The Journal of Clinical Endocrinology & Metabolism

The acute cytokine response to 30 min exercise bouts before and after 8-week endurance training in subjects with obesity

--Manuscript Draft--

Manuscript Number:	jc.2022-01772R1
Article Type:	Clinical Research Article
Full Title:	The acute cytokine response to 30 min exercise bouts before and after 8-week endurance training in subjects with obesity
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Section/Category:	Obesity and Adipocyte Biology
Manuscript Classifications:	Exercise; Metabolism / Obesity
Abstract:	Background: One acute bout of exercise leads to a rapid increase in the systemic cytokine concentration. Regular exercise might alter the cytokine response, in particular in beforehand untrained and obese subjects. Methods: Using a proximity extension assay, we studied the effects of acute exercise as well as endurance training on a panel of 92 cytokines related to inflammation. 22 subjects (30±9 years; VO2peak 25.2±4.2 ml/(kg*min); BMI 31.7±4.4 kg/m2) participated in an 8-week endurance exercise intervention. Blood samples were collected before and immediately after 30 min ergometer exercise at 80% VO2peak. Results: Before and after the training intervention, 40 and 37 cytokines, respectively, were acutely increased more than 1.2-fold (BH-adjusted $p<0.05$). The exercise intervention did not change the acute increase in cytokines nor the resting cytokine levels, whereas fitness was improved and adiposity reduced. The increase in fitness led to a slight increase in power output when exercising at the same heart rate, which might explain the comparable increase in cytokines before and after the intervention. The largest acute increase was found for OSM, TGFA, CXCL1 and 5 and TNFSF14 (≥ 1.9-fold, BH-adjusted p-values < 0.001). The transcript levels of these proteins in whole blood were also elevated, particularly in the trained state. Only the acute increase in IL6 (1.3-fold) was related to the increase in lactate, confirming the lactate-driven secretion of IL6. Conclusion: Our comprehensive proteomics approach detected several underexplored

	serum exerkines with up to now less understood function in the adaptation to exercise.
Funding Information:	Bundesministerium für Bildung und Forschung (01GI0925) — Not applicable Deutsche Diabetes Gesellschaft — Anja Moller
Author Comments:	DISCLOSURE SUMMARY: The authors have nothing to disclose.
Additional Information:	
Question	**Response**
REPORTING GUIDELINES Does this manuscript report on the results of a clinical trial or an observational trial? If so, we encourage the authors to comply with the appropriate reporting guidelines, detailed in the author guidelines. For more information on the CONsolidated Standards of Reporting Trials (CONSORT) guidelines, please see http://www.consort-statement.org/consort-2010. For more information on the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) guidelines, please see https://www.strobe-statement.org/index.php?id=strobe-home.	Yes, this reports on the results of an observational study and complies with the STROBE guidelines
DATA REPOSITORIES AND DATA REGISTRATION I have read and agree to take appropriate action to comply with the following Data Repositories and Data Registration guidelines and confirm that I have included the appropriate registration numbers / information in the text of the manuscript being submitted.	Not Applicable
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Options for these statements are below:	

Dear Editor-in-Chief, Prof. Paul M. Stewart, and dear Timothy M. Beardsley

Thank you for the opportunity to resubmit a revised version of our manuscript to the Journal of Clinical Endocrinology & Metabolism. We appreciate the effort and time you and the reviewers carried out in order to revise our work. We are grateful to the reviewers for their valuable feedback and are delighted to respond to the reviewer's comments.

In the following is a point-by-point response to the comments. All comments were also addressed in the revised manuscript.

Comments from reviewer 1

- **Comment 1:** *Some statements in the introduction and discussion miss references (lines 64-68, 248-253)*
  Response: Thank you for pointing this out and we fully agree with it. Therefore, we have added additional references to verify out statements (line 67 and 261 in the new version).
- **Comment 2:** *Please provide the details for preparation of the serum samples (coagulation time and centrifugation speed/time)*
  Response: These details have been added to the methods section (lines 131-135).
- **Comment 3:** *Line 139-142: these results should be moved to results section or included as supplementary data*
  Response: Thank you for this good suggestion. The results comparing ELISA and PEI are now presented as supplementary figure.
- **Comment 4:** *Line 150-151: which RT-PCR instrument was used? What was the normalization strategy/housekeeping genes? Please have a look at (Bustin et al., 2009)*
  Response: We agree with this comment. For the correction, we extended the paragraph on "*Whole blood RNA isolation, cDNA and gene expression analysis*" in the methods section. We added more detailed information concerning the protocols employed for cDNA synthesis and pPCR and the device and software used. Additionally, we provided information on the processing of qPCR data, which was normalized to the housekeeping gene TBP after concentration determination employing standard curves (lines 162-166).
- **Comment 5:** *Line 210-212: It is very bad practice to try to compare mRNA qPCR data from whole blood with proximity extension assay from serum samples. If any comparison should be made between whole blood and serum, both types of samples should be included in the respective assays. There is no evidence in the data linking circulating leukocytes to the release of the cytokines*
  Response: Thank you for this important comment. We are aware that quantifying mRNA in whole blood is by no means suitable to demonstrate that circulating cytokines originate from blood cells. But it is at least conceivable that the same stimulus and signaling process that induces the secretion of a cytokine also triggers its production by upregulating gene transcription or increasing transcript stability. However, we agree with this reviewer that our previous wording might have been misleading. We have therefore changed the respective passages in the abstract (line 48) and results (lines 219-222).

- **Comment 6:** *Another limitation to the study is that rest samples were obtained in a fasted state while the acute exercise samples were obtained following a light breakfast. Consequently, some of the response in the released cytokines could be attributed to the effect of the diet.*
  Response: Again, this is a valid and important point. Since the subjects received a light standardized breakfast between the first blood draw and the commencement of exercise, we cannot exclude acute effects from food intake. However, the intake of meals with varying composition of carbohydrates and fat has been shown to acutely lower, rather than increase, IL6 and have no effect on TNF levels (1). Comparable results were obtained after an OGTT (2). A systematic review supported that common inflammatory markers showed no consistent alteration in the post-prandial state (3). Only IL6 was found to be consistently increased 4-6 h after meal consumption (3,4). Thus, an additive effect of breakfast and exercise on the acute increase of cytokines in our samples collected 80 min after food intake and 5 min after the subsequent exercise bout appears less likely. (lines 377-382).

