

Medicine & Science IN Sports & Exercise

The Official Journal of the American College of Sports Medicine

www.acsm-msse.org

... Published ahead of Print

Exercise Improves Insulin Sensitivity in the Absence of Changes in Cytokines

Rebecca J.H.M. Verheggen¹, Fleur Poelkens¹, Sean H.P.P. Roerink², Rob E.F.S. Ramakers¹,
Milène Catoire¹, Ad R.M.M. Hermus², Dick H.J. Thijssen^{1,3}, and Maria T.E. Hopman¹

¹Department of Physiology, ²Department of Internal Medicine, Division of Endocrinology;
Radboud university medical center, Radboud Institute for Health Sciences, Nijmegen, the
Netherlands; ³Research Institute for Sport and Exercise Sciences, Liverpool John Moores
University, Liverpool, United Kingdom

Accepted for Publication: 10 June 2016

Medicine & Science in Sports & Exercise®. Published ahead of Print contains articles in unedited manuscript form that have been peer reviewed and accepted for publication. This manuscript will undergo copyediting, page composition, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered that could affect the content.

Exercise Improves Insulin Sensitivity in the Absence of Changes in Cytokines

REBECCA J.H.M. VERHEGGEN, MD, MSc¹

FLEUR POELKENS, MD, PhD¹

SEAN H.P.P. ROERINK, MD, MSc²

ROB E.F.S. RAMAKERS, BSc¹

MILÈNE CATOIRE, PhD¹

AD R.M.M. HERMUS, MD, PhD²

DICK H.J. THIJSEN, PhD^{1,3}

MARIA T.E. HOPMAN, MD, PhD¹

¹*Department of Physiology*, ²*Department of Internal Medicine*, Division of Endocrinology; Radboud university medical center, Radboud Institute for Health Sciences, Nijmegen, the Netherlands; ³*Research Institute for Sport and Exercise Sciences*, Liverpool John Moores University, Liverpool, United Kingdom

Author for correspondence:

Maria Hopman MD, PhD, Dept. of Physiology (392), Radboud university medical center, Radboud Institute for Health Sciences, PO Box 9101, 6500 HB Nijmegen, The Netherlands

e-mail: maria.hopman@radboudumc.nl

Tel. (+31) (0)24 36 14906

Fax. (+31) (0)24 354 0535

None of the authors have any conflict of interest to declare, nor are funded by any organisation for the submitted work. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

SHORT TITLE: insulin sensitivity and cytokines in exercise

ACCEPTED

ABSTRACT

Purpose. The benefits of aerobic exercise training on insulin sensitivity in subjects with the metabolic syndrome (MetS) are, at least in part, associated with changes in cytokines. Recent studies identified novel cytokines (e.g. fractalkine, omentin and osteopontin) that are strongly involved in glucose homeostasis and therefore potentially contribute in the exercise-induced changes in insulin sensitivity. Therefore, we aim to examine changes in skeletal muscle RNA expression and plasma levels of novel cytokines after exercise training, and correlate these changes to the exercise-induced changes in insulin sensitivity. **Methods.** Women with the metabolic syndrome (MetS, n=11) and healthy women (n=10) participated in a 6-month aerobic exercise training intervention (3/week, 45min per session at 65%-85% of individual heart rate reserve). Before and after training, we examined insulin sensitivity (M-value during hyperinsulinemic euglycaemic clamp), circulating blood levels of cytokines (venous blood sample; leptin, adiponectin, omentin, fraktalkin, osteopontin). Skeletal muscle RNA-expression of these cytokines (muscle biopsy) was examined in two subgroups (MetS n=6; healthy women n=6). **Results.** At baseline, plasma levels of omentin (85.8 ± 26.2 ng/ml) and adiponectin (5.0 ± 1.7 μ g/ml) levels were significantly higher in controls compared to MetS (51.1 ± 27.1 ; 3.6 ± 1.1 respectively), and leptin levels were lower in controls (18.7 ± 11.5 ng/ml vs 53.0 ± 23.5). M-value was significantly higher in controls (8.1 ± 1.9 mg/kg/min) than in MetS (4.0 ± 1.7). Exercise training significantly improved M-values in both groups ($P < 0.01$). Exercise training did not alter plasma and skeletal muscle RNA-expression levels of cytokines, whilst no correlation was observed between changes in cytokine level/RNA-expression and M-values ($P > 0.05$). **Conclusion.** Whilst exercise training successfully improves insulin sensitivity in MetS and healthy women, we found no change in plasma and mRNA expression levels of novel cytokines. **Keywords:** metabolic syndrome, exercise training, RNA-expression

INTRODUCTION

The metabolic syndrome (MetS) comprises a set of interrelated risk factors resulting in an increased risk for development of type 2 diabetes mellitus and cardiovascular diseases.(13, 26) The pathophysiology of MetS is characterized by the presence of peripheral insulin resistance, which is caused by a low physical activity level and excessive central adiposity.(11) Several previous intervention studies have demonstrated that exercise training is a powerful, non-pharmacological tool to improve insulin sensitivity via a number of metabolic adaptations.(18, 32) Part of these adaptations may be related to exercise-induced alterations in cytokines. These pro- and anti-inflammatory factors play a pivotal role in the presence of low-grade inflammation that underlies a decline in insulin sensitivity.(19, 37) Therefore, the measurement of cytokines prior to and after exercise training may provide potential insight in the mechanisms underlying the benefits of exercise training on insulin sensitivity.

Recent studies have identified novel cytokines, which may play an important role in glucose homeostasis and the presence of inflammation. Fractalkine is secreted in both adipose tissue and skeletal muscle,(5, 36) and plays a role in the regulation of pancreatic islet β cell function. In humans, fractalkine has been proven to modulate monocyte adhesion in adipose tissue, thereby influencing chronic inflammation processes,(36) and is independently associated with markers of insulin resistance (HOMA-IR).(10) Omentin is a cytokine mainly secreted by visceral adipose tissue. Circulating omentin levels are negatively correlated with insulin resistance and are decreased in obesity and type 2 diabetes.(39) Osteopontin is biosynthesized in numerous cell types including macrophages and myoblasts in skeletal muscle.(41) In rodents, osteopontin influences macrophage recruitment in adipose tissue and thereby contributes to the inflammatory

state. In obesity models in mice, circulating osteopontin levels are increased, whilst mice with a lack in osteopontin display improved insulin sensitivity.(28) Finally, vaspin is a cytokine that is mainly secreted in visceral adipose tissue and is reported to have insulin-sensitizing effects in rodents. (2, 45) Currently, it is not known whether the benefits of exercise training on insulin sensitivity are related to changes in these novel cytokines.

