin resistance in obese subjects results from impaired post-receptor insulin-signaling, causing reduced insulin-induced glucose uptake. Recent studies have demonstrated a strong link between chronic low-grade inflammation and the development of insulin resistance[3, 4,] but the underlying mechanism remains elusive. Exercise training is a well-established method to improve insulin resistance and obesity-associated cardiovascular risk factors. Although the beneficial effects of exercise training on the human metabolic and cardiovascular system are strongly established, the mechanisms behind the positive effects of exercise training are still largely unknown[5]. In this thesis, we first explored molecular mechanisms related to insulin resistance and subsequently studied the molecular mechanisms involved in exercise-induced improvements in insulin resistance and vascular remodeling. Our population of interest consisted of women with the metabolic syndrome. In this final chapter, results are summarized, related to previous studies, and future perspectives are discussed.

Molecular mechanisms of insulin resistance

The most commonly accepted hypothesis in the pathophysiology of the metabolic syndrome is insulin resistance[6], which may be caused by excessive central obesity and/or physical inactivity. Activation of the immune system in adipose tissue of obese subjects results in chronic low-grade inflammation. It is hypothesized that adipose tissue-secreted inflammatory signals affect target organs like skeletal muscle and thereby explain the link between obesity and insulin resistance [3, 7, 8]. In chapter 2, we indeed found that expression of genes involved in inflammatory pathways is upregulated in skeletal muscle of women with the metabolic syndrome, who are insulin resistant and have chronic low-grade systemic inflammation. The upregulated genes were significantly enriched for the gene ontology (GO) term 'inflammatory response', and expression levels of three genes, interleukin 6 receptor (IL6R), histone deacetylase 9 (HDAC9), and CD97 molecule (CD97), correlated with insulin resistance. This suggests an important role of these genes in the development of skeletal muscle insulin resistance. Interleukin 6 (IL-6) plasma levels are elevated in subjects with the metabolic syndrome[9]. In contrast to our findings of an IL6R up-regulation on the mRNA level, a recent study reported a marked down-regulation of the IL-6 receptor protein in skeletal muscle tissue of obese subjects with and without diabetes[10]. This apparent discrepancy between IL-6 receptor mRNA and protein levels has previously been noticed in human exercise physiology studies[11, 12]. HDAC9 has been associated with inflammation[13] and seems to have

an important role in the transcriptional regulation of skeletal muscle fiber type[14, 15]. HDAC9 can bind to transcription factor myocyte enhancing factor 2 (MEF2), thereby inhibiting its normal function of maintaining slow (type I) muscle fiber type. CD97 is mainly expressed on activated lymphocytes at sites of tissue inflammation. To our knowledge, CD97 has not directly been linked with insulin resistance yet, and also the role of lymphocytes in the development of skeletal muscle insulin signaling remains elusive. Infiltration of lymphocytes may represent an important initial step in tissue inflammation and the subsequent development of insulin resistance[16].

Taken together, these results lead to the concept (as depicted in Figure 2, left panel) in which central adiposity and associated chronic low-grade inflammation results in chronic elevated levels of circulating inflammatory markers. This probably also includes IL-6 and the IL-6 receptor. At the same time, a transition from slow insulin sensitive to fast, more insulin resistant, skeletal muscle fibers occurs. In addition, lymphocytes infiltrate the muscle and initiate tissue inflammation, which , similar to adipose tissue inflammation, is likely to play a role in the development of insulin resistance.

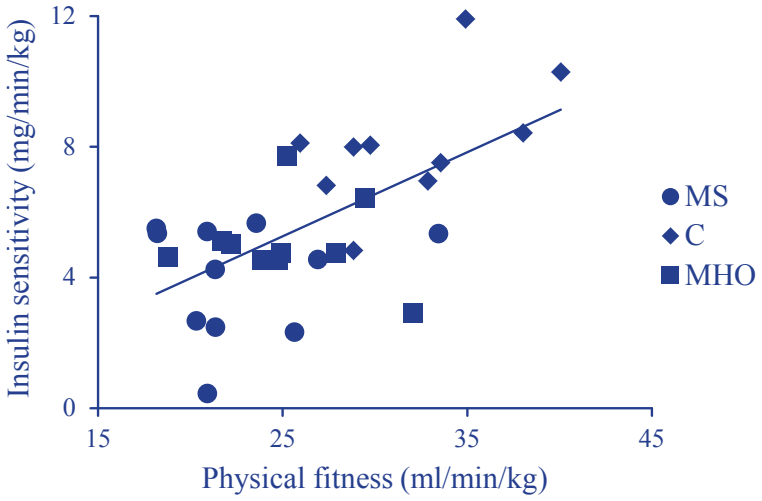
Metabolically healthy obese phenotype

Approximately 30% of obese subjects have a 'metabolically healthy obese phenotype'[17]. Following the same lines of reasoning as in our first study, we wondered whether metabolically healthy obese women have a different metabolic activity of fat tissue i.e. low-grade inflammatory profile, compared to obese women with the metabolic syndrome. The metabolic syndrome may be caused by excessive obesity and/or physical inactivity, so we extended the study by including physical fitness as a potential explanation for the metabolically healthy obese phenotype. In *chapter 3*, we found that metabolically healthy obese women have a higher cardio-respiratory fitness (VO2max) compared to age- and weight-matched obese women with the metabolic syndrome. They also appeared physically more active as they tended to take more steps per day. In addition, we found lower circulating TNF-α levels and a tendency for lower circulating hsCRP levels in obese women with the metabolically healthy obese phenotype, despite corresponding levels of adipose tissue mass in both groups of obese women. This findings are consistent with a lower inflammatory profile of fat tissue. The lower TNF-α levels found in our metabolically healthy obese women could not be explained by their quantity of fat tissue or degree of insulin resistance since both variables did not differ between groups. Our results suggest that in obese women TNF-α levels are influenced by cardio-respiratory fitness (VO_{2max}), which corresponds with the observation that exercise training can lower TNF-α levels in diabetic patients [18]. Taken together, our results show that a

higher physical fitness and lower circulating TNF-α levels may partly explain why metabolically healthy obese women are protected against the detrimental effects of obesity.

Physical fitness levels seem more important for a the metabolic phenotype than for body composition (*chapter 3*). This is in agreement with results of a large observational cohort study, which showed that unfit-lean subjects have a higher cardiovascular risk than fit-obese subjects[19]. When pooling data of all investigated women in this thesis (metabolic syndrome n=18; metabolic healthy obese n=10; lean women n=11), we found a significant positive correlation between physical fitness (VO_{2max}) and insulin sensitivity (gold standard M-value), as depicted in Figure 1.

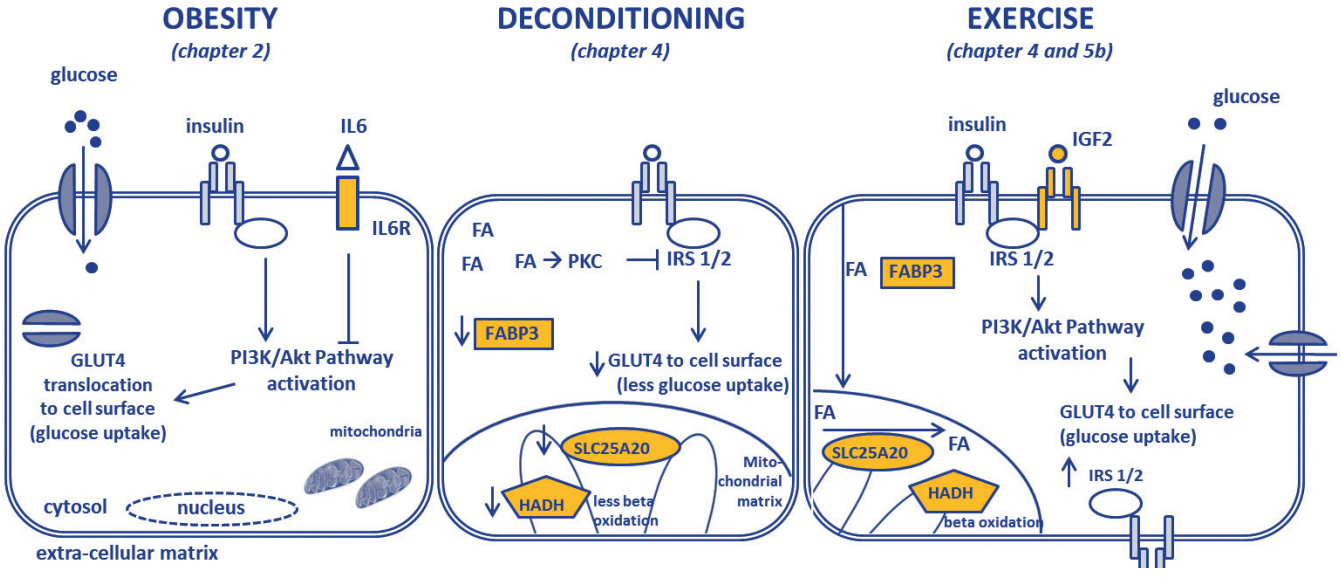
Figure 1.