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The acute cytokine response to 30 min exercise bouts before and after 8-week endurance training in subjects with obesity

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NCT03151590
Short title: cytokine response to exercise
Key words: cytokines, acute exercise, proximity extension assay, IL6, OSM, lactate
Disclosure: The authors declare no competing interests.

Abstract

Background: One acute bout of exercise leads to a rapid increase in the systemic cytokine concentration. Regular exercise might alter the cytokine response, in particular in beforehand untrained and obese subjects.

Methods: Using a proximity extension assay, we studied the effects of acute exercise as well as endurance training on a panel of 92 cytokines related to inflammation. 22 subjects (30±9 years; VO_2peak 25.2±4.2 ml/(kg*min); BMI 31.7±4.4 kg/m^2) participated in an 8-week endurance exercise intervention. Blood samples were collected before and immediately after 30 min ergometer exercise at 80% VO_2peak.

Results: Before and after the training intervention, 40 and 37 cytokines, respectively, were acutely increased more than 1.2-fold (BH-adjusted $p<0.05$). The exercise intervention did not change the acute increase in cytokines nor the resting cytokine levels, whereas fitness was improved and adiposity reduced. The increase in fitness led to a slight increase in power output when exercising at the same heart rate, which might explain the comparable increase in cytokines before and after the intervention. The largest acute increase was found for OSM, TGFA, CXCL1 and 5 and TNFSF14 (≥ 1.9-fold, BH-adjusted p-values < 0.001). The transcript levels of these proteins in whole blood were also elevated, particularly in the trained state. Only the acute increase in IL6 (1.3-fold) was related to the increase in lactate, confirming the lactate-driven secretion of IL6.

Conclusion: Our comprehensive proteomics approach detected several underexplored serum exerkines with up to now less understood function in the adaptation to exercise.

Introduction

The acute cytokine response to one single bout of exercise was first observed in 1983 (1) in the form of pyrogenic activity of plasma obtained from humans after exercise. Later on, this effect on body temperature was attributed to the increase in plasma IL6 which was found to be released from the exercising leg (2). This kicked off not only one but two emerging research fields. The first is the immunomodulatory function of exercise and its relevance in the prevention of chronic metabolic diseases (3) and of aging-associated disorders (4). The second is the field of myokines and other exercise-induced secreted factors which support the functional adaptation of the organism to repeated bouts of exercise and contribute to the beneficial effect of regular physical activity on human health (5). The enormous interest for this research area led to the discovery that several cytokines are elevated in the blood stream after one acute bout of exercise including the interleukins IL1beta, IL4, IL13, IL8, IL10, and IL15, interleukin receptor antagonist IL1Ra, colony stimulating factors (CSF), tumor necrosis factor (TNF), and chemokine ligands, e.g. CCL2 and CX3CL1 (6-8). The acute release of cytokines with mainly anti-inflammatory properties, such as IL10, IL1Ra and IL6, is considered one of the potent mechanisms by which exercise modulates the function of monocytes and macrophages directing them towards a less pro-inflammatory phenotype (6). Notably, many cells are capable of producing cytokines. The systemic concentration always reflects the sum of the release from all sources minus the clearance rate. Measuring the arterio-venous difference over the exercising leg validated the release of some, but not all cytokines showing increased systemic blood levels from skeletal muscle (5). Other sources for cytokine release into the circulation in response to exercise are natural killer cells (9), monocytes, macrophages and T-cells (10).

Most previous studies have focused on small, pre-defined sets of cytokines rather than evaluating the broad cytokine response in an unbiased approach. Moreover, low physical fitness as well as an increased amount of visceral and subcutaneous fat, which harbors a relevant number of macrophages and T-cells, might influence the cytokine pattern after acute exercise. Both, fitness and adiposity are improved by endurance training, which might change

the acute cytokine response. To address this question, we made use of the recently developed proximity extension analysis which allows the simultaneous measurement of a large number of inflammatory cytokines (11). We applied this technique to blood samples collected before and 5 minutes after one 30 min ergometer exercise bout at 80% VO2peak. The participants were middle-aged, untrained, and had overweight or obesity (12). After the first exercise bout, they conducted an 8-week endurance exercise training with three 60 min-sessions per week followed by the second 30 min ergometer exercise bout with collection of blood samples. We investigated the acute regulation of a cytokine panel covering 92 analytes and assessed whether this response is altered after the 8-week training period. Furthermore, we analyzed the influence of fitness and metabolic parameters on the cytokine response.

Material and Methods

Study participants and design

Participants and design of the study were described recently (12) (NCT03151590 at Clinicaltrials.gov). Healthy individuals with <120 min of physical activity per week and BMI >27 kg/m^2 were recruited. They underwent incremental cardiopulmonary exercise testing before and after the intervention. The individual anaerobic threshold (IAT) was calculated as the lactate concentration of 1.0 mmol/L above the lowest value of the lactate to performance ratio (13). Peak VO_2 was defined as the mean VO_2 over the last 20 seconds prior to cessation of exercise and assessed by metabolic gas analysis (MetaLyzer 3B and MetaMax 3B, Cortex Biophysics GmbH, Leipzig, Germany). On the day after the fitness test, the participants performed 30 min of bicycle ergometer exercise at their individual training intensity set to 80% of VO_2peak. 45 min before this acute exercise visit, the participants had a standardized breakfast (1 bun, 20 g butter, 1 slice of cheese, 150 g apple puree, water ad libitum). Blood samples were collected in the fasted state and 5 min after the exercise bout. After the first acute exercise day, the participants performed an 8-week supervised aerobic endurance training at 80% VO_2peak (3 sessions per week consisting of 30 min treadmill walking and 30 min bicycle ergometer exercise). The intervention was concluded by a second fitness test and a second 30 min ergometer exercise which were performed on two successive days. Exercise intensity was adjusted to and controlled by the heart rate corresponding to 80% VO_2peak. The heart rate was kept constant throughout the intervention, resulting in an increase in exercise intensity during the intervention in most subjects, as detailed below. From 22 participants, samples of the four timepoints of the two acute exercise bouts were available and used for the analysis of cytokines. All participants gave written informed consent and the study protocols were approved by the ethics committee of the University of Tübingen and in accordance with the declaration of Helsinki.