The first aim of this study was to examine whether 6 months of aerobic exercise training alters circulating levels as well as RNA-expression levels in skeletal muscle of a set of selected novel (i.e. fractalkine, omentin, osteopontin, vaspin) and traditional (i.e. adiponectin, leptin and interleukin-6) cytokines, and whether these changes are associated with exercise training-induced adaptations in insulin sensitivity in subjects with MetS and healthy controls. We hypothesize that the benefits of aerobic exercise training on insulin sensitivity in MetS may be associated with alterations in levels of these cytokines.

METHODS

Subjects

Eleven women with MetS and ten lean, age-matched, sedentary control women were included 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, including waist circumference >88 cm, triglycerides >1.7 mmol/l, High Density Lipoprotein (HDL)-cholesterol <1.3 mmol/l, blood pressure >130/85 mmHg and/or the use of antihypertensive medication, and fasting glucose levels >6.1 mmol/l.(1) Lean women were defined as having a body mass index (BMI) <25 kg/m² and the absence of all metabolic syndrome criteria. Pre-, peri- and

postmenopausal women were included. Women were considered peri-menopausal when they experienced a persistent change in menstrual cycles of at least seven days, or a period of amenorrhea of 60 days or more. Post-menopause was defined as a period of amenorrhea of 12 months or more.⁽¹⁵⁾ We excluded women with a medical history of known diabetes mellitus and/or cardiovascular diseases, liver or renal diseases, smoking, who consume more than two units of alcohol (10 g) a day, or perform regular physical activity >2 hours a week. Before participation, written informed consent was obtained. This study was approved by the Medical Ethical Committee of the Radboud university medical center, and was conducted in accordance with the Declaration of Helsinki (2000).

Study design

All subjects who participated in this study were engaged in a six month aerobic exercise training intervention. Before and after the intervention, a venapuncture, hyperinsulinemic, euglycemic clamp, vastus lateralis muscle biopsy and a maximal cycling test was conducted in each participant.

Exercise training

During this training study, all women trained three times a week under the supervision of an experienced researcher. Training consisted of cycling exercise on an 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 ending with a cooling-down of 5 minutes. Workload was increased based on improvements in physical fitness level across the six month intervention. Exercise intensity was monitored and documented with the use of heart rate

monitors (Polar). Women had to attend at least 90% of the training session during this six month period to be eligible for inclusion of the statistical analysis.

Measurements

Insulin sensitivity. Peripheral tissue sensitivity to exogenous insulin was measured using a hyperinsulinemic euglycemic clamp as previously described.(8) The clamp was performed at least 48 hours after cessation of the last exercise bout. After an overnight fast (10 hours), the subject was placed in the supine position in a quiet, temperature controlled (22 – 24 °C) room. Insulin (Actrapid, Novo-Nordisk, Copenhagen, Denmark) was infused intravenously in a dose of $430 \text{ pmol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ($60 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) for 120 minutes. Insulin $50 \text{ U} \cdot \text{ml}^{-1}$ was diluted in 48 ml NaCl 0.9% with the addition of 2 ml blood from the subject to a concentration of $1 \text{ U} \cdot \text{ml}^{-1}$. Venous plasma glucose concentrations were clamped at $5.0 \text{ mmol} \cdot \text{L}^{-1}$ 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, with use of the glucose oxidation method (Beckman Glucose Analyzer 2, Beckman Instruments Inc, Fullerton, CA 92634, USA). Insulin was measured in duplicate conform international standard 83/500 by an in-house radio-immunoassay (RIA) with the use of an anti-human insulin antiserum raised in guinea pig and radio-iodinated 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 $33 \text{ mU} \cdot \text{L}^{-1}$. Whole body glucose disposal during the last 30 minutes of the euglycemic clamp was calculated as the M-value.

Plasma cytokine levels. Fasting venous blood samples collected prior to the start of the hyperinsulinemic euglycemic clamp were used to determine glucose, cholesterol and triglycerides via standard laboratory methods. Fasting venous blood was sampled at least 48 hours after cessation of the last exercise bout. 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 marker C-reactive protein by Dako high-sensitivity ELISA (Glostrup, Denmark) and fractalkine, omentin, osteopontin, vaspin and interleukin 6 (IL-6) by Luminex assay (Austin, Texas, USA).

Skeletal muscle cytokine RNA gene expression. Before and after the six month training period, biopsies from the vastus lateralis muscle were taken after a standardized 250 kcal breakfast (79% carbohydrates, 11.2% protein, 9.8% fat). The muscle biopsies after the exercise training period, were taken at least 48 hours after the last exercise session. From muscle biopsies of six women of each group total RNA was isolated and purified. The physiological characteristics of these subgroups were representative for the whole group (see Table, Supplemental Digital Content 1, Characteristics of the entire group versus the subgroups, <http://links.lww.com/MSS/A722>). RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was analyzed on an Agilent Bioanalyser (Santa Clara, CA, USA). 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 average fluorescence intensity of all genes was calculated using the Robust Multiarray Analysis (RMA) Algorithm, including a quantile normalization and using a background correction for GC-content.(20) Microarray analysis was performed using

MADMAX pipeline for statistical analysis of microarray data.(24) Quality control was performed and all arrays met our criteria, except arrays from three participants. Data of these participants was excluded from further analysis. Microarray data were filtered, and probe sets with expression values higher than 20 on more than 5 arrays were considered to be expressed and were selected for further statistical analysis. Significant differences in expression levels were assessed using Intensity-Based Moderated T-statistics (IBMT).(35) Gene expression was defined as significantly changed when the p-value was <0.01 , an alpha that is commonly used in gene expression studies.(5, 35) The protocol used was compliant with the MIAMI guidelines, and data have been submitted to the Gene Expression Omnibus (GEO) repository under no. GSE43760.

Antropometry. Before and after six months of endurance training we examined height and body weight (Seca 888 Scale, Seca, Hamburg) to calculate body mass index (BMI). Waist and abdominal circumference were measured with a measuring tape (Seca 201, Chino, USA) to calculate waist-to-hip ratio. Plasma cholesterol, triglyceride and glucose were determined in fasting venous blood samples using standard laboratory methods. Before and after the training period a total body Dual-X-ray Absorptiometry (DXA) scan was performed to determine lean body mass, total fat mass and trunk fat mass (QDR 4500 densitometer, Hologic Inc. Waltham, MA).