Correlation between physical fitness and insulin sensitivity in women with the metabolic syndrome (MS), lean women (C), and metabolically healthy obese women (MHO).

While physical inactivity seems associated with the development of insulin resistance, exercise training effectively counteracts this development by stimulating the insulin-signaling cascade. To gain more insight into the mechanisms of insulin resistance, we compared in *chapter 4* muscle gene expression levels after physical deconditioning to gene expression levels after exercise training. When we analyzed

Figure 2.



Hypothetical representation in skeletal muscle cells of results (orange highlighted) obtained from studies presented in this thesis. Obesity (left panel) and associated low-grade inflammation induces upregulation of inflammatory markers including IL-6 receptor (IL6R) which inhibits Phosphatidylinositide-3 Kinase (PI3K)/Akt activation. Deconditioning (middle panel) reduces fatty acid (FA) transport and results in accumulation of intracellular FAs. This activates protein kinase C (PKC), which inhibits insulin receptor substrate 1/2 (IRS 1/2), resulting in less translocation of Glut4 to the cell membrane and a reduced insulin-induced glucose uptake. Exercise training in insulin sensitive lean subjects (right panel) results in the upregulation of FABP3 that transports FAs between the cellular plasma membrane and the membranes of the mitochondria. At the inner mitochondrial membrane, SLC25A20 is upregulated and transports FAs over the membrane. Inside the mitochondria, HADH is upregulated and involved in the beta oxidation of FAs. Exercise training in women with the metabolic syndrome results in upregulation of IGF2, which interacts with IRS 1/ 2 and leads to PI3K/Akt activation resulting in translocation of Glut4 vesicles to the cell surface enabling glucose uptake.

the subset of genes that were downregulated after deconditioning and upregulated after exercise training, we found two significantly enriched pathways, namely 'insulin action' and 'adipocytokine signaling'. These pathways included 18 unique genes of which muscle fatty acid binding protein 3 (FABP3), the fatty acid oxidizing enzyme hydroxyacyl-CoA dehydrogenase (HADH), and the mitochondrial fatty acid transporter solute carrier 25 family member A20 (SLC25A20) showed the most contrasting expression levels. Integrating our findings with existing models results in a proposed working mechanism of physical activity on insulin resistance, as depicted in Figure 2; right panel. Exercise training upregulates FABP3, the enzyme that transports fatty acids from the cellular plasma membrane to the mitochondrial membranes. SLC25A20 is also upregulated and transports the fatty acids over the inner mitochondrial membrane. Inside the mitochondria, upregulated HADH is involved in the beta oxidation of fatty acids. Physical deconditioning, on the other hand, results in downregulation of these genes, which leads to the accumulation of intracellular fatty acids (Figure 2; middle panel). This activates protein kinase C (PKC), which inhibits insulin receptor substrate 1/2 (IRS 1/2), resulting in less translocation of Glut4 to the cell membrane and thus a reduced insulin-induced glucose uptake. Additional experiments and measurements at the protein level are required to prove the validity of this model.

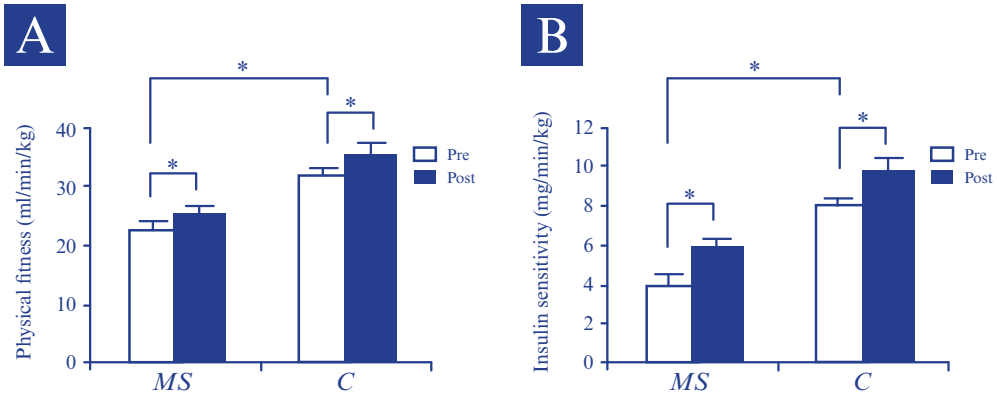
Exercise in women with the metabolic syndrome

Six months/three times a week of endurance leg exercise training improved cardiorespiratory fitness (VO_{2max}) by 11% in both women with the metabolic syndrome as well as in healthy lean women (Figure 3A)(*chapter 5* and *6*). The endurance exercise training reversed the metabolic syndrome in three out of eleven women, which is in accordance with previous studies[20-24] *(Chapter 5* and *6*). Approximately 60% of the beneficial effects of exercise training on total CVD risk can be explained by improvements of traditional CVD risk factors, including glucose levels, haemostatic and inflammation factors, lipid profile, and body weight[5]. Several hypothesis have been postulated about the remaining ~40%, the so called 'risk factor gap' (Figure 5). In *chapter 5* and *chapter 6* we used microarrays to analyse expression levels of virtually all known genes after six months of exercise training. This provided the opportunity to identify whole genome transcriptional responses to exercise training and subsequently generated new hypothesis regarding the beneficial effects of exercise training that may partly explain the 'risk factor gap'.

Molecular mechanisms of exercise training on insulin resistance

On the metabolic level, as expected, six months of endurance exercise training significantly improved insulin sensitivity (M-value) in both insulin resistant women (45%) with the metabolic syndrome as well as in insulin sensitive lean women (22%), even without changes in body weight and/or composition (Figure 3B) (*chapter 5*). The extent of this improvement in insulin resistance after the training program is large compared to the effect of glucose lowering medication, such as metformin[25]. This emphasizes the beneficial effects of exercise. Although, as described in *chapter 2*, we found an upregulation of expression of genes involved in inflammatory pathways in women with the metabolic syndrome, the exercise training program did not result in a downregulation of these pathways and identified genes (*Chapter 5*). We actually found an upregulation of expression of genes involved in the immune- and inflammatory response in insulin sensitive lean women. These apparent contradictory findings require further investigation.

Figure 3.



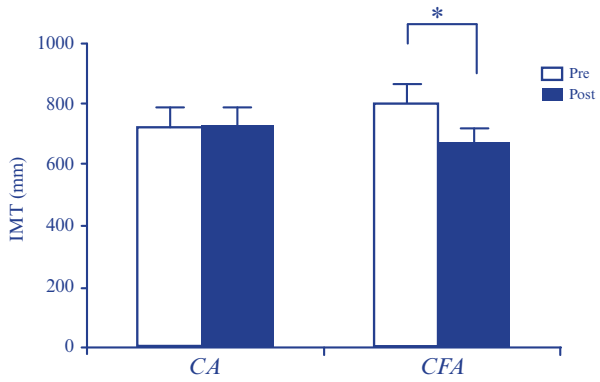
Effects of six months endurance exercise on A, physical fitness; and B, insulin sensitivity in women with the metabolic syndrome (MS) and lean control women (C). Mean ± SEM. *p<0.05.

Molecular mechanisms of exercise training on vascular remodeling

On the vascular level, six months of leg exercise training in women with the metabolic syndrome induced structural changes in peripheral conduit arteries supplying both the active leg regions (i.e. wall thickness (IMT) common femoral artery (CFA) and diameter (D) superficial femoral artery (SFA)) as well as the inactive arm regions (i.e. diameter brachial artery (BA)) (*chapter 6*) (Figure 4). Our

findings of an increase in peripheral artery diameter are in accordance with previous aerobic exercise intervention studies of similar duration in healthy women26. Our study extend these observations with the finding of a decrease in peripheral artery wall thickness (i.e. IMT CFA) after exercise training. In marked contrast to remodeling in peripheral arteries, no significant changes were observed in the central carotid artery diameter and wall thickness, suggesting that exercise training has more effect on remodeling of the arterial wall of peripheral conduit arteries than on central arteries[27].