Clinical analyses and anthropometry

Glucose and lactate were measured with an ADVIA XPT clinical chemistry analyzer and insulin with an ADVIA Centaur XPT immunoassay system (both Siemens Healthineers, Eschborn, Germany). HbA1c was measured by HPLC (Tosoh Bioscience, Griesheim, Germany). Peripheral blood cell counts were performed using an XN-10 hematology analyzer (Sysmex Norderstedt, Germany). Body fat mass and distribution were measured by magnetic resonance imaging (14). Subcutaneous and visceral adipose tissue was measured from femoral head to humeri. Insulin sensitivity was assessed after an overnight fast by two 75g-OGTTs, performed pre-intervention and 5 days after the last exercise bout, respectively. The insulin sensitivity index was estimated by the method of Matsuda and DeFronzo (ISI_{Mats}) (15). Blood samples for clinical routine parameters were obtained in the fasted state. Serum was obtained by drawing blood into serum collection tubes (no. 03.1397, Sarstedt, Nümbrecht, Germany), incubating for 30 min on the top of crushed ice to reduce variations caused by seasonal differences in room temperature, and centrifuging 10 min at 2000 g, 4°C. Aliquots were immediately prepared on ice and stored at -80 °C.

Proximity extension assay

Serum samples were analyzed with the OLINK Target 96 Inflammation panel (OLINK Proteomics, Uppsala, Sweden) that cover 92 pro- and anti-inflammatory cytokines, chemokines, growth factors, and factors involved in acute inflammatory and immune responses, angiogenesis, fibrosis, and endothelial activation. The assay allows for a relative quantification of analyte concentrations, which are given as normalized protein expression (NPX) based on real-time qPCR cycle values on a log2 scale (11). Only the 69 proteins with ≥ 80% values above the limit of detection at one or more timepoints were used for analysis. OLINK assay data from one sample were missing. Two cytokines, IL6 and VEGFA, were also measured using ELISAs (R&D Systems, Minneapolis, MN, USA; VEGFA cat no.: DVE00, Antibody Registry: AB_2800364; IL6 cat. no.: HS600C, Antibody Registry: AB_2893335). NPX values (PEA) and log-transformed absolute protein concentrations (ELISA) were highly

correlated for both IL-6 (r = 0.86, P < 0.001) and VEGFA (r = 0.93, P < 0.001) Supplementary Fig. 1 (16).

Whole blood RNA isolation, cDNA and gene expression analysis

Whole blood was collected in PAXgene tubes after overnight fasting and total cellular RNA was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). Subsequently, 1 µg of total RNA was reverse transcribed into cDNA utilizing the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions and using an 1:1 mix of random hexamer primers in a volume of 20 µl. Quantitative real-time PCR was performed using 1 µl of the resulting cDNA and the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions in a total volume of 20 µl using the following QuantiTect Primer Assays (Qiagen): CXCL1 (QT02559186), CXCL5 (QT00095431), IL6 (QT00083720), OSM (QT00209286), TGFA (QT00033887), TNF (QT01079561), TNFSF14 (QT01011682). Results were normalized using the housekeeper TBP (QT00000721). Concentrations was determined using the standard curve method. qPCR analysis was performed using the LightCycler 480 System and analyzed using the LightCycler 480 Software (v.1.5.0). Two qPCR data outliers were detected by principal component analysis and removed.

Statistical analyses

Analyses were performed using R4.0.2/RStudio (v1.3.959). Olink data were processed in their original $\log_2$ format. Other data were tested for normality by Shapiro-Wilk test from the R package 'stats' (v4.0.2) and non-normal data were log-transformed. Paired t-tests were used to compare cytokine responses between two time points and Pearson's r was used for correlation analysis. Benjamini-Hochberg (BH) correction was used to account for multiple testing as indicated. Graphs were plotted using the R packages 'ggplot2' (v3.3.5), 'ggsignif' (v0.6.3) and 'ggrepel' (v0.9.1).

Results

Study population

The anthropometric, metabolic, and fitness parameters of the 22 (14 female and 8 male) participants from whom serum samples at the two acute exercise sessions were available are shown in Table 1. The subjects were obese (BMI 31.7±4.4 kg/m^2) and untrained (less than 120 min structured exercise per week; VO_2peak 25.2±4.2 ml/(kg*min)). Resting IL6 plasma levels quantified by ELISA were ≤ 4 pg/ml in all participants (mean 1.86±0.85 pg/ml), indicating an absence of subclinical inflammatory processes. After the 8-week intervention, subjects showed significant improvements in IAT, VO_2peak and exercise performance, and significant reductions in body mass, BMI, total adipose tissue, and subcutaneous and visceral adipose tissue volume.

Acute cytokine response to exercise

After the first acute exercise session, 40 out of the 69 included cytokines were increased more than 1.2-fold (BH-adjusted p<0.05) (Fig. 1A, Table 2 and Suppl Table 1 ((16)). The highest increases were found for oncostatin M (OSM) (2.31-fold), transforming growth factor alpha (TGFA) (2.07-fold), C-X-C motif chemokine ligand (CXCL)5 (1.99-fold), TNF superfamily member (TNFSF)14 (1.93-fold), and CXCL1 (1.89-fold), all of which exhibited a BH-adjusted p<0.001. Several cytokines showed an increase of >1.5-fold. Interestingly, the prototypic exercise-regulated cytokine, IL6, was increased only 1.29-fold (BH-adjusted p<0.001) (Fig. 1A). The pro-inflammatory cytokine TNF was increased to a similar extend as IL6 (1.26-fold, BH-adjusted p<0.001).

After the second acute exercise session, which was conducted after the 8-week training, 37 cytokines were increased more than 1.2-fold (BH-adjusted p<0.05) (Fig. 1B, Table 2 and Suppl. Table 1). The mean increase was highly comparable to the increase caused by the first, pre-training, exercise session (Fig. 1C). Again, OSM, TGFA, CXCL1 and 5 and TNFSF14 were the cytokines with the largest increase (>1.9-fold, BH-adjusted p-values<0.001). Comparing

the response to the acute exercise session before and after the 8 week-training revealed no significant difference in the change in any of the 69 cytokines ($p>0.05$ paired t-test).