Daily activity levels. At baseline, daily activity levels were assessed with an accelerometer (SenseWear Pro3 Armband, Body Media Inc., Pittsburgh, PA, USA). From each 24 hour interval, data were analyzed from 0700 to 2300 h with a minimum on-body time of 85%. At least three days had to fulfill these criteria to be used for analysis. Time per day spent in vigorous

intensity activities (>6.0 METs) and time per day in very vigorous intensity activity (>9.0 METs) were calculated.(16)

Dietary intake. During the training intervention, women were instructed not to change their caloric intake. To assess potential changes in daily food intake, women were asked to record 3-day dietary intake records before and in the last week of the training intervention. Dietary records were analyzed with Eetmeter Software (Voedingscentrum, the Hague, Netherlands). Only data of women who completed the dietary records on at least three days before and at the end of the training period was included for analysis.

Cardio-respiratory fitness level. Women performed a maximal exercise test on an electrically braked leg-cycling ergometer (Lode, Angio 300, Groningen, the Netherlands) using an incremental protocol, to assess their cardio-respiratory fitness level. 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 ($\text{VO}_{2\text{max}}$) 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, capillary blood lactate level (Roche Diagnostics GmbH, Mannheim, Germany) was measured.

Statistical analysis

All statistical analyses were conducted in SPSS 20 (Statistical Package for Social Sciences 20.0, SPSS Inc., Chicago, Illinois, USA). The sample size calculation to achieve statistical power were based on data from previous research published on the effects of exercise training on circulating

leptin levels in obese individuals.(23) The estimated sample size was 10 participants in order to detect a difference of 4.1 ng/ml in leptin levels after training ($\alpha=0.05$, $\beta=0.85$). Baseline characteristics of the groups were compared with the use of an unpaired t-test. A Two-way repeated ANOVA was used to examine the impact of exercise training on VO_{2max} , insulin sensitivity and cytokines. Correlations between circulating levels of cytokines and the M-value were assessed with the use of Spearman's correlation coefficient. The level of statistical significance for data except the microarray data was defined at $\alpha=0.05$. In the microarray analysis, genes were defined as significantly changed at $\alpha=0.01$. Data are presented as mean \pm SD, unless stated otherwise.

RESULTS

Subject characteristics

The physical characteristics of the women with the MetS and the lean sedentary controls are presented in Table 1. In both groups pre-, peri- and post-menopausal women were included (Table 1). None of the included women used hormone replacement therapy, since this is known to directly influence circulating cytokine levels(38). As a consequence of the selection procedure, women with MetS demonstrated a significantly higher body weight, BMI, blood pressure and triglycerides at baseline (Table 1). Analysis of physical activity data at baseline demonstrated that the women with MetS spent 10 minutes per day in vigorous intensity activities and 0 minutes per day in very vigorous intensity activities. Lean controls spent on average 19 minutes per day in vigorous intensity activities and 0 minutes per day in very vigorous intensity.

Exercise training intervention

Eleven women with MetS and ten lean control women all successfully completed the exercise intervention, with a training compliance of 92%. Cardio-respiratory fitness, determined as the peak oxygen uptake, improved significantly in both MetS and the lean control women, but improvement did not differ between the two groups (Table 1). Waist circumference improved significantly in both groups, whereas other body composition measurements showed no change over time, but remained different between the two groups. Analysis of the dietary records in a subgroup of women (MetS $n = 4$; lean control women $n = 5$) showed no significant change in caloric intake during the training intervention (pre: 7.6 ± 1.2 MJ/day; post: 7.2 ± 1.2 MJ/day; $p = 0.12$). Circulating markers of glucose homeostasis (i.e. fasting glucose and insulin) and lipids showed no change over time in both groups, whilst blood pressure decreased (Table 1). Insulin sensitivity (M-value) improved significantly in both groups, with no differences in the magnitude of improvement between both groups (Figure 1). Three out of eleven women with the metabolic syndrome (27%) did not meet the criteria for the metabolic syndrome anymore after six months of cycling training.

Cytokines

Plasma cytokine levels. At baseline, plasma levels of omentin and adiponectin were significantly higher in healthy control women, whilst leptin levels were lower in healthy women compared to women with MetS. No differences between the groups were found for the other cytokines (Figure 2). Exercise training did not change plasma levels of cytokines in both groups (Figure 2). At baseline, circulating vaspin did not reach the minimum detecting value of 0.16 ng/ml in $n=8$ women with the metabolic syndrome and in $n=6$ lean control women. Plasma levels of IL-6 did

not meet the minimal detection value of 3 pg/ml at baseline in all lean control women (n=10) and in most women with the metabolic syndrome (n=9). After the training period, levels of both vaspin and IL-6 remained below our minimal detection limit in these subjects. Since a large proportion of the individuals included in this study did not show detectable circulating IL-6 and vaspin levels, data on both IL-6 and vaspin were excluded from further analysis.

Skeletal muscle mRNA expression. Skeletal muscle gene expression levels of novel and known cytokines were compared before and after the training intervention in five women with the metabolic syndrome and four lean control women. In both groups, no differences were found in gene expression levels of the cytokines before and after exercise training (Table 2).

Correlation analysis

At baseline we found a significant inverse correlation between insulin sensitivity (i.e. M-value) and circulating levels of leptin ($R=0.65$, $P=0.002$), whereas no correlation was found between the M-value and adiponectin ($R=0.35$, $P=0.124$), fractalkine ($R=-0.22$, $P=0.33$), omentin ($R=0.38$, $P=0.09$), or osteopontin ($R=0.14$, $P=0.55$). When exploring the exercise training induced changes in insulin sensitivity (Δ M-value) and the changes in cytokines (Δ Fractalkine ($R=-0.36$, $P=0.18$); Δ Omentin ($R=0.22$, $P=0.34$) ; Δ Osteopontin ($R=-0.13$, $P=0.59$), Δ Leptin ($R=-0.24$, $P=0.29$), Δ Adiponectin($R=0.37$, $P=0.10$)), no significant correlations were found. Furthermore, no correlation between weight change (Δ weight) and changes in Δ Fractalkine ($R=-0.20$, $P=0.40$) ; Δ Omentin ($R=-0.25$, $P=0.27$); Δ Osteopontin ($R=0.10$, $P=0.68$), Δ Leptin ($R=-0.22$, $P=0.34$) or Δ Adiponectin ($R=0.06$, $P=0.79$) was present.