Figure 4.



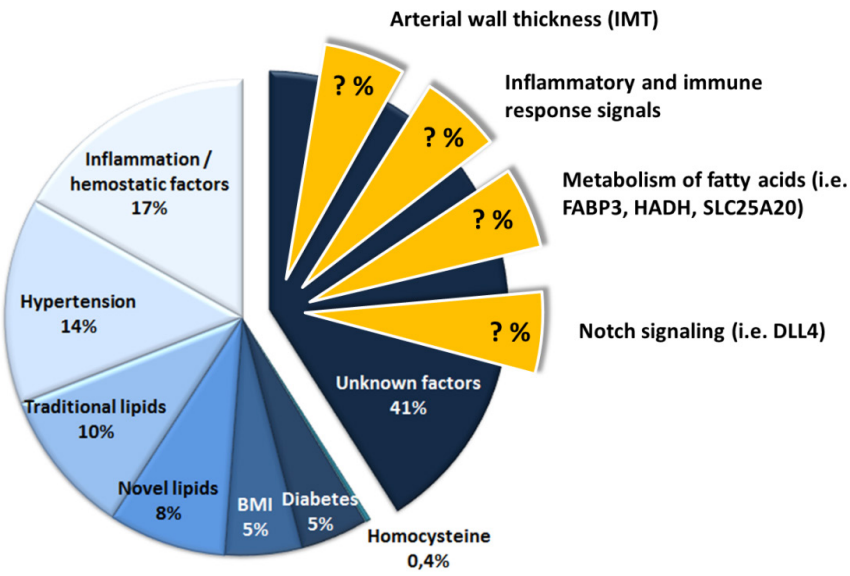
Effect of six months endurance exercise on Intima-media Thickness (IMT) of the carotid artery (CA) and common femoral artery (CFA) in women with the metabolic syndrome. Mean ± SEM. *p<0.05.

After the exercise training program, we found in skeletal muscle tissue surrounding the peripheral vasculature, an upregulation of genes which were enriched for gene ontology (GO) terms related to vascular remodeling including 'vasculature development', 'blood vessel development', 'blood vessel morphogenesis' and 'angiogenesis', which included a total of 17 unique genes. The most significantly changed gene was delta like protein 4 (DLL4), an endothelial cell-specific Notch ligand which, once activated by vascular endothelial growth factor (VEGF), can induce Notch signaling. Notch signaling is known to play a critical role in physiological arterial remodeling[28] via maturation and stabilization of endothelial cells[29, 30]. Our study is the first to suggest a role of DLL4 in exercise-induced adaptations in arterial wall structure in humans. The other significantly upregulated gene after exercise training was collagen type 1 alpha 1 (COL1A1). COL1A1 is an extra-cellular matrix component which contributes to the stiffness of an artery.[31] Initial degradation of the

extra-cellular matrix surrounding the artery is necessary to allow outward arterial remodeling. When a new stable arterial structure has been established strengthening of the extracellular matrix occurs.[32]

Taken together, six months of leg exercise training improves cardio-respiratory fitness in women with the metabolic syndrome. But more importantly, it improves insulin resistance and leads to arterial remodeling including a decrease in wall thickness (i.e IMT CFA) in peripheral arteries. These physiological improvements may substantially reduce the risk for future development of diabetes and CVD. In relation to the 'risk factor gap' mentioned before, we propose change in arterial wall thickness (IMT) as a potential explanation for the 'risk factor gap' (Figure 5) By comparing skeletal muscle gene expression (RNA microarrays), we identified several key genes with enhanced expression after exercise training including those genes related to the transport and metabolism of fatty acids (*chapter 3*: FABP3, HADH, SLC25A20), inflammatory and immune response signals (*chapter 5*), and Notch signaling (*chapter 6*: DLL4). Obviously, additional measurements at the protein levels of the identified genes are required to prove the validity of the proposed factors.

Figure 5.



Exercise-induced improvements in previously established risk factors associated with a reduction in CVD events[5] extended with factors identified in studies performed in this thesis.

Future perspectives

Results of this thesis show that physical fitness plays an important role in metabolic and cardiovascular health. Higher physical fitness levels seem, at least partly, able to prevent obesity-associated risk factors and diseases such as diabetes. Investigating people with obesity who are metabolically healthy can provide more insight into the pathophysiological emergence of metabolic risk factors and diseases. Future studies should focus on the physiological phenotype of metabolically healthy obese subjects, such as the activity of visceral adipose tissue including adipocytokines and immunological markers. At the same time modern high-throughput genetic techniques can contribute to the discovery of gene single-nucleotide polymorphisms associated with the interesting metabolically healthy obese phenotype.

Besides prevention of obesity-associated metabolic and cardiovascular risk factors, increasing physical fitness levels with an exercise training program can improve the obesity-associated risk factors, as described in this thesis. In our studies we focused on long term endurance exercise training. It is however known, that approximately 5-10% of the general population does not respond to this type of exercise training[33]. Future studies should focus on other training strategies such as resistance training, interval training or a combination of these to optimize beneficial training effects. We investigated the effects of exercise training on insulin resistance and vascular structure. Since there is growing awareness and scientific evidence for the involvement of adipose tissue, adipocytokines and the immune response system, future studies may aim for these topics.

Despite large physiological differences in insulin resistance between 1) lean and obese women (cross-sectional) and, 2) the inter-individual variation in improvement in insulin resistance after the training program, the RNA gene expression differences were surprisingly small. The highest fold-change in our studies was 3.15, with the annotation that after stringent correction for multiple testing, no single gene reached statistical significance. Although there is probably a publication bias regarding negative outcome gene expression studies, other research groups also found relative small gene expression differences in skeletal muscle tissue after an exercise training program. There are several explanations for these small gene expression differences. First, in particular initial exercise training stimuli and changes in physical activity levels may induce larger changes in RNA gene expression, which in turn contribute to the formation of relevant proteins responsible for the initial adaptation process. After several weeks or months of training, a new equilibrium may have been and reversal of the increase in gene expression. Thus, after longer exercise training periods, final changes in gene expression may be minimal. This phenomenon was noticed in a study from Egan et al.[34] who performed time-course analysis during an exercise training program in human skeletal muscle.

Second, exercise training may result in the formation of more stable proteins with lower degradation rates, and in that way less RNA transcription. Third, the intra-individual gene expression differences in skeletal muscle tissue may be much larger than the effect of an intervention, whereby studies with small subject numbers in combination with the stringent statistical correction for multiple testing may fail to detect significant differences. Stringent correction for multiple testing prevents type 1 errors (false positive results) to occur but too stringent correction may introduce type 2 errors (false negative results). Longitudinal exercise intervention studies with multiple time-course assessments of gene expression and protein levels should be a next step. To reach statements of causality between changes in gene expression level and whole body physiological effects, innovative research is needed in which conditions at the gene level are controlled and manipulated, while measuring at the functional physiological level, and vice versa.

New techniques such as next generation RNA/DNA sequencing will allow faster, cheaper and more in-depth analysis and holds promises for even more reliable expression level analysis. In the future, a combination of in vivo and whole body (patho-)physiological scientific research should ideally lead to optimal individualized patient-specific treatment options to prevent the massive medical, social and economic burden of risk factors such as physical inactivity and obesity.