Acute increase in OSM, TGFA, and CXCL1 transcripts in cellular RNA from whole blood

An acute increase in circulating leukocytes, in particular NK cells, in response to exercise has been demonstrated which can contribute to an acute increase in cytokines in blood (17). We have no information about the acute regulation of the number of blood leukocytes in our study, but instead we analyzed the cellular transcripts of OSM, TGF alpha, and TNFSF14 together with CXCL1, CXCL5, TNF and IL6 in whole blood samples collected immediately after the acute exercise sessions together with the serum samples. The transcript levels of TNF and IL6 were unchanged, while OSM, TGFA and CXCL1 transcripts were increased in whole blood after both the first and the last acute exercise session (Fig. 2). Notably, the leukocyte mRNA response was more pronounced after the 8 weeks training. The latter effect was also seen for CXCL5 and TNFSF14 transcripts, which were acutely upregulated in whole blood after the training period but not in untrained individuals. While the increase in transcript levels in whole blood cannot prove a direct contribution of circulating leukocytes to the increase in the respective cytokines in serum, a role for leukocytes is at least conceivable based on these data.

Effect of the 8-week training intervention on cytokines in the resting state

To assess potential training effects on circulating cytokine levels, the cytokine levels in the fasting blood samples taken before the exercise bouts were compared. Despite the clear effects of the intervention on fitness and adiposity, no change in the 69 cytokines was observed in the resting serum values before and after the 8-week intervention (BH-adjusted $p>0.05$) (Table 2, Suppl. Table 1).

Effect of adipose tissue compartments on the acute cytokine response

Next, we performed exploratory correlation analyses to test whether the individual cardiorespiratory fitness (quantified as VO_2peak), or metabolic parameters such as subcutaneous or visceral adipose tissue volume and systemic insulin sensitivity might have an influence on the acute cytokine response to exercise (Fig.3). The fitness level did not correlate with any of the acute increases. BMI and visceral adipose tissue, but not subcutaneous adipose tissue volume was positively correlated to the increase of several cytokines in the untrained subjects before the intervention. After the 8-week training, the correlation remained for visceral adipose tissue and CXCL10 and sulfotransferase family 1A member 1 (ST1A1). Systemic insulin sensitivity assessed by the Matsuda insulin sensitivity index correlated negatively to the increase in IL6 and positively to ST1A1 before the intervention.

Increase in IL6 correlates with increase in lactate

Lastly, we studied whether the increase of lactate was associated to the increase in cytokines. Blood lactate concentrations increase depending on the intensity of the performed ergometer exercise and correlate also with perceived exertion (18). In our study, plasma lactate concentrations were increased to a similar extend after both acute exercise sessions with no effect of the training intervention (3.87 and 3.82-fold, $p<0.001$ paired t-test) (Fig. 4A). Only IL6 was correlated to the increase in lactate after the first exercise bout ($p<0.03$, $r=0.48$) and the exercise bout after training ($p=0.045$, $r=0.43$) (Fig. 4B, C). The increase in CST5 ($p<0.0085$, $r=0.57$) and MCP1 ($p<0.036$, $r=0.47$) was correlated to the increase in lactate only after the first exercise bout.

Discussion

Proximity extension assay analysis of the acute cytokine response to exercise in overweight to obese subjects confirmed the increase in several cyto- and chemokines, chemokine receptor ligands, monocyte chemoattractant proteins that were previously reported in ELISA-

based studies of lean and physically active humans (8). The physiological function of this increase in cytokines is to induce leukocyte chemotaxis, to activate T-cells, to regulate adaptive immune responses and to promote angiogenesis and smooth cell proliferation. Prototypical pro-inflammatory cytokines (TNF) showed only a minor increase compared with other cytokines or were below the limit of detection even after exercise (IL1alpha). Thus, our data underline the potential of exercise to modulate immune responses and to support anti-inflammatory pathways in a highly specific manner.

In addition, our approach revealed a robust increase in cytokines which are yet not well characterized as exercise-regulated secreted circulating proteins such as OSM, TGFA, and TNFSF14. OSM has been shown to increase in serum after one bout of 60 min cycling in young and old subjects (19). OSM increased also in serum of mice after 60 min of swimming, which was paralleled by enhanced OSM transcript levels in skeletal muscle, but not in liver, adipose tissues or spleen, leading to its denomination as a myokine (20). While OSM was also detected in the supernatant of human differentiated skeletal muscle cells by a cytokine antibody array (21), it is mainly expressed in immune cells including T cells, monocytes, macrophages, dendritic cells, granulocytes and mast cells (22). The pronounced increase in OSM transcripts in cellular RNA isolated from whole blood immediately after exercise also argues for a contribution of circulating immune cells to the increase in serum OSM concentration. Of notice, OSM and conditioned serum of exercised mice inhibit tumor cell growth and induce apoptosis (20), making it a promising candidate for exercise-related risk reduction in development, progression and recurrence of certain cancers (23). TNFSF14 is also widely expressed in immune cells (24), but only the regulation of TNFSF14 transcripts in whole blood in trained subjects followed the pronounced increase in TNFSF14 protein after acute exercise. This might indicate an additional source of the circulating protein or different kinetics of transcript and protein regulation. TNFSF14 also known as LIGHT has shown some potential in cancer immunotherapy (25), making it also a candidate for the therapeutic anti-tumor potential of exercise. TGFA showed a robust increase in both whole blood transcript and in serum protein levels after acute exercise in untrained and trained subjects, suggesting a