DISCUSSION

Our results confirm that exercise training successfully improves insulin sensitivity, in both women with the metabolic syndrome and lean, sedentary controls. The improvement in insulin sensitivity after exercise training was not accompanied by changes in circulating levels of novel (i.e. fractalkine, omentin, osteopontin and vaspin) and traditional (i.e. leptin, adiponectin and IL-6) cytokines. Furthermore, gene expression levels of these cytokines in skeletal muscle did not change after exercise training. Taken together, these results suggest that cytokines are not associated with exercise-induced improvements in insulin sensitivity in women.

Baseline

In this study, we confirmed with use of the M-value as a gold standard technique, that insulin sensitivity in women with the MetS is significantly lower compared to age-matched lean controls. Furthermore, we found that lean controls show lower leptin levels, and higher omentin and adiponectin levels than women with MetS. Whilst previous research showed that omentin is lower in subjects with obesity and type 2 diabetes, this study is the first to show that omentin is also lower in women with MetS compared to controls.(34, 39) Previous studies found that fractalkine, osteopontin and vaspin are associated with insulin resistance and obesity.(2, 10, 36) However, no baseline differences in circulating levels of cytokines were found between women with MetS and controls in our study. Furthermore, we found no correlation between insulin sensitivity and levels of these cytokines. One important difference with previous studies is that we used the gold standard to measure insulin sensitivity rather than the less invasive, but also less reliable oral glucose tolerance test (OGTT). Such measures for insulin sensitivity, that use fasting levels of glucose and insulin have clear limitations,(30) and should therefore be

interpreted with caution to examine the relationship between cytokines and insulin sensitivity. With the use of M-value, our study tempers the results of previous work that suggested an association between serum levels of fractalkine, omentin, osteopontin, vaspin with insulin sensitivity.

Training

Six months of aerobic exercise training significantly improved VO_{2max} in both women with MetS and sedentary control women. In both groups, no changes in body mass and waist to hip ratio were observed. Furthermore, circulating levels of cytokines and their gene expression in skeletal muscle did not change after training. The effect of aerobic exercise training on leptin and adiponectin is not clear and conflicting results have been reported.(4, 40) Some studies showed a decrease in leptin levels after aerobic exercise training in obese women,(14, 23, 31) whilst others demonstrated no change.(31) Some studies have shown that adiponectin increases after exercise training in obese subjects,(3, 6) but also an absence of a response has been reported.(17, 31) In our study we found no change in leptin and adiponectin both in circulating serum levels and on gene expression level in skeletal muscle, despite the significant improvement in M-value. Furthermore, our correlation analysis was unable to demonstrate an association between changes in leptin and adiponectin and changes in M-value. Taken together, it is unlikely that leptin or adiponectin play a pivotal role in the mechanisms underlying a change in M-value by exercise training.

The response of omentin, fractalkine and osteopontin to exercise training in humans have scarcely been studied and evidence is inconclusive. Omentin has been studied most extensively

in relation to exercise training of non-diabetic individuals and in combination with parameters of glucose homeostasis. Saremi et al. reported an increase in circulating omentin levels after 12 weeks of aerobic exercise (5x/week, 50-60 minutes), which was accompanied by a significant decrease in HOMA-IR and significant weight loss.(34) Another study reported no effect of 3 months (3x/week, 30 minutes) of exercise on circulating omentin levels women, whilst BMI and HOMA-IR decreased significantly.(34, 43) One previous study by Catoire et al. examined the response of fractalkine to combined exercise training (2x/ week endurance exercise; 1x/week resistance exercise; 45 minutes each) of 12 weeks and found no change in RNA expression levels and circulating levels of fractalkine.(5) Our study confirms these findings after a 6 month endurance training intervention in women. Two previous studies investigated circulating osteopontin levels before and after an aerobic training intervention, without assessing measurements of insulin sensitivity and both found no change. (9) (44) In line with previous work our study demonstrates that omentin, osteopontin and fractalkine do not change after training. An important difference between studies that did find an effect on cytokines versus those who did not is the presence of weight loss. It has been suggested in previous reviews that considerable weight loss (of at least 5%) is needed to achieve a change in cytokine expression,(22, 40) and thereby circulating levels. However, in exercise training studies the relation between weight loss and change in cytokines seems less clear. In some exercise studies significant weight loss is present, whilst no change in cytokines occurred.(9, 34, 43) Furthermore, endurance training does not necessarily result in weight loss. During endurance training adipose tissue depots may decrease, whilst lean body mass and/or circulating blood volume may increase.(7, 21, 33) These counteracting processes may result in the absence of weight loss during exercise training. Indeed, in our study no correlation was present between

weight change and change in circulating cytokines. Furthermore, we demonstrated significant and clinically relevant improvements in $\text{VO}_{2\text{max}}$ and insulin sensitivity in the absence of weight loss and changes in plasma cytokine levels and skeletal muscle RNA expression. This suggests that exercise-induced improvements in glycemic control are not accompanied by a change in cytokines.

Another explanation for the differences between training studies on cytokine responses might be related to training frequency and intensity(42) Whilst we implemented a training frequency of three times per week, Saremi implemented a frequency of five training sessions per week and found a significant increase in omentin levels.(34) Urbanova et al. implemented a 3x/week protocol and found no change in omentin levels.(43) Several studies that investigated leptin and adiponectin applied similar training frequency and found conflicting cytokine responses.(3, 6, 14, 23) Possibly, a higher training frequency may be needed to cause a change cytokines.

A more plausible explanation for the absence of changes in circulating cytokines and their expression in skeletal muscle in our study relates to a difference in time-dependent responses of cytokines and M-value to exercise training. Each training bout elicits acute changes in energy metabolism and homeostasis, and also in circulating cytokine levels and their expression. Some acute changes in cytokines and their expression will persist for hours, whilst others quickly disappear after cessation of exercise.(12, 25) Potentially, the cytokine response to an acute bout of exercise can influence metabolic adaptations that contribute to a change in insulin sensitivity. However, with our study design acute changes in cytokines after exercise were not examined. In the discussed exercise training studies, blood (and tissue) samples were collected at 24 hours,(9,

14) 48 hours(34, 44) or 72 hours(31) after the last exercise session, whilst the majority of the authors did not report the timing of sampling. Considering the responses of cytokines to an acute exercise bout, this heterogeneity among study designs might be an explanation for differences found in the response of cytokines to training. Very little is known about the specific time course of expression levels and circulating levels of well-investigated and recently discovered cytokines after an exercise bout. This knowledge is needed to interpret each study on its merits.