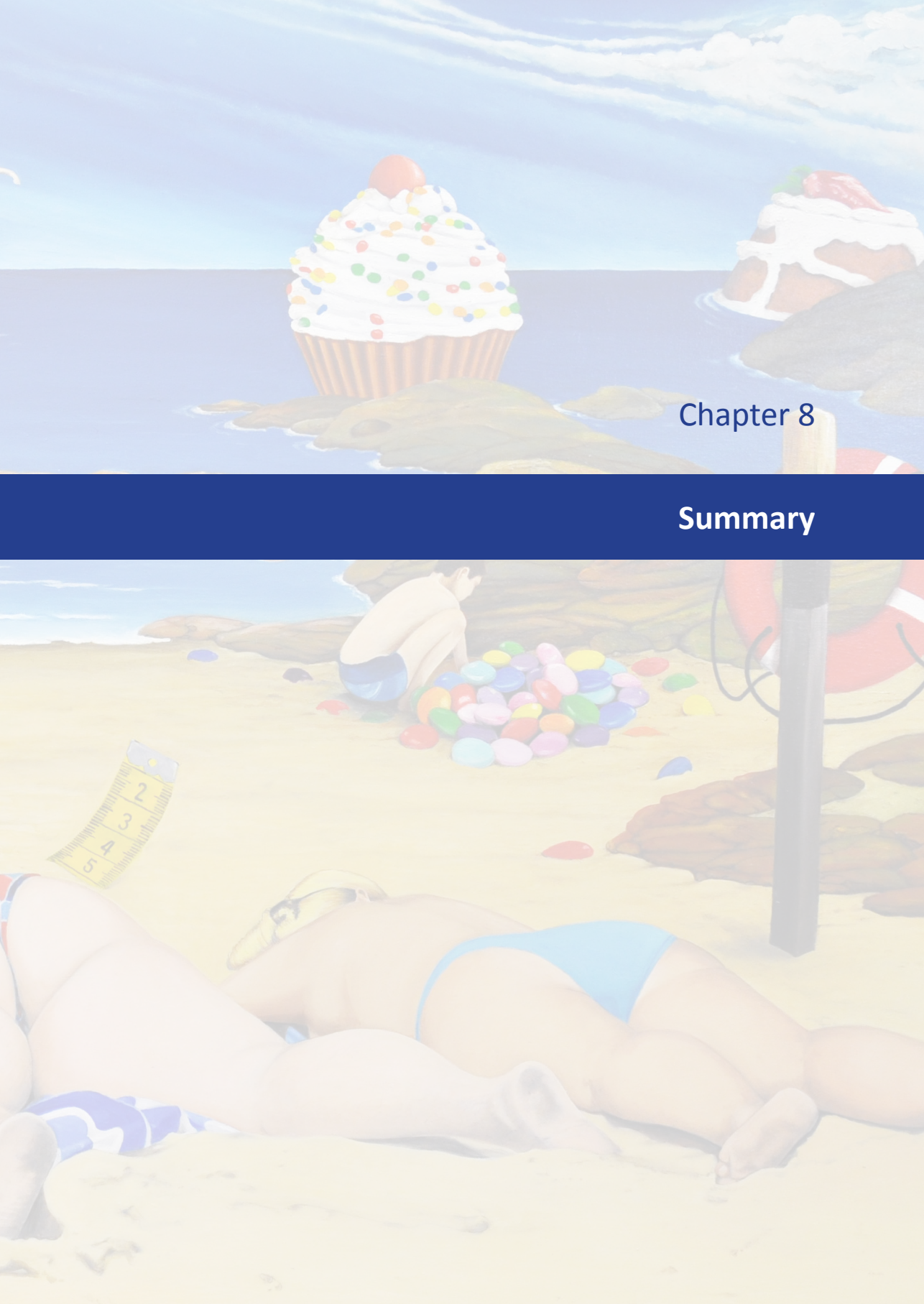
References

1. Wilson PW, D'Agostino RB, Parise H, Sullivan L, Meigs JB. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*. 2005;112:3066-3072
2. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care*. 2001;24:683-689
3. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA, Hooiveld GJ, Hijmans A, Vroegrijk I, van den Berg S, Romijn J, Rensen PC, Joosten LA, Netea MG, Kanneganti TD. Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci U S A*. 2011;108:15324-15329
4. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest*. 2006;116:1793-1801
5. Mora S, Cook N, Buring JE, Ridker PM, Lee IM. Physical activity and reduced risk of cardiovascular events: Potential mediating mechanisms. *Circulation*. 2007;116:2110-2118
6. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet*. 2005;365:1415-1428
7. Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes*. 2008;57:3239-3246
8. Kalupahana NS, Moustaid-Moussa N, Claycombe KJ. Immunity as a link between obesity and insulin resistance. *Mol Aspects Med*. 2012;33:26-34
9. Pickup JC, Mattock MB, Chusney GD, Burt D. Niddm as a disease of the innate immune system: Association of acute-phase reactants and interleukin-6 with metabolic syndrome x. *Diabetologia*. 1997;40:1286-1292
10. Scheele C, Nielsen S, Kelly M, Broholm C, Nielsen AR, Taudorf S, Pedersen M, Fischer CP, Pedersen BK. Satellite cells derived from obese humans with type 2 diabetes and differentiated into myocytes in vitro exhibit abnormal response to il-6. *PloS one*. 2012;7:e39657
11. Keller C, Steensberg A, Hansen AK, Fischer CP, Plomgaard P, Pedersen BK. Effect of exercise, training, and glycogen availability on il-6 receptor expression in human skeletal muscle. *J Appl Physiol*. 2005;99:2075-2079
12. Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J, Pedersen BK. Interleukin-6 receptor expression in contracting human skeletal muscle: Regulating role of il-6. *Faseb J*. 2005;19:1181-1183
13. Verdin E, Dequiedt F, Kasler HG. Class ii histone deacetylases: Versatile regulators. *Trends Genet*. 2003;19:286-293
14. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: Implications for disease and therapy. *Nat Rev Genet*. 2009;10:32-42
15. Potthoff MJ, Wu H, Arnold MA, Shelton JM, Backs J, McAnally J, Richardson JA, Bassel-Duby R, Olson EN. Histone deacetylase degradation and mef2 activation promote the formation of slow-twitch myofibers. *J Clin Invest*. 2007;117:2459-2467
16. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, Hauner H, Bluher M, Unger T, Wolf AM, Knippschild U, Hombach V, Marx N. T-lymphocyte infiltration in visceral adipose tissue: A primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol*. 2008;28:1304-1310

17. Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, Sowers MR. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: Prevalence and correlates of 2 phenotypes among the us population (nhanes 1999-2004). *Arch Intern Med*. 2008;168:1617-1624
18. Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, Fallucca S, Alessi E, Letizia C, Jimenez A, Fallucca F, Pugliese G. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr Metab Cardiovasc Dis*. 2010;20:608-617
19. Lee CD, Blair SN, Jackson AS. Cardiorespiratory fitness, body composition, and all-cause and cardiovascular disease mortality in men. *Am J Clin Nutr*. 1999;69:373-380
20. Tjonna AE, Lee SJ, Rognmo O, Stolen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slordahl SA, Kemi OJ, Najjar SM, Wisloff U. Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: A pilot study. *Circulation*. 2008;118:346-354
21. Stensvold D, Tjonna AE, Skaug EA, Aspenes S, Stolen T, Wisloff U, Slordahl SA. Strength training versus aerobic interval training to modify risk factors of the metabolic syndrome. *J Appl Physiol*. 2010
22. Katzmarzyk PT, Leon AS, Wilmore JH, Skinner JS, Rao DC, Rankinen T, Bouchard C. Targeting the metabolic syndrome with exercise: Evidence from the heritage family study. *Med Sci Sports Exerc*. 2003;35:1703-1709
23. Kemmler W, Von Stengel S, Engelke K, Kalender WA. Exercise decreases the risk of metabolic syndrome in elderly females. *Med Sci Sports Exerc*. 2009;41:297-305
24. Bateman LA, Slentz CA, Willis LH, Shields AT, Piner LW, Bales CW, Houmard JA, Kraus WE. Comparison of aerobic versus resistance exercise training effects on metabolic syndrome (from the studies of a targeted risk reduction intervention through defined exercise - strride-at/rt). *Am J Cardiol*. 2011;108:838-844
25. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346:393-403
26. Sabatier MJ, Schwark EH, Lewis R, Sloan G, Cannon J, McCully K. Femoral artery remodeling after aerobic exercise training without weight loss in women. *Dyn Med*. 2008;7:13
27. Maiorana A, O'Driscoll G, Dembo L, Cheetham C, Goodman C, Taylor R, Green D. Effect of aerobic and resistance exercise training on vascular function in heart failure. *Am J Physiol Heart Circ Physiol*. 2000;279:H1999-2005
28. Hofmann JJ, Iruela-Arispe ML. Notch signaling in blood vessels: Who is talking to whom about what? *Circ Res*. 2007;100:1556-1568
29. Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. Regulation of notch1 and dll4 by vascular endothelial growth factor in arterial endothelial cells: Implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*. 2003;23:14-25
30. Corada M, Nyqvist D, Orsenigo F, Caprini A, Giampietro C, Taketo MM, Iruela-Arispe ML, Adams RH, Dejana E. The wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating dll4/notch signaling. *Dev Cell*. 2010;18:938-949
31. Schmidt A, Lorkowski S, Seidler D, Breithardt G, Buddecke E. Tgf-beta1 generates a specific multicomponent extracellular matrix in human coronary smc. *Eur J Clin Invest*. 2006;36:473-482
32. Fleenor BS, Marshall KD, Durrant JR, Lesniewski LA, Seals DR. Arterial stiffening with ageing is associated with transforming growth factor-beta1-related changes in adventitial collagen: Reversal by aerobic exercise. *J Physiol*. 2010;588:3971-3982