289 contribution from leukocytes to the increase in circulating protein levels. TGFA has many
290 mitogenic functions but less is known about its relevance as a systemic factor in the adaptation
291 to exercise.
292 The overall response in cytokines to a 30 min ergometer exercise bout before and after a
293 structured and supervised 8-week endurance training was remarkably stable. Exercise
294 intensity was individually set and monitored as the heart rate corresponding to 80% VO_2peak
295 determined before the training period. As a consequence, absolute exercise performance
296 assessed in [Watts] was higher during the second 30 min exercise bout compared to the first
297 one while individual relative exercise intensity was unchanged. The constant relative exercise
298 intensity was reflected by a similar increase in lactate before and after the training period. Thus,
299 our results indicate that the increase in cytokines is proportional to the relative, rather than
300 absolute, exercise intensity. The effect of training on the acute exercise-induced regulation of
301 plasma proteins was also addressed in a recent study taking the improvement in exercise
302 performance into account (26). When the second exercise bout after a six-week endurance
303 training period was performed at the same absolute intensity as the first exercise bout before
304 training, levels of some cytokines increased to a lesser extent, but this effect was not found
305 when the intensity of the second acute exercise bout was adjusted to the improved fitness (26).
306 Similarly, the increase in IL6 was reduced after 6 weeks of high-intensity interval running when
307 comparing the response to one acute high-intensity interval running test at the same absolute
308 workloads before and after training (27). When the workload of the acute exercise bout was
309 adjusted to the improvement in maximal power output after 10 weeks of knee extensor
310 endurance training, the acute response in plasma IL6 was similar before and after training
311 (28). These results together with the data of our study clearly showed that it is important for
312 the comparison of the cytokine response to acute exercise to consider a potential training-
313 induced change in the individual exercise performance.
314 This is even more evident when comparing the acute exercise response in individuals with
315 different training status. The increase in IL6 and IL8 was not different between endurance-
316 trained and sedentary young healthy males in a study of 30 min of treadmill running at 75 %

317 of the individual VO_2max, albeit cardiorespiratory fitness was very different (VO_2max of 70 vs.
318 47 ml/kg/min) (29). No data on the actual individual exercise performance were reported.
319 Studying the response to a 60 min ergometer test at 80% VO_2max in endurance trained and
320 sedentary young healthy males revealed that the trained individuals had even more
321 pronounced increases in some of the analyzed proteins (e.g. IL8, MCP1, IL10, TNF, TNFR2,
322 MMP2, MMP3) and higher levels of the muscle damage markers creatine kinase and FABP
323 after the exercise bout (30). Notably, exercise performance at 80%VO_2max was considerably
324 higher in the trained group (254 vs. 156 Watts) despite a comparable heart rate in the trained
325 and sedentary group (30). Thus, physical performance in relation to individual fitness is
326 decisive for the cytokine response and an attenuated increase after training can be masked by
327 the capacity to exercise at a higher absolute intensity.
328 In contrast to circulating protein, the transcript levels of CXCL1, CXCL5, OSM, TGFA and
329 TNFSF14 were higher after the acute exercise bout in the trained state compared to before
330 the intervention. This might show a higher responsiveness in the expression of these genes in
331 circulating leukocytes after training, which, however was not translated to higher serum protein
332 levels in samples collected at the same timepoint. We did not collect samples at a later
333 timepoint which would allow us to study whether the altered expression results in a prolonged
334 increase of the respective cytokines in blood.
335 Another factor influencing the acute increase in several cytokines might be the visceral adipose
336 tissue volume. We found a specific correlation for this fat compartment, but not for
337 subcutaneous adipose tissue. Visceral adipose tissue has a higher content of NK cells,
338 macrophages and T-cells which is associated to a higher release of inflammatory cytokines
339 (31). The mobilization or activation of the resident immune cells in visceral adipose tissue might
340 contribute to the increase in serum cytokines in response to exercise. The correlation for most
341 cytokines was not found after the 8-week training, except for CXCL10 and ST1A1. While this
342 would argue for a training-induced adaptation of visceral adipose tissue immune cells resulting
343 in a reduction in the release of certain cytokines after acute exercise, the comparable increase
344 in cytokines before and after training and comparable resting values despite the reduction of

visceral fat after training does not support the hypothesis. Further studies are needed to clarify the contribution of visceral fat to the cytokine response to exercise. The robust correlation of the increase in ST1A1 to visceral fat volume suggests the exercise-induced release of the protein from this compartment. The sulfotransferase ST1A1 is classified as intracellular protein, but was already detected by mass spectrometry-based proteomics in plasma (32,33). Its function as secreted protein is unknown.

A clear result of our study is that the 8-week training intervention did not change the resting levels of cytokines related to inflammatory processes. While this would argue against the regularly described anti-inflammatory potential of training, it must be considered that our participants were in average obese but also relatively young and otherwise healthy. Resting IL6 plasma levels do not indicate subclinical inflammatory processes. Thus, despite considerably high BMI values, our participants could still have been too healthy to show a pro-inflammatory phenotype, which might explain why training could not alter their resting cytokine levels.

Previous studies already described the association of the response of serum IL6 and lactate after acute exercise (34,35), a correlation also reported for the release of IL6 and lactate from electrically stimulated human myotubes (34). Since the systemic increase in IL6 has also been linked to exercise duration and intensity and muscular glycogen depletion (36,37), a common mechanism depending on muscular energy status might be assumed. In 2019, a study by Hojman et al. showed that the muscular production of lactate triggers the release of IL6 from intracellular vesicles involving the activity of matrix metalloproteases (34). This study validated previous results demonstrating the existence of preformed vesicles containing IL6 protein in skeletal muscle fibers allowing a rapid release of IL6 into the bloodstream from the contracting skeletal muscle (38). Our data supported the concept of a specific, lactate-dependent regulation of the systemic levels of the myokine IL6, since only the increase in IL6, but not of any other analyzed cytokines, was correlated to the increase in lactate after both exercise bouts. We found this correlation despite an only moderate increase in serum IL6 after the 30 min ergometer exercise bout.

There are limitations to our study. The increase in fitness and reduction in adiposity was moderate after the 8-week intervention and might not be sufficient to change the resting cytokine levels or to modify the acute response. By design and as discussed, the participants performed the two acute exercise bouts at the same individual heart rate which masked a potential reduction in the acute response of cytokines after training. Since the subjects received a light standardized breakfast between the first blood draw and the commencement of exercise, we cannot exclude acute effects from food intake. However, data on alterations in systemic cytokine levels in the post-prandial phase are not consistent (39) and food consumption or OGTT has been shown to acutely lower, rather than increase, IL-6 and have no effect on TNF levels in individuals with obesity (40,41). Finally, due to the limited sample size, sex-specific differences were not be investigated in the current study.