Limitations. Microarray analysis was performed in a subgroup of nine women, which limited statistical power. Both circulating levels and mRNA expression levels of cytokines in skeletal muscle were analyzed. Since these subgroups were a good representation of the whole group, we do not expect larger numbers would have altered the main outcomes of our study. In this study pre-, peri- and post-menopausal women were included, which provides a well representation of the entire female population in this age category. Although the menopause affects fat distribution, its effects on cytokines is less clear.(27, 29) We therefore do not believe that the main outcome of this study is influenced by menopausal status. In this study we explored cytokine expression levels in skeletal muscle, whilst adiponectin, omentin and vaspin might not be expressed in this tissue. However, gene expression analysis allows us to examine the potential role of skeletal muscle as source for circulating cytokines. Since white adipose tissue is also an important source for circulating cytokines, future work should examine expression changes in this tissue.(40)

Conclusion

In conclusion, this study shows that exercise training successfully improves insulin sensitivity and physical fitness in both women with the metabolic syndrome and lean controls. The improvement in insulin sensitivity after exercise training was not accompanied by or correlated with changes in circulating levels or gene expression levels in skeletal muscle of novel (i.e. fractalkine, omentin, osteopontin and vaspin) and traditional (i.e. leptin, adiponectin and IL-6) cytokines. Our data may suggest that exercise-induced improvements in insulin sensitivity are not accompanied by a change in cytokines.

Acknowledgements

None of the authors have any conflict of interest to declare, nor are funded by any organisation for the submitted work.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

ACCEPTED

References

1. Alberti KG, Eckel RH, Grundy SM et al. 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(16):1640-5.
2. Bluher M. Vaspilin in obesity and diabetes: pathophysiological and clinical significance. *Endocrine*. 2012;41(2):176-82.
3. Bluher M, Bullen JW, Jr., Lee JH et al. Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training. *The Journal of clinical endocrinology and metabolism*. 2006;91(6):2310-6.
4. Bouassida A, Zalleg D, Bouassida S et al. Leptin, its implication in physical exercise and training: a short review. *Journal of sports science & medicine*. 2006;5(2):172-81.
5. Catoire M, Mensink M, Kalkhoven E, Schrauwen P, Kersten S. Identification of human exercise-induced myokines using secretome analysis. *Physiological genomics*. 2014;46(7):256-67.
6. Christiansen T, Paulsen SK, Bruun JM, Pedersen SB, Richelsen B. Exercise training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects: a 12-week randomized intervention study. *American journal of physiology. Endocrinology and metabolism*. 2010;298(4):E824-31.
7. Convertino VA. Blood volume: its adaptation to endurance training. *Med Sci Sports Exerc*. 1991;23(12):1338-48.
8. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *The American journal of physiology*. 1979;237(3):E214-23.
9. Duggan C, Xiao L, Wang CY, McTiernan A. Effect of a 12-month exercise intervention on serum biomarkers of angiogenesis in postmenopausal women: a randomized controlled trial. *Cancer epidemiology, biomarkers & prevention : a publication of the*

- American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* 2014;23(4):648-57.
10. Ebert T, Hindricks J, Kralisch S et al. Serum levels of fractalkine are associated with markers of insulin resistance in gestational diabetes. *Diabetic medicine : a journal of the British Diabetic Association.* 2014;31(8):1014-7.
 11. Eckel RH, Alberti KG, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet.* 2010;375(9710):181-3.
 12. 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(9):e74098.
 13. Ford ES, Li C, Sattar N. Metabolic syndrome and incident diabetes: current state of the evidence. *Diabetes care.* 2008;31(9):1898-904.
 14. Friedenreich CM, Neilson HK, Woolcott CG et al. Changes in insulin resistance indicators, IGFs, and adipokines in a year-long trial of aerobic exercise in postmenopausal women. *Endocrine-related cancer.* 2011;18(3):357-69.
 15. Harlow SD, Paramsothy P. Menstruation and the menopausal transition. *Obstetrics and gynecology clinics of North America.* 2011;38(3):595-607.
 16. Haskell WL, Lee IM, Pate RR et al. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Medicine and science in sports and exercise.* 2007;39(8):1423-34.
 17. Hayashino Y, Jackson JL, Hirata T et al. Effects of exercise on C-reactive protein, inflammatory cytokine and adipokine in patients with type 2 diabetes: a meta-analysis of randomized controlled trials. *Metabolism: clinical and experimental.* 2014;63(3):431-40.
 18. Henriksen EJ. Invited review: Effects of acute exercise and exercise training on insulin resistance. *Journal of applied physiology (Bethesda, Md. : 1985).* 2002;93(2):788-96.
 19. Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006;444(7121):860-7.

20. Irizarry RA, Hobbs B, Collin F et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)*. 2003;4(2):249-64.
21. Janssen I, Fortier A, Hudson R, Ross R. Effects of an energy-restrictive diet with or without exercise on abdominal fat, intermuscular fat, and metabolic risk factors in obese women. *Diabetes Care*. 2002;25(3):431-8.
22. Klimcakova E, Kovacikova M, Stich V, Langin D. Adipokines and dietary interventions in human obesity. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. 2010;11(6):446-56.
23. Kondo T, Kobayashi I, Murakami M. Effect of exercise on circulating adipokine levels in obese young women. *Endocrine journal*. 2006;53(2):189-95.
24. Lin K, Kools H, de Groot PJ et al. MADMAX - Management and analysis database for multiple ~omics experiments. *Journal of integrative bioinformatics*. 2011;8(2):160.
25. 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 journal : official publication of the Federation of American Societies for Experimental Biology*. 2005;19(11):1498-500.
26. Malik S, Wong ND, Franklin SS et al. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. *Circulation*. 2004;110(10):1245-50.
27. McKane WR, Khosla S, Peterson JM, Egan K, Riggs BL. Circulating levels of cytokines that modulate bone resorption: effects of age and menopause in women. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 1994;9(8):1313-8.
28. Nomiya T, Perez-Tilve D, Ogawa D et al. Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *The Journal of clinical investigation*. 2007;117(10):2877-88.
29. Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. *Endocrine reviews*. 2002;23(1):90-119.