33. Bouchard C, An P, Rice T, Skinner JS, Wilmore JH, Gagnon J, Perusse L, Leon AS, Rao DC. Familial aggregation of vo(2max) response to exercise training: Results from the heritage family study. *J Appl Physiol (1985)*. 1999;87:1003-1008

34. Egan B, O'Connor PL, Zierath JR, O'Gorman DJ. Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle. *PloS one*. 2013;8:e74098



Chapter 8

Summary

Summary

Obesity is an enormous world-wide health threat. It leads to insulin resistance, hypertension, low-grade inflammation and dyslipidemia, and ultimately results in type 2 diabetes mellitus and cardiovascular diseases. It is caused by a complex interplay between genetic predisposition, nutrient overconsumption and a sedentary lifestyle. Lifestyle modification by increasing physical fitness level is a well-established intervention to improve these obesity-associated risk factors, including insulin resistance. However, the molecular mechanisms behind the positive effects of improved physical fitness level are still largely unknown. Gene expression microarray analysis, a modern technology in human genetic research, can be used as a rapid and comprehensive approach for the first identification of exercise-induced molecular changes. Therefore, the general aim of this thesis was to perform human, translational studies which would provide more insight into the molecular mechanism of insulin resistance and into the molecular mechanisms of exercise-induced improvements in insulin resistance and arterial remodeling in obesity, with a special interest in women with the metabolic syndrome.

Obese women often show insulin resistance. In **chapter 2** we investigated the link between obesity-induced insulin resistance and chronic low-grade inflammation in skeletal muscle tissue. In comparison to healthy controls, in insulin resistant women with the metabolic syndrome the expression of genes involved in inflammatory pathways was upregulated. The three most significantly upregulated inflammatory genes we identified were Histone Deacetylase 9 (Hdac9), Interleukin-6 Receptor (Il6r), and CD97 molecule (Cd97); all three genes showed a correlation with insulin resistance. Hdac9 gene expression plays a role in the transcriptional regulation of skeletal muscle fiber type, where Hdac9 upregulation induces a transition from slow insulin-sensitive skeletal muscle fibers to fast, more insulin-resistant, skeletal muscle fibers. Elevated plasma levels of interleukin-6, a pro-inflammatory cytokine, has been associated with insulin resistance, but a discrepancy between IL-6 receptor mRNA and protein levels exists in human exercise physiology studies. The Cd97 molecule has never before been linked to insulin resistance. It is expressed on activated lymphocytes and its overexpression in this subject group may represent an important step in tissue inflammation and the subsequent development of insulin resistance in skeletal muscle.

Interestingly, some 30% of women with obesity have a metabolically healthy phenotype. In **chapter 3** we examined the role of physical fitness level and low-grade systemic inflammation profile as an explanation for this healthy obese phenotype. In comparison to corresponding obese women with the metabolic

syndrome, metabolically healthy obese women had higher cardio-respiratory fitness levels and lower circulating TNF-α levels, suggesting a lower inflammatory profile of adipose tissue. Our regression model revealed that cardio-respiratory fitness was the strongest predictor for having the metabolically healthy obese phenotype.

To gain more insight into the molecular mechanisms underlying the prevention and development of insulin resistance, in **chapter 4** we compared skeletal muscle gene expression in two subject groups: after physical deconditioning and after an exercise training program. The most interesting subset of genes were those that were both downregulated after deconditioning *as well as* upregulated after exercise training. This subset contained 18 genes related to both the 'insulin action' and 'adipocytokine signaling' pathway. Of these genes, the three with the strongest upregulation after exercise as well as downregulation after deconditioning were the muscular fatty acid binding protein 3 (FABP3), the fatty acid oxidizing enzyme hydroxyacyl-CoA dehydrogenase (HADH), and the mitochondrial fatty acid transporter solute carrier 25 family member A20 (SLC25A20). These results indicate an important role for decreased transport and metabolism of fatty acids in skeletal muscle tissue, providing a possible molecular link between physical activity levels and insulin signaling.

In **chapter 5** we subsequently analysed exercise-induced changes in insulin sensitivity with concomitant changes in the global gene expression profile in women with, and without metabolic syndrome. Six months of exercise training improved the insulin sensitivity in both groups. In obese women with the metabolic syndrome insulin sensitivity improved by 45% and in lean control women by 22%. This improvement was independent of changes in body weight and/or composition. Pathway analysis revealed four enriched pathways in women with the metabolic syndrome, in which insulin-like growth factor 2 (IGF2) showed the most significant upregulation after the training program. In contrast, in lean women, expression of genes related to the immune system and inflammatory response were most upregulated. These results indicate that different molecular mechanisms are involved in exercise-induced improvements in insulin sensitivity in women with and without the metabolic syndrome.

In the penultimate chapter **(chapter 6)**, we investigated the effects of an exercise training program on central and peripheral arterial remodeling with simultaneous assessment of (muscle) gene expression in women with the metabolic syndrome. Six months of endurance leg exercise training induced structural changes in the peripheral conduit arteries supplying both the active leg regions (i.e. common femoral artery wall thickness and superficial femoral artery diameter) as well as the

inactive arm regions (i.e. brachial artery diameter). In marked contrast to remodeling in peripheral arteries, no significant changes were observed in the central carotid artery diameter and wall thickness, suggesting that exercise training has more effect on remodeling of the arterial wall of peripheral conduit arteries than on central arteries. Microarray analyses identified upregulation of genes enriched for molecular pathways related to arterial remodeling (Gene Ontology term: vasculature development, blood vessel development, blood vessel morphogenesis, and angiogenesis), including 17 unique genes. Two of the unique genes showed upregulated expression, namely delta-like-protein-4 (DLL4) and collagen-type-1-alpha-1 (COL1A1). Both DLL4 and COL1A1 are involved in stabilizing the arterial wall and are for the first time identified as possible factors in exercise-induced arterial remodeling in humans.

In **chapter 7,** current knowledge of the molecular mechanisms of insulin resistance and the effects of exercise training on insulin resistance and arterial remodeling was reviewed and discussed in conjunction with the novel data from this thesis. We speculated on the potential molecular mechanisms of exercise-induced improvements in insulin resistance and arterial remodeling, with a special focus on obese women with the metabolic syndrome. Although obesity is strongly correlated with several cardiovascular and metabolic risk factors, the level of physical fitness seems crucial. Being obese but physically fit is associated with a metabolically healthy phenotype, lowering the risk for developing diabetes and cardiovascular diseases. Finally, we provide molecular and physiological evidence for the powerful therapeutic effects exercise training has on insulin resistance and arterial remodeling.

Chapter 9

Nederlandse Samenvatting

In dit hoofdstuk wordt een overzicht gegeven van de resultaten van de studies die worden beschreven in dit proefschrift. Daarbij worden medisch-wetenschappelijke termen zoveel mogelijk achterwege gelaten en beschrijf ik de onderzoeksresultaten voor zover mogelijk in begrijpelijke alledaagse taal.

Ernstig overgewicht, ook wel obesitas genoemd, leidt vaak tot een verstoorde suikerstofwisseling (hierna genoemd insulineresistentie), een verhoogde bloeddruk, laaggradige ontsteking en een verstoord vetspectrum in het bloed. Veel mensen met obesitas ontwikkelen dan ook uiteindelijk suikerziekte en/of hart- en vaatziekten. Obesitas wordt veroorzaakt door een complexe interactie tussen genetische aanleg, de overconsumptie van voeding en een inactieve levensstijl. Aanpassing van de levensstijl door te sporten, en dientengevolge een verbetering van de lichamelijke conditie, is een bewezen methode om de obesitas-geassocieerde risicofactoren waaronder insulineresistentie te verbeteren. Het is echter nog grotendeels onbekend wat het precieze werkingsmechanisme van het positieve effect van sporten is. Moderne genetische technologieën, waaronder microarrays, kunnen worden ingezet voor een snelle en complete analyse van het werkingsmechanisme op het niveau van lichaamscellen. Iedere cel in ons lichaam bevat hetzelfde genetische DNA materiaal. Het DNA vormt de genetische code voor het maken van eiwitten, welke de vorm en functie van een cel bepalen. RNA zorgt voor het kopiëren van de genetische informatie die is opgeslagen in het DNA en zorgt uiteindelijk voor de vertaling naar een eiwit. Met behulp van een microarray kan het totale patroon van RNA genexpressie in een cel zichtbaar worden gemaakt en kunnen verschillen in genexpressie tussen weefsels worden opgespoord maar ook in hetzelfde weefsel voor en na een periode van sporten. Het doel van dit proefschrift was om meer inzicht te krijgen in het werkingsmechanisme van insulineresistentie en meer inzicht in het werkingsmechanisme van een sportprogramma op insulineresistentie en de structuur van een slagader. Hiertoe hebben wij obese vrouwen, die tenminste drie obesitas-geassocieerde risicofactoren hadden, onderzocht. Deze vrouwen hebben het zo genoemde metabool syndroom.