In conclusion, our results provide a comprehensive insight into the acute cytokine response to one acute bout of exercise in overweight to obese subjects. Targeting a broad panel of cytokines strongly supports the immune modulatory function of exercise. We identified a number of still underexplored exerkines that showed a robust increase in serum and could contribute to adaptation mechanism to and clinical benefits from exercise.

Acknowledgements

The authors thank all study participants. The authors are grateful for the excellent technical support provided by Alke Guirguis, Karin Schweitzer and Nadine Sanabria Valdés (Institute for Clinical Chemistry and Pathobiochemistry, University Hospital Tübingen).

Data availability

The data are available from the corresponding author on reasonable request.

Funding

This study was supported in part by grants from the German Federal Ministry of Education and Research (BMBF) to the German Centre for Diabetes Research (DZD e.V.; No. 01GI0925)

and by a grant from the German Diabetes Association to AM. AM is currently funded by a clinician scientist program from the medical faculty of the University of Tübingen.

Disclosures

The authors declare no competing interests.

Author Contributions

TG and MH analyzed most of the data, interpreted the data and wrote the manuscript with the contribution from all coauthors. LF collected metabolic data. PS conduced exercise training. JM performed whole-body MRI. AP and SH performed and analyzed proteomics data and provided scientific guidance. AF, AB, AP, MH provided scientific guidance and experimental design and contributed to discussion. AN designed study and contributed to discussion. AM designed study and analyzed metabolic data. CW designed study, supervised the whole project, interpreted the data and wrote the manuscript and is guarantor of work.

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Table 1. Anthropometric, fitness and metabolic data

Parameter	Pre	Post-8-week	p-value
Sex	14 female / 8 male		-
Age [years]	30 ± 8.7 (19 – 59)	30.1 ± 8.65 (19 – 59)	-
Height [cm]	171 ± 9.47 (157 – 193)		-
Body mass [kg]	92.6 ± 16.1 (69.9 – 132)	91.4 ± 16.6 (67.2 – 130)	**0.008**
BMI [kg/m²]	31.7 ± 4.38 (27.5 – 45.5)	31.3 ± 4.58 (26.3 – 45.2)	**0.006**
Waist to hip ratio	0.89 ± 0.06 (0.76 – 1.03)	0.89 ± 0.06 (0.74 – 0.98)	>0.1
Total AT volume [L]	40.4 ± 11.5 (25.3 – 74.2)	39.4 ± 11.6 (22.8 – 73.9)	**0.004**
Subcutaneous AT [L]	15.3 ± 5.73 (8.42 – 32.2)	14.7 ± 5.93 (7.20 – 33.1)	**0.006**
Visceral AT [L]	3.53 ± 1.61 (0.81 – 7.26)	3.38 ± 1.54 (0.94 – 6.68)	**0.012**
IAT_{ergo}/BM [W/kg]	1.11 ± 0.22 (0.77 – 1.55)	1.32 ± 0.26 (0.89 – 1.87)	**<0.001**
VO_2peak_{ergo}/BM [mL/(kg*min)]	25.0 ± 4.15 (18.3 – 32.3)	26.5 ± 4.55 (16.0 – 34.9)	**0.042**
Performance Cycling [W]	119 ± 29.8 (67.5 – 180)	127 ± 24.9 (77.5 – 175)	**0.011**
Glucose fasting [mmol/L]	5.09 ± 0.39 (4.61 – 6.00)	5.02 ± 0.39 (4.33 – 5.61)	>0.1
Glucose $OGTT_{120\ min}$ [mmol/L]	5.70 ± 1.17 (3.44 – 8.00)	5.52 ± 1.58 (3.50 – 11.6)	0.089
Insulin fasting [pmol/L]	110 ± 40.4 (38.0 – 188)	103 ± 36.9 (50.0 – 170)	>0.1
Insulin $OGTT_{120\ min}$ [pmol/L]	546 ± 400 (65.0 – 1766)	472 ± 341 (61.0 – 1345)	0.095
ISI_{Mats}	8.35 ± 4.90 (3.12 – 27.0)	8.86 ± 4.20 (4.47 – 21.4)	>0.1
HbA_{1c} [mmol/mol Hb]	34.2 ± 2.41 (28.0 – 39.0)	33.5 ± 2.64 (26.0 – 37.0)	**0.050**
HbA_{1c} [%]	5.28 ± 0.22 (4.71 – 5.72)	5.22 ± 0.24 (4.53 – 5.54)	**0.050**
Leukocytes	6890 ± 1513 (4740 – 10200)	6485 ± 1437 (4220 – 9220)	>0.1
Interleukin 6 [pg/mL]	1.86 ± 0.85 (0.77 – 4.06)	1.90 ± 1.12 (0.77 – 5.84)	>0.1

AT: adipose tissue, IAT: individual anaerobic threshold, BM: body mass, ergo: bicycle ergometer, ISI: insulin sensitivity index. Paired t-tests or Wilcoxon signed rank tests when data were not normally distributed. N = 22 or N = 21 for VO_2peak_{ergo}/BM, mean ± SD (range of values). Participants were a subgroup of the study published in (12).