30. Pisprasert V, Ingram KH, Lopez-Davila MF, Munoz AJ, Garvey WT. Limitations in the use of indices using glucose and insulin levels to predict insulin sensitivity: impact of race and gender and superiority of the indices derived from oral glucose tolerance test in African Americans. *Diabetes care*. 2013;36(4):845-53.
31. Polak J, Klimcakova E, Moro C et al. Effect of aerobic training on plasma levels and subcutaneous abdominal adipose tissue gene expression of adiponectin, leptin, interleukin 6, and tumor necrosis factor alpha in obese women. *Metabolism: clinical and experimental*. 2006;55(10):1375-81.
32. Rockl KS, Witczak CA, Goodyear LJ. Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. *IUBMB life*. 2008;60(3):145-53.
33. Ross R, Rissanen J, Pedwell H, Clifford J, Shragge P. Influence of diet and exercise on skeletal muscle and visceral adipose tissue in men. *Journal of applied physiology (Bethesda, Md. : 1985)*. 1996;81(6):2445-55.
34. Saremi A, Asghari M, Ghorbani A. Effects of aerobic training on serum omentin-1 and cardiometabolic risk factors in overweight and obese men. *Journal of sports sciences*. 2010;28(9):993-8.
35. Sartor MA, Tomlinson CR, Wesselkamper SC, Sivaganesan S, Leikauf GD, Medvedovic M. Intensity-based hierarchical Bayes method improves testing for differentially expressed genes in microarray experiments. *BMC bioinformatics*. 2006;7:538.
36. Shah R, Hinkle CC, Ferguson JF et al. Fractalkine is a novel human adipochemokine associated with type 2 diabetes. *Diabetes*. 2011;60(5):1512-8.
37. Stienstra R, van Diepen JA, Tack CJ et al. Inflammasome is a central player in the induction of obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(37):15324-9.
38. Straub RH, Hense HW, Andus T, Scholmerich J, Riegger GA, Schunkert H. Hormone replacement therapy and interrelation between serum interleukin-6 and body mass index in postmenopausal women: a population-based study. *The Journal of clinical endocrinology and metabolism*. 2000;85(3):1340-4.

39. Tan BK, Adya R, Randeve HS. Omentin: a novel link between inflammation, diabetes, and cardiovascular disease. *Trends in cardiovascular medicine*. 2010;20(5):143-8.
40. Thompson D, Karpe F, Lafontan M, Frayn K. Physical activity and exercise in the regulation of human adipose tissue physiology. *Physiological reviews*. 2012;92(1):157-91.
41. Uaesoontrachoon K, Yoo HJ, Tudor EM, Pike RN, Mackie EJ, Pagel CN. Osteopontin and skeletal muscle myoblasts: association with muscle regeneration and regulation of myoblast function in vitro. *The international journal of biochemistry & cell biology*. 2008;40(10):2303-14.
42. Umpierre D, Ribeiro PA, Schaen BD, Ribeiro JP. Volume of supervised exercise training impacts glycaemic control in patients with type 2 diabetes: a systematic review with meta-regression analysis. *Diabetologia*. 2013;56(2):242-51.
43. Urbanova M, Dostalova I, Trachta P et al. Serum concentrations and subcutaneous adipose tissue mRNA expression of omentin in morbid obesity and type 2 diabetes mellitus: the effect of very-low-calorie diet, physical activity and laparoscopic sleeve gastrectomy. *Physiological research / Academia Scientiarum Bohemoslovaca*. 2014;63(2):207-18.
44. Venojarvi M, Korkmaz A, Wasenius N et al. 12 weeks' aerobic and resistance training without dietary intervention did not influence oxidative stress but aerobic training decreased atherogenic index in middle-aged men with impaired glucose regulation. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2013;61:127-35.
45. Wada J. Vaspin: a novel serpin with insulin-sensitizing effects. *Expert opinion on investigational drugs*. 2008;17(3):327-33.

FIGURE Captions

FIGURE 1. A. Insulin sensitivity (M-value) in women with the metabolic syndrome (MetS) before (□) and after (■) exercise training in women and in lean control women (C) before (○) and after (●) training. * **P-value < 0.05**

Figure 2. Circulating levels of cytokines before (□) and after (■) exercise training in women with the metabolic syndrome (MetS) and in lean control women (C) before (○) and after (●) training. A. Fractalkine; B. Omentin. C. Osteopontin. D. Vaspin (only baseline values shown). E. Leptin. F. Adiponectin. * **P-value < 0.05.**

List of Supplemental Digital Content

Supplemental Digital Content 1 .docx—Characteristics of the entire group versus the subgroups