Vrouwen met obesitas zijn vaak resistent voor insuline. In **hoofdstuk 2** onderzochten we het verband tussen obesitas-geïnduceerde insulineresistentie en chronische laaggradige ontsteking in spierweefsel. In vergelijking met het spierweefsel van gezonde slanke vrouwen, komen in spierweefsel van insuline resistente vrouwen met het metabool syndroom genen tot expressie die betrokken zijn bij ontstekings-pathways. De drie meest significante genen die tot expressie kwamen waren histondeacetylase 9 (HDAC9), interleukine-6 receptor (IL-6R) en het CD97 molecuul (CD97). Alle drie deze genen lieten een correlatie zien met insulineresistentie. Expressie van het HDAC9 gen speelt een rol bij de regulatie van

spiervezeltypering. Meer expressie van het HDAC9 gen veroorzaakt een verschuiving van langzame, insuline-gevoelige spiervezels naar snelle, insulineresistente spiervezels. Interleukine-6 is een pro-inflammatie cytokine die geassocieerd is met insulineresistentie. Er bestaat op dit moment echter een wetenschappelijke discrepantie tussen genexpressie van deze cytokine en de eiwitconcentraties. Het CD97 molecuul is nog nooit eerder in verband gebracht met insulineresistentie. Het molecuul komt tot expressie op geactiveerde witte bloedcellen en zou op deze manier een rol kunnen spelen bij spierweefselontsteking en dientengevolge bij het ontstaan van insulineresistentie in spierweefsel.

Opmerkelijk is dat ongeveer 30% van de vrouwen met obesitas geen obesitas-geassocieerde risicofactoren heeft, zij hebben een metabool gezond fenotype. In **hoofdstuk 3** hebben we onderzocht wat hier een mogelijke verklaring voor zou kunnen zijn. Vergeleken met obese vrouwen met het metabool syndroom hebben vrouwen met dezelfde mate van obesitas, maar met een metabool gezond fenotype, een betere lichamelijk conditie en lagere bloedwaarde van de ontstekings cytokine TNF-α. Dit geeft een indicatie dat het vetweefsel van obese vrouwen met een gezond fenotype mogelijk een lagere ontstekingsactiviteit heeft. De sterkste voorspeller voor een metabool gezond obees fenotype was het niveau van de lichamelijke conditie.

Om meer inzicht te krijgen in het werkingsmechanisme van insulineresistentie hebben we in **hoofdstuk 4** de genexpressie in spierweefsel na een periode van fysieke inactiviteit vergeleken met de genexpressie na een sportprogramma. De meest interessante genen zijn de genen die minder tot expressie komen na een periode van lichamelijke inactiviteit en meer tot expressie komen na een sportprogramma. Op deze manier identificeerden we 18 genen welke gerelateerd bleken aan het ‘insulin action’ en ‘adipocytokine signaling’ pathway. Die genen met de hoogste opwaartse expressie na sporten en hoogste neerwaartse expressie na een periode van lichamelijke inactiviteit waren muscular fatty acid binding protein 3 (FABP3), de fatty acid oxidizing enzyme hydroxyacyl-CoA dehydrogenase (HADH) en de mitochondrial fatty acid transporter solute carrier 25 family member A20 (SLC25A20). Deze resultaten wijzen op een belangrijke rol voor een verminderd transport en metabolisme van vetzuren in spierweefsel, wat mogelijk een moleculaire verklaring zou kunnen zijn voor de relatie tussen lichamelijke activiteit en insulineresistentie.

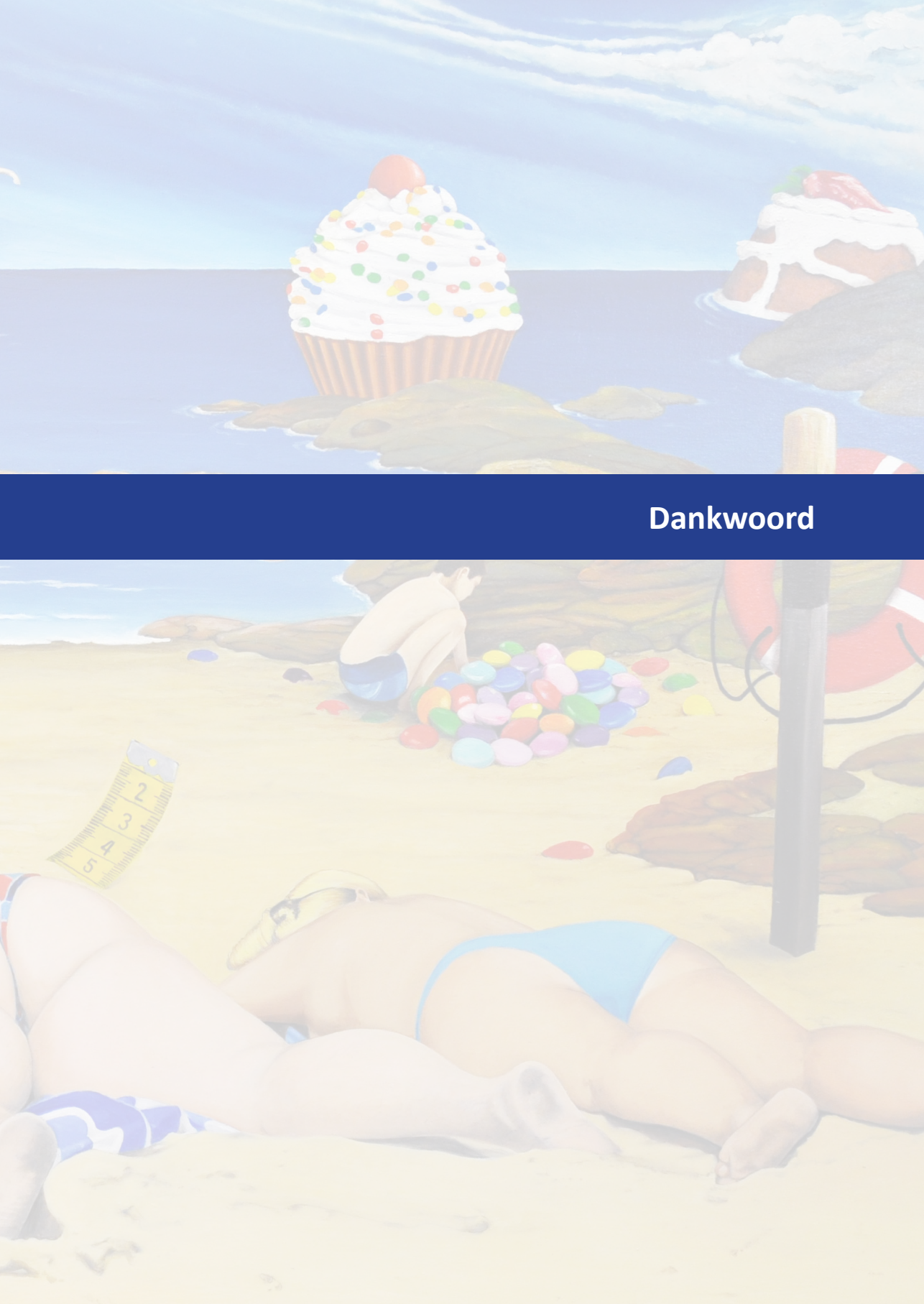
In **hoofdstuk 5** hebben we vervolgens gekeken naar het effect van een zes maanden durend sportprogramma op de insulineresistentie en gelijktijdige veranderingen in genexpressie in het spierweefsel van vrouwen met en zonder het metabool syndroom. Ondanks de afwezigheid van gewichtsverlies verbeterde het sportprogramma de insulineresistentie aanzienlijk in zowel vrouwen met het

metabool syndroom (-45%) als in slanke vrouwen (-22%). Tegelijkertijd bleken in het spierweefsel van vrouwen met het metabool syndroom geselecteerde genen verrijkt aanwezig te zijn in 4 'pathways', waarbij insulin-like growth factor 2 (IGF2) de hoogste opwaartse genexpressie liet zien. In het spierweefsel van slanke vrouwen daarentegen kwamen genen tot verhoogde expressie die betrokken zijn bij het immuunsysteem en onstekingsreacties. Deze resultaten duiden op een verschillend werkingsmechanisme van sporten tussen slanke vrouwen en vrouwen met het metabool syndroom.