Table 2. Response of serum cytokines to acute exercise and training intervention

Protein	Untrained Acute Ex. Adj.p-value	Untrained Acute Ex. FC+Range	Trained Acute Ex. Adj.p-value	Trained Acute Ex. FC+Range	Training Intervention Adj.p-value	Training Intervention FC+Range	Protein	Untrained Acute Ex. Adj.p-value	Untrained Acute Ex. FC+Range	Trained Acute Ex. Adj.p-value	Trained Acute Ex. FC+Range	Training Intervention Adj.p-value	Training Intervention FC+Range
ADA	0.047	1.10 (0.68 – 1.49)	0.005	1.11 (0.85 – 1.38)	>0.1	1.06 (0.62 – 1.51)	HGF	<0.001	1.59 (1.00 – 2.53)	<0.001	1.65 (0.94 – 2.4)	>0.1	1.03 (0.52 – 2.04)
AXIN1	0.047	1.56 (0.34 – 6.15)	0.005	1.40 (0.21 – 3.50)	>0.1	1.24 (0.16 – 9.23)	IFNG	<0.001	0.99 (0.79 – 1.54)	<0.001	0.96 (0.67 – 1.32)	>0.1	1.00 (0.31 – 8.38)
CASP8	0.008	1.21 (0.87 – 1.88)	0.061	1.21 (0.81 – 1.79)	>0.1	1.02 (0.55 – 1.80)	IL10	<0.001	1.12 (0.73 – 1.49)	0.003	1.10 (0.81 – 1.71)	>0.1	1.06 (0.82 – 2.92)
CCL11	0.002	1.35 (1.02 – 2.06)	0.002	1.39 (0.96 – 1.94)	>0.1	1.01 (0.66 – 1.77)	IL10RA	>0.1	1.18 (0.96 – 1.51)	0.029	1.14 (0.88 – 1.36)	>0.1	0.97 (0.71 – 1.25)
CCL13	<0.001	1.75 (1.17 – 5.44)	<0.001	1.76 (0.86 – 3.73)	>0.1	1.01 (0.31 – 3.83)	IL10RB	<0.001	1.15 (0.93 – 1.34)	<0.001	1.12 (0.90 – 1.38)	>0.1	1.03 (0.85 – 1.31)
CCL19	<0.001	1.21 (0.96 – 1.54)	<0.001	1.19 (0.91 – 1.64)	>0.1	1.03 (0.67 – 2.05)	IL12B	0.006	1.15 (0.91 – 1.37)	0.024	1.16 (0.89 – 1.52)	>0.1	1.03 (0.75 – 1.49)
CCL2	>0.1	1.35 (0.98 – 3.06)	0.026	1.38 (0.71 – 2.06)	>0.1	1.03 (0.56 – 2.67)	IL15RA	<0.001	1.15 (0.95 – 1.46)	<0.001	1.12 (0.85 – 1.40)	>0.1	1.06 (0.84 – 1.37)
CCL20	<0.001	1.10 (0.34 – 3.28)	<0.001	1.19 (0.61 – 2.62)	>0.1	0.91 (0.21 – 4.23)	IL17C	<0.001	1.08 (0.75 – 1.74)	0.003	1.10 (0.75 – 1.48)	>0.1	1.05 (0.32 – 1.95)
CCL23	<0.001	1.40 (1.07 – 1.92)	<0.001	1.37 (0.99 – 1.93)	>0.1	1.17 (0.73 – 1.82)	IL18	<0.001	1.18 (0.88 – 1.44)	<0.001	1.17 (0.86 – 1.49)	>0.1	1.01 (0.83 – 1.35)
CCL25	<0.001	1.20 (0.93 – 1.72)	<0.001	1.22 (0.90 – 1.88)	>0.1	0.97 (0.75 – 1.24)	IL18R1	0.008	1.21 (0.95 – 1.44)	0.023	1.21 (0.94 – 1.55)	>0.1	0.98 (0.77 – 1.21)
CCL28	<0.001	1.31 (0.93 – 2.06)	<0.001	1.31 (0.95 – 1.85)	>0.1	1.00 (0.64 – 1.47)	IL6	<0.001	1.29 (0.86 – 1.90)	<0.001	1.21 (0.78 – 2.10)	>0.1	0.97 (0.51 – 2.38)
CCL3	<0.001	1.22 (0.81 – 1.77)	<0.001	1.28 (0.86 – 2.32)	>0.1	1.01 (0.51 – 1.83)	IL7	<0.001	1.47 (0.84 – 3.31)	<0.001	1.47 (0.69 – 2.89)	>0.1	1.02 (0.41 – 2.20)
CCL4	<0.001	1.45 (0.98 – 2.42)	<0.001	1.47 (0.92 – 2.32)	>0.1	1.03 (0.43 – 2.3)	IL8	<0.001	1.21 (0.87 – 2.24)	<0.001	1.20 (0.59 – 2.17)	>0.1	1.08 (0.64 – 2.20)
CCL8	<0.001	1.66 (1.18 – 3.14)	<0.001	1.70 (1.00 – 3.33)	>0.1	0.99 (0.40 – 2.17)	LIFR	<0.001	1.18 (0.99 – 1.42)	<0.001	1.16 (0.89 – 1.46)	>0.1	1.05 (0.81 – 1.43)
CD244	<0.001	1.22 (0.95 – 1.44)	<0.001	1.21 (0.91 – 1.52)	>0.1	1.04 (0.85 – 1.33)	MMP1	<0.001	1.73 (1.10 – 4.78)	<0.001	1.79 (1.01 – 4.09)	>0.1	0.94 (0.34 – 3.17)
CD274	<0.001	1.19 (0.90 – 1.41)	<0.001	1.18 (0.89 – 1.59)	>0.1	1.00 (0.54 – 1.35)	MMP10	<0.001	1.14 (0.94 – 1.43)	<0.001	1.16 (0.90 – 1.59)	>0.1	1.15 (0.77 – 2.86)
CD40	<0.001	1.29 (1.00 – 1.82)	0.002	1.32 (0.92 – 1.67)	>0.1	1.05 (0.75 – 1.42)	OPG	<0.001	1.21 (1.00 – 1.52)	<0.001	1.22 (0.92 – 1.68)	>0.1	1.00 (0.80 – 1.31)
CD5	<0.001	1.22 (0.97 – 1.54)	<0.001	1.17 (0.87 – 1.52)	>0.1	1.04 (0.86 – 1.44)	OSM	<0.001	2.31 (0.94 – 10.6)	<0.001	2.36 (0.95 – 5.92)	>0.1	1.15 (0.21 – 9.07)
CD6	<0.001	1.25 (0.96 – 1.67)	<0.001	1.24 (0.90 – 1.61)	>0.1	1.00 (0.71 – 1.49)	PLAU	<0.001	1.17 (1.00 – 1.37)	<0.001	1.17 (0.89 – 1.86)	>0.1	1.07 (0.88 – 1.33)
CD8A	0.034	1.18 (0.9 – 1.48)	0.009	1.15 (0.80 – 1.72)	>0.1	1.04 (0.68 – 1.49)	S100A12	<0.001	1.37 (0.15 – 2.94)	<0.001	1.49 (0.60 – 2.69)	>0.1	0.72 (0.05 – 2.63)
CDCP1	<0.001	1.2 (0.87 – 1.61)	<0.001	1.18 (0.98 – 1.47)	>0.1	1.01 (0.81 – 1.34)	SCF	<0.001	1.17 (0.99 – 1.33)	<0.001	1.15 (0.97 – 1.40)	>0.1	1.09 (0.86 – 1.67)
CSF1	<0.001	1.13 (1.00 – 1.33)	<0.001	1.13 (0.95 – 1.40)	>0.1	1.00 (0.81 – 1.19)	SLAMF1	<0.001	1.15 (0.93 – 1.45)	0.005	1.13 (0.81 – 1.49)	>0.1	1.03 (0.80 – 1.48)
CST5	<0.001	1.34 (0.90 – 15.0)	<0.001	1.32 (0.95 – 8.97)	>0.1	0.98 (0.74 – 1.24)	ST1A1	0.006	1.6 (0.40 – 6.61)	0.024	1.47 (0.37 – 7.34)	>0.1	1.21 (0.28 – 5.08)
CX3CL1	<0.001	1.23 (0.98 – 1.62)	<0.001	1.20 (0.89 – 1.61)	>0.1	1.12 (0.93 – 1.51)	STAMBP	>0.1	1.04 (0.22 – 3.10)	>0.1	1.03 (0.36 – 3.67)	>0.1	1.12 (0.28 – 3.77)
CXCL1	<0.001	1.89 (1.00 – 5.05)	<0.001	1.94 (0.98 – 6.04)	>0.1	0.95 (0.22 – 4.07)	TGFA	<0.001	2.07 (0.89 – 5.32)	<0.001	2.17 (0.79 – 4.96)	>0.1	1.04 (0.29 – 3.91)
CXCL10	<0.001	1.34 (1.04 – 2.15)	<0.001	1.35 (0.80 – 2.15)	>0.1	1.16 (0.30 – 3.44)	TGFB1	<0.001	1.52 (0.77 – 2.37)	<0.001	1.64 (0.87 – 2.87)	>0.1	0.99 (0.44 – 2.31)
CXCL11	<0.001	1.7 (1.18 – 3.25)	<0.001	1.81 (1.05 – 3.35)	>0.1	1.07 (0.35 – 2.75)	TNF	<0.001	1.26 (1.06 – 1.85)	<0.001	1.26 (0.90 – 1.69)	>0.1	1.06 (0.73 – 1.54)
CXCL5	<0.001	1.99 (1.14 – 7.70)	<0.001	1.98 (1.00 – 6.05)	>0.1	1.05 (0.21 – 4.68)	TNFB	0.011	1.15 (0.94 – 1.38)	0.033	1.14 (0.91 – 1.54)	>0.1	1.05 (0.82 – 1.44)
CXCL6	0.034	1.62 (0.97 – 3.47)	<0.001	1.81 (0.97 – 3.09)	>0.1	0.99 (0.42 – 2.68)	TNFRSF9	<0.001	1.09 (0.90 – 1.56)	<0.001	1.08 (0.84 – 1.33)	>0.1	1.03 (0.75 – 1.39)
CXCL9	>0.1	1.24 (1.02 – 1.61)	>0.1	1.23 (0.84 – 1.48)	>0.1	1.21 (0.45 – 3.87)	TNFSF12	<0.001	1.43 (1.04 – 2.23)	<0.001	1.41 (0.94 – 1.88)	>0.1	1.07 (0.64 – 1.85)
DNER	>0.1	1.14 (0.97 – 1.34)	>0.1	1.14 (0.97 – 1.44)	>0.1	1.08 (0.89 – 1.26)	TNFSF14	0.062	1.93 (0.77 – 5.56)	0.024	2.15 (0.81 – 6.63)	>0.1	0.98 (0.29 – 2.15)
EIF4EBP1	<0.001	0.85 (0.09 – 5.53)	<0.001	0.84 (0.13 – 10.4)	>0.1	1.27 (0.23 – 8.92)	TRAIL	<0.001	1.11 (0.87 – 1.34)	<0.001	1.11 (0.91 – 1.44)	>0.1	1.06 (0.77 – 1.26)
FGF19	<0.001	0.87 (0.43 – 4.16)	<0.001	0.91 (0.43 – 3.04)	>0.1	0.8 (0.33 – 2.32)	TRANCE	<0.001	1.06 (0.83 – 1.28)	0.001	1.09 (0.83 – 1.49)	>0.1	1.05 (0.73 – 1.51)
FGF21	>0.1	1.02 (0.51 – 2.25)	>0.1	0.92 (0.51 – 1.62)	>0.1	1.04 (0.20 – 5.61)	VEGFA	<0.001	1.45 (1.01 – 2.90)	<0.001	1.49 (0.87 – 2.49)	>0.1	0.98 (0.44 – 1.74)
FLT3LG	<0.001	1.37 (1.05 – 1.75)	<0.001	1.41 (1.08 – 2.00)	>0.1	0.95 (0.67 – 1.20)							