Figure 1

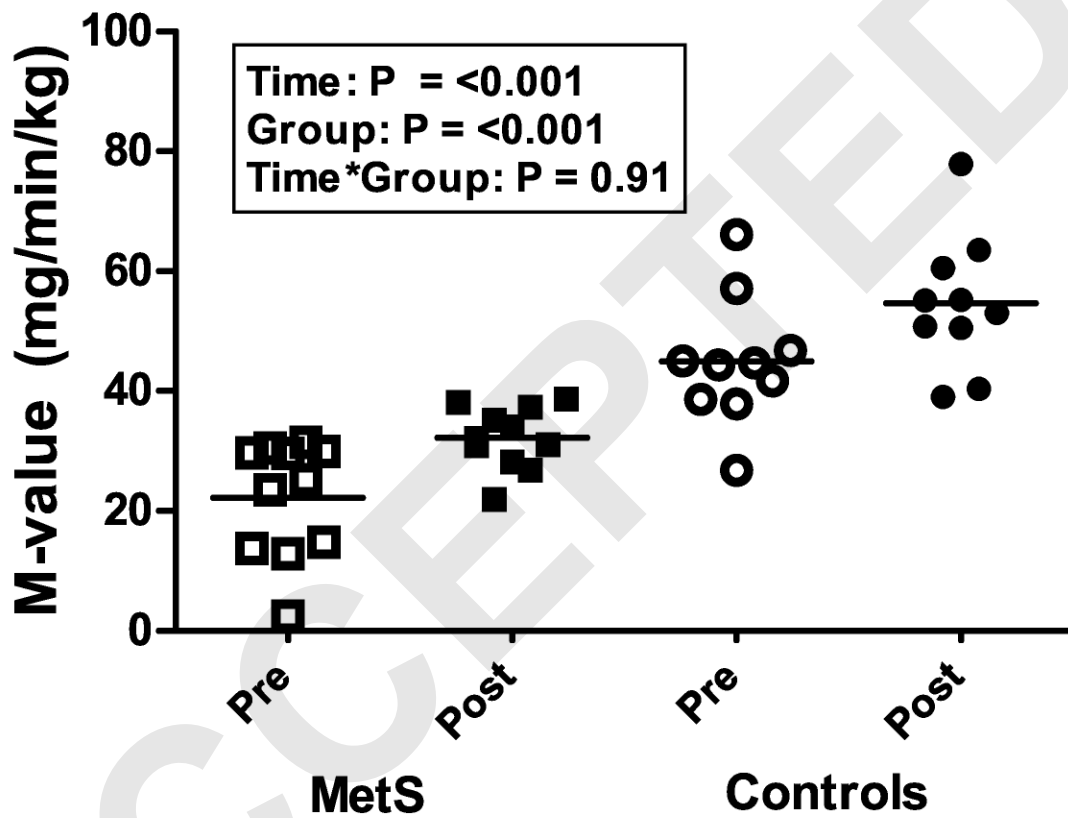


Figure 2

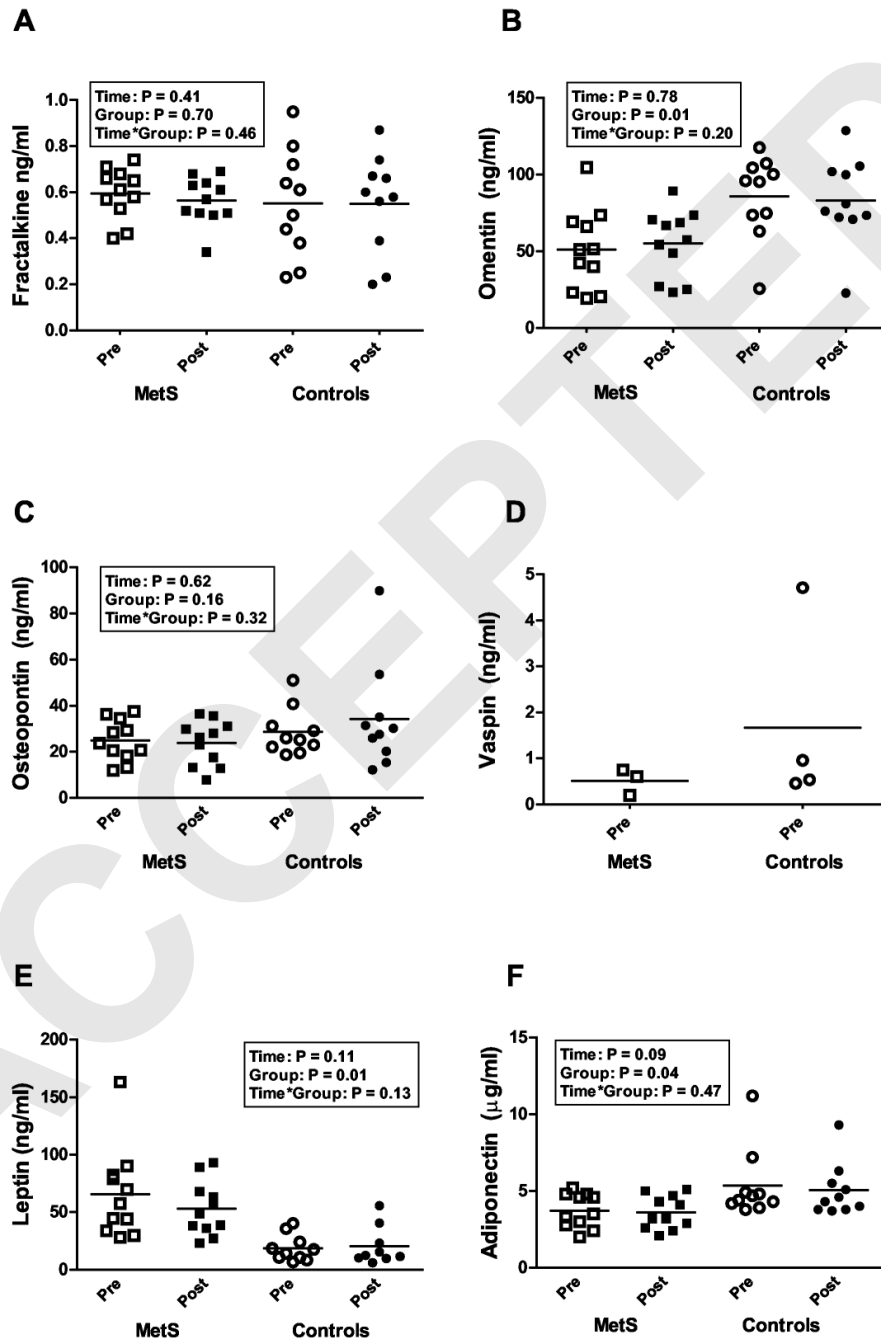


TABLE 1. Physiological characteristics before and after exercise training.