In **hoofdstuk 6** onderzochten we het effect van een sportprogramma op de diameter en wanddikte van slagaders bij vrouwen met het metabool syndroom. De dikte van een slagaderwand wordt bepaald door de mate van aderverkalking (atherosclerose), waarbij een dikkere wand duidt op meer aderverkalking. Wederom beoordeelden we tegelijkertijd de effecten van het sportprogramma op genexpressieveranderingen in spierweefsel. Zes maanden intensief sporten induceerde een toename in de diameter van de slagaders en een afname van de wanddikte van de slagader in het been. Er was geen veranderingen opgetreden in de structuur van de halsslagader. Dit suggereert dat sporten meer invloed heeft op de structuur van slagaders in de actieve ledematen dan in de halsslagaders. In het spierweefsel kwamen na afloop van het sportprogramma genen tot expressie die gerelateerd zijn aan de ontwikkeling van bloedvaten ('vasculature development'; 'blood vessel development'; 'blood vessel morphogenesis'; 'angiogenesis'). De genen met de hoogste opwaartse expressie waren delta-like protein-4 (Dll4) en collageen type-1-alfa-1 (COL1A1). Zowel Dll4 en COL1A1 zijn betrokken bij het stabiliseren van de structuur van een bloedvat en zijn nu voor het eerst geïdentificeerd als mogelijke genen betrokken bij aanpassingen in de structuur van een bloedvat door sporten.

In **hoofdstuk 7** wordt een samenvatting gegeven van de huidige kennis, inclusief resultaten van studies uit dit proefschrift, met betrekking tot het werkingsmechanisme van insulineresistentie en het effect van een sportprogramma op de insulineresistentie en structuur van een slagader. We speculeerden over mogelijke werkingsmechanismen betrokken bij de door het sporten-geïnduceerde verbeteringen in insulineresistentie en structuur van een slagader. Ondanks het feit dat obesitas sterk gecorreleerd is met verscheidene metabole en cardiovasculaire risicofactoren, lijkt het hebben van een goede fysieke conditie cruciaal. Obese vrouwen met een metabool gezond fenotype bleken namelijk een betere lichamelijke conditie te hebben en hebben uiteindelijk dus een lager risico op het ontwikkelen van suikerziekte en hart- en vaatziekten. Tevens is het verbeteren van de lichamelijke

conditie door een sportprogramma uiterst effectief als therapie voor insulineresistentie en verbetering van de dikte van de slagaderwand.



Dankwoord

Het einde van dit proefschrift en mijn promotie traject is genaderd. Nog het laatste hoofdstuk schrijven en het boek kan letterlijk en figuurlijk dicht. Wat een tijd in mijn leven. Blijkbaar heeft het zo moeten zijn dat alle life-events in pak ‘m beet 8 jaar moesten gebeuren; afstuderen en mijn eerste baan als arts, de opleiding beginnen tot medisch specialist, een huis kopen, moeder worden, trouwen, mijn geliefde verliezen en dan ook nog eens proberen te promoveren. Dit proefschrift stond om eerlijk te zijn af en toe als laatste op mijn ‘to do list’. Zonder de hulp van een heleboel mensen was dit proefschrift er dan ook niet gekomen. Mijn dank gaat uit naar iedereen die aan de totstandkoming ervan heeft bijgedragen en een aantal wil ik in het bijzonder noemen.

Drie volle onderzoeksdagen voorafgaand aan de trainingen, drie volle onderzoeksdagen na afloop van de trainingen, 6 maanden lang - 3 keer per week - 45 minuten fietsen. Als proefpersoon meedoen aan mijn promotieonderzoek vergde veel fysieke inzet en mentaal doorzettingsvermogen. Mijn dank gaat dan ook allereerst uit naar de deelnemende proefpersonen, zonder wie de onderzoeken niet plaats hadden kunnen vinden. Bedankt voor jullie waardevolle tijd, enthousiasme en soms ook geduld!

Prof. dr. M.T.E. Hopman, beste Maria, ondertussen alweer heel wat jaren geleden hebben we elkaar leren kennen toen ik als student bewegingswetenschappen aan je vroeg of jij eventueel een stageplek voor me had in het buitenland. Ik kwam terecht in Hamilton (Canada) bij Maureen MacDonald. Nadien ben ik altijd de Heintje Davids van de afdeling Fysiologie gebleven. Mijn dank is groot voor je enthousiasme voor mijn onderzoek, maar met name voor de vrijheid die je me hebt gegeven en je onvoorwaardelijke geloof in de goede afloop van mijn promotieonderzoek.

Prof. dr. C.J. Tack, beste Cees, tijdens mijn allerlaatste coschap interne geneeskunde was jij mijn beoordelaar, niet wetende dat we elkaar daarna nog veel vaker zouden treffen. Ik heb het altijd fijn gevonden om met je samen te werken en te luisteren naar je bevlogen verhalen over ons vakgebied. Dank voor je altijd snelle respons op mijn vragen, je betrokkenheid en medisch wetenschappelijke kunde. Mede op jou aanraden heb ik toch maar besloten deze promotie groots te vieren.

De manuscript commissie bestaande uit professor J.W.A. Smit, professor J.A. Veltman en professor M.K.C. Hesselink wil ik bedanken voor hun kritische beschouwing en enthousiaste goedkeuring van het manuscript.

Alle collega's van de afdeling Fysiologie met wie ik in de loop der jaren heb samengewerkt wil ik bedanken voor de inhoudelijke discussies maar ook om wie jullie zijn.

Gerwen, ik vind het geweldig dat je mijn copromotor bent geworden gedurende het traject. Je bent, samen met Linda, van onschatbare waarde geweest dankzij je kennis en kunde op moleculair fysiologisch gebied. Dankzij jullie is het gelukt om deze onderzoekslijn op te zetten en tot enkele mooie publicaties te komen, dank daarvoor. Beste Bregina, bedankt voor alle echo-metingen die je hebt verricht bij mijn proefpersonen, maar nog meer voor je vrolijkheid en je aanstekelijke schaterlach. Jos, jij bént fysiologie en ondanks je afscheid na het bereiken van je pensioenleeftijd, bleef je enthousiast betrokken bij de trainingen van de deelnemers. Noortje, als partner op dezelfde onderzoekslijn en ook als "het altijd in te huren biopten duo" wil ik je bedanken voor de prettige samenwerking. Als er iemand een doorzetter is, dan ben jij het wel Madelijn! Vaak was je ontzettend serieus met je eigen promotie bezig maar op de ontspannen momenten kun jij het hardste lachen van ons allemaal. Ik vond het een eer je paranimf te mogen zijn. Tim, de grapjas van de afdeling met zijn angstaanjagende T-shirts. In het begin kon ik je niet goed inschatten, maar na de eerste indruk heb ik een bijzonder iemand leren kennen. Bedankt voor het geouwehoer maar ook voor de serieuze gesprekken over het leven en de liefde. Ralph, Matthijs, Martijn, Joost, Nathalie en Rebecca, heel veel succes en plezier bij het afronden van jullie promotieonderzoek. Dick, Thijs, Jan, Patricia, Michiel en Miriam jullie zijn mijn voorbeeld en inspiratie als onderzoekers geweest. Dank voor de collegialiteit en gezellige momenten. Tevens wil ik alle studenten die bij mijn projecten betrokken waren bedanken voor hun inzet: Sanne, Anke, Anne, Margo, Paul en Duby. Veel succes met jullie eigen carrières. Anja en Karin van de afdeling Klinische Fysiologie wil ik bedanken voor alle hulp en inzet bij de insuline clamp studies.

Beste collega's van de afdeling Interne Geneeskunde van het RadboudUMC en het Slingeland ziekenhuis. Graag wil ik jullie bedanken voor de betrokkenheid bij mijn promotie en privé leven. De gezamenlijke carpool tochten van en naar Doetinchem waren uitermate heilzaam voor mijn geestelijke gezondheid en met een grote glimlach denk ik hieraan terug. In het bijzonder wil ik professor J. de Graaf bedanken, Jacqueline dank voor je warme hart en betrokkenheid van de afgelopen jaren.

Yvonne en Ben, met name het laatste jaar hebben we weer veel contact en ik waardeer het erg dat ik op jullie kan rekenen en dat Elise altijd bij jullie terecht kan. Stijn, bedankt voor al je goede promotie adviezen en de gezellige diners samen met Malaika. Douwe, ooit, lang geleden zijn we samen begonnen aan ons studentenleven.

Fijn dat je nu, samen met Dorien, mijn paranimf wilt zijn op deze bijzondere dag. Eindelijk de definitieve afsluiting van mijn 'studenten' periode.

Mijn liefste vriendinnen Dorien, Heleen en Jorien. Vanaf het eerste jaar van onze studies zijn we dikke mik en delen we lief en (soms helaas teveel) leed met elkaar. Ik vind het heerlijk om bij jullie te zijn en beschouw onze band als onvoorwaardelijk. Ik hoop jullie te blijven zien en in mijn leven te hebben totdat we allemaal oud, grijs, tandeloos en gerimpeld zijn.

Evelyn en Ineke, veel mensen vragen me vaak of ik nog contact met jullie heb na het overlijden van Koen. Wat een vraag! De afgelopen tien jaar is onze band zeer hecht geworden en ik zou wel tien van zulke schoonmoeders willen hebben.

Zonder de steun van mijn lieve familie zou ik veel minder bereikt hebben dan mogelijk was geweest. Pap en mam, jullie lieten me altijd vrij om mijn eigen keuzes te maken. De voltooiing van dit proefschrift was niet gelukt zonder jullie praktische en emotionele steun. Het is altijd fijn om naar het oosten te rijden voor complete ontspanning en vertroeteling. Ome Hans, bedankt voor het prachtige ontwerp van de cover en je vormgevingsadviezen.

En dan mijn lieve meisje Elise. Het is niet voor te stellen wat wij samen in de relatief korte periode van je leven al hebben meegemaakt. Jij bent mijn vrolijkheid met je grapjes, het altijd maar willen stoeien en oneindig knutselen met eierdozen, loom bandjes en WC rollen. Ik hou van je zoals je bent en zult worden.

Lieve Koen, ondanks dat ik cognitief weet dat je er niet meer bent, ben je elke dag bij mij. Ik weet dat je ontzettend trots op mij en Elise zult zijn geweest en dat geeft me de kracht om door te gaan en nog iets moois van mijn leven te maken. Ik mis je elke dag en hou van je.

List of Publications

List of publications

1. **Poelkens F**, Eijsvogels THM, Brussee P, Verheggen RJHM, Tack CJ, Hopman MTE. Physical fitness can partly explain the metabolically healthy obese phenotype in women. Exp Clin Endocrinol Diabetes. 2014 Feb;122(2):87-91

2. **Poelkens F**, Lammers G, Pardoel EM, Tack CJ, Hopman MTE. Upregulation of skeletal muscle inflammatory genes links inflammation with insulin resistance in women with the metabolic syndrome. Exp Physiol. 2013. 98.10 pp1485-94

3. Lammers G, **Poelkens F**, Duijnhoven NT, Pardoel EM, Hoenderop JG, Thijssen DH, Hopman MT. The expression of genes involved in fatty acid transport and insulin signaling is altered by physical inactivity and exercise training in human skeletal muscle. Am J Physiol Endocrinol Metab. 2012 Nov 15;303(10):E1245-51.

4. Oudegeest-Sander MH, Eijsvogels TH, Verheggen RJ, **Poelkens F**, Hopman MT,Jones H, Thijssen DH. Impact of physical fitness and daily energy expenditure on sleep efficiency in young and older humans. Gerontology. 2013;59(1):8-16.

5. Eijsvogels TM, Veltmeijer MT, Schreuder TH, **Poelkens F**, Thijssen DH, Hopman MT. The impact of obesity on physiological responses during prolonged exercise. Int J Obes (Lond). 2011 Nov 35(11). 1404-12

6. **Poelkens F**, Hopman MT, Tack CC. Letter by Poelkens et al regarding article, "Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study". Circulation 2009 mar3, 119 (8). E225

7. Eijsvogels TM, Thijssen DH, **Poelkens F**, Binkhorst M, Wouters CW, Schouwenberg BJ, Hopman MT. Physical risks whilst walking the Nijmegen

Four Day Marches in 2007: electrolyte imbalance in 1 to 5 walkers. Ned tijdschr Geneeskd. 2008. Jul 12, 152(28).1571-8

8. Groothuis JT, **Poelkens F,** Wouters CW, Kooijman M, Hopman MT. Leg intravenous pressure during head-up tilt. J appl physiol. 2008. Sep, 105(3). 811-5

9. **Poelkens F**, Rakobowchuk M, Burgomaster KA, Hopman MT, Phillips SM, MacDonald MJ. Effect of unilateral resistance training on arterial compliance in elderly men. Appl Physiol Nutr Metab. 2007. Aug 32(4). 670-6.

10. Kooijman M, **Poelkens F**, Rongen GA, Smits P, Hopman MT. Leg blood flow measurements using venous occlusion plethysmography during head-up tilt. Clin Auton Res. 2007. Apr 17 (2). 106-11.

11. **Poelkens F**, Thijssen DH, Kersten B, Scheurwater H, van Laarhoven EW, Hopman MT. Counteracting venous stasis during acute lower limb immobilization. Acta Physiol (Oxf). 2006. Feb 186(2). 111-8.
12. Bleeker MWP, de Groot PCE, **Poelkens F**, Rongen RA, Smits P, Hopman MTE. Vascular adaptations to 4 wk of deconditioning by unilateral lower limb suspension. Am J Physiol Heart Circ Physiol. 2005. 288. 1747-1755.

13. De Groot PC, **Poelkens F**, Kooijman M, Hopman MTE. Preserved flow-mediated dilation in the inactive legs of spinal cord-injured individuals. Am J Physiol Heart Circ Physiol.2004. 287. H374-H380.

Other publications

1. Den Otter RA, Geurts ACH, Van Duijnhoven NT, **Poelkens F**, Duysens J. Changes in lower extremity muscle activity at very slow walking speeds. In Duysens J, Smits-Engelsman BCM, Kingma H, eds. Control of posture and Gait, pp 550-3. 2001.

Curriculum Vitae

Curriculum Vitae

Fleur Poelkens werd op donderdag 27 juli 1978 geboren te Hengelo (Ov). Na het behalen van het VWO diploma aan de openbare scholengemeenschap Bataafse Kamp te Hengelo in 1996 begon zij in datzelfde jaar aan de studie Biologie aan de Katholieke Universiteit Nijmegen (de huidige Radboud Universiteit Nijmegen). Na het behalen van de propedeuse Biologie stapte zij over naar de studie Biomedische Gezondheidswetenschappen, waar zij zich specialiseerde in de bewegingswetenschappen. In het kader van de specialisatie tot bewegingswetenschapper liep zij stage bij het kenniscentrum Research, Development and Education van de Sint Maartenskliniek te Nijmegen (2000) en bij de afdeling Kinesiology van de McMaster University te Hamilton, Canada (2001) onder leiding van dr. Maureen MacDonald. Na haar afstuderen in 2001, stroomde zij door in het verkorte doctoraal programma van de studie Geneeskunde aan de Radboud Universiteit Nijmegen. Tijdens deze periode participeerde zij als onderzoekster van de afdeling Fysiologie onder leiding van professor Maria Hopman in verschillende lopende onderzoeksprojecten. Begin 2006 behaalde zij haar artsexamen, waarna ze begon als arts-assistent in opleiding bij de interne geneeskunde (opleiders: dr. A. Mudde, prof. dr. J.W.M. van der Meer, prof. dr. J.W.A. Smit en prof. dr. J. de Graaf) gecombineerd met haar promotieonderzoek '*Exercise in women with the metabolic syndrome; molecular mechanisms of exercise-induced changes in insulin resistance and vascular structure'* in het Radboudumc middels een AGIKO stipendium van ZonMw. Haar promotieonderzoek werd begeleid door professor Maria Hopman en professor Cees Tack, een samenwerking van de afdelingen Fysiologie en Algemene Interne Geneeskunde. De onderzoeksresultaten staan in dit proefschrift beschreven en werden gepresenteerd op (inter)nationale congressen. Ondertussen heeft zij haar (voor-) opleiding interne geneeskunde afgerond en is zij vanaf januari 2014 gestart met de specialisatie tot reumatoloog in de Sint Maartenskliniek en het Radboudumc.

Fleur is weduwe van Koen Brand (2012) en samen hebben zij één dochter, Elise (2009).