| | Women with MetS (n=11) | | Lean control women (n=10) | | P-value | | |
|---|------------------------|-----------|---------------------------|-----------|---------|--------|-------------|
| | Pre | Post | Pre | Post | Time | Group | Interaction |
| Age (years) | 53.2±7.2 | | 48.5±10.2 | | - | 0.24 | - |
| Body composition (anthropometry) | | | | | | | |
| Weight (kg) | 96.4±11.3 | 94.8±9.6 | 67.0±6.6 [#] | 66.9±6.2 | 0.18 | <0.001 | 0.201 |
| Body mass index (kg/m ²) | 34.5±3.2 | 34.0±2.9 | 22.8±1.7 [#] | 22.7±1.5 | 0.18 | <0.001 | 0.23 |
| Waist (cm) | 108.6±9.4 | 105.0±8.3 | 80.4±6.4 [#] | 78.7±4.7 | 0.03 | <0.001 | 0.97 |
| Waist-to-hip ratio | 0.91±0.08 | 0.90±0.07 | 0.80±0.05* | 0.78±0.04 | 0.57 | <0.001 | 0.22 |
| Body composition (DXA) | | | | | | | |
| Lean body mass (kg) | 56.6±7.2 | 55.9±6.4 | 47.2±4.3* | 47.4±4.1 | 0.62 | 0.002 | 0.24 |
| Total fat mass (kg) | 38.6±6.5 | 37.6±5.8 | 19.2±4.3 [#] | 18.7±3.9 | 0.08 | <0.001 | 0.61 |
| Trunk fat mass (kg) | 20.2±4.0 | 19.5±3.1 | 8.4±2.8 [#] | 8.0±2.4 | 0.09 | <0.001 | 0.54 |
| Trunk fat percentage (%) | 52.4±5.1 | 52.0±4.8 | 42.9±7.1* | 42.6±7.0 | 0.47 | 0.002 | 0.91 |
| Blood markers | | | | | | | |
| Fasting glucose (mmol/l) | 5.5±0.6 | 5.7±0.8 | 4.5±0.3 [#] | 4.7±0.4 | 0.10 | <0.001 | 0.85 |
| Insulin (mE/l) | 17.4±10.4 | 15.4±5.2 | 8.9±3.0 [#] | 10.0±3.8 | 0.76 | 0.013 | 0.24 |
| HDL-cholesterol (mmol/l) | 1.21±0.30 | 1.38±0.29 | 1.59±0.29 | 1.58±0.19 | 0.49 | 0.007 | 0.29 |
| Triglycerides (mmol/l) | 1.98±0.84 | 1.83±0.66 | 0.87±0.27* | 0.92±0.22 | 0.61 | <0.001 | 0.22 |
| Free fatty acids (mmol/l) | 0.58±0.15 | 0.45±0.18 | 0.52±0.16 | 0.43±0.23 | 0.01 | 0.86 | 0.69 |
| Blood pressure & heart rate | | | | | | | |
| Systolic blood pressure (mmHg) | 138±11 | 132±11 | 120±9* | 114±9 | 0.02 | <0.001 | 0.99 |
| Diastolic blood pressure (mmHg) | 84±5 | 80±7 | 76±5* | 73±6 | 0.03 | 0.001 | 0.90 |
| Resting heart rate (bpm) | 68±5 | 59±7 | 60±7* | 58±5 | 0.003 | 0.03 | 0.06 |
| Physical fitness | | | | | | | |
| VO ₂ max (ml/min/kg) | 22.8±4.5 | 25.3±3.8 | 32.0±4.7 [#] | 35.6±5.5 | <0.001 | <0.01 | 0.32 |
| VO ₂ max (ml/min/kg FFM) | 38.6±6.5 | 42.6±5.6 | 45.5±7.1* | 50.4±7.8 | <0.001 | 0.023 | 0.48 |
| Power (Watt) | 158±28 | 185±23 | 180±21 | 205±27 | <0.001 | 0.05 | 0.83 |
| Menopausal status | | | | | | | |

| | | | | | | | |
|----------------------------|---|---|---|---|---|---|---|
| Pre-menopausal (n) | 2 | - | 3 | - | - | - | - |
| Peri-menopausal (n) | 4 | - | 4 | - | - | - | - |
| Post-menopausal (n) | 5 | - | 4 | - | - | - | - |

significantly different from Mets at baseline * P < 0.05; #P < 0.001

ACCEPTED

TABLE 2. Micro-array results for gene-expression in skeletal muscle.

| | Women with MetS (n=5) | | | Healthy control women (n=4) | | |
|----------------------------|-----------------------|-------------------|-------------|-----------------------------|-------------------|-------------|
| | P-value | Corrected P-value | Fold Change | P-value | Corrected P-value | Fold Change |
| Fractalkine (ng/ml) | 0.492269 | 0.997875 | -1.16 | 0.544348 | 0.957122 | -1.13 |
| Omentin (ng/ml) | 0.970832 | 0.999025 | -1.02 | 0.930327 | 0.99287 | 1.00 |
| Osteopontin (ng/ml) | 0.514872 | 0.997875 | 1.06 | 0.40586 | 0.941683 | -1.07 |
| Vaspin (ng/ml) | 0.778275 | 0.997875 | 1.02 | 0.727007 | 0.974637 | 1.03 |
| Leptin (ng/ml) | 0.0950706 | 0.997875 | -1.20 | 0.485465 | 0.952995 | 1.03 |
| Adiponectin (µg/ml) | 0.145784 | 0.997875 | -1.91 | 0.973873 | 0.996383 | 1.02 |

Table, Supplemental Digital Content 1
Characteristics of the entire group versus the subgroups

| | Metabolic syndrome | | Controls | |
|--|------------------------|-----------------------|------------------------|-----------------------|
| | All subjects (n=11) | Array cohort (n=6) | All subjects (n=10) | Array cohort (n=6) |
| <i>Age (years)</i> | 53 ± 7 | 58 ± 5 | 49 ± 10 | 50 ± 9 |
| <i>Weight (kg)</i> | 96.4 ± 11.3 | 105.8 ± 7.1 | 67.0 ± 6.6 | 64.5 ± 6.0 |
| <i>BMI (kg/m²)</i> | 34.5 ± 3.2 | 36.7 ± 2.0 | 22.8 ± 1.7 | 22.2 ± 1.0 |
| <i>Waist-to-hip ratio</i> | 0.91 ± 0.08 | 0.95 ± 0.08 | 0.80 ± 0.05 | 0.78 ± 0.04 |
| <i>M-value (mg/min/kg)</i> | 4.0 ± 1.8 | 3.1 ± 2.0 | 8.1 ± 1.9 | 8.6 ± 2.1 |
| <i>HDL-cholesterol (mmol/l)</i> | 1.21 ± 0.30 | 1.38 ± 0.40 | 1.59 ± 0.29 | 1.53 ± 0.32 |
| <i>Triglycerides (mmol/l)</i> | 1.98 ± 0.84 | 1.83 ± 0.89 | 0.87 ± 0.27 | 0.98 ± 0.23 |
| <i>Systolic blood pressure (mmHg)</i> | 138 ± 11 | 143 ± 13 | 120 ± 9 | 117 ± 8 |
| <i>Diastolic blood pressure (mmHg)</i> | 84 ± 5 | 83 ± 5 | 76 ± 5 | 75 ± 5 |
| <i>Resting heart rate (bpm)</i> | 68 ± 5 | 68 ± 4 | 60 ± 7 | 59 ± 5 |