Fold changes (FC) of protein abundance is given as mean (range of values). P-values were adjusted for multiple testing based on Benjamini-Hochberg. Acute Ex: Acute exercise.

Figure legends

Figure 1: Acute exercise-induced changes in circulating cytokines. (A-B): Volcano plots showing changes in serum levels of cytokines caused by 30 min cycling in (A) untrained subjects (n = 21) and (B) after an 8-week endurance training (n = 22). Red color highlights a fold change >1.2. IL6 and all cytokines with fold change >1.5 are annotated. (C): Correlation between acute exercise-induced cytokine changes before and after endurance training. The dotted line represents equality. P-values were adjusted for multiple testing according to Benjamini-Hochberg.

Figure 2: Transcript levels of cytokines in leukocytes. Boxplots showing the expression of (A) *CXCL1*, (B) *CXCL5*, (C) *IL6*, (D) *OSM*, (E) *TGFA*, (F) *TNF* and (G) *TNFSF14* in leukocytes from untrained and trained subjects before and after acute exercise. Results were normalized using TBP transcript levels. The box represents the interquartile range (IQR, 25th to 75th percentile), the black line represents the median. The whiskers extend the box to the lowest or largest value no further than 1.5*IQR. AU: arbitrary units. N = 20–22.

Figure 3: Correlation of acute exercise-induced changes in circulating cytokines with fitness and clinical data. The acute exercise-induced fold changes of serum cytokine concentrations in untrained or trained subjects were correlated to pre-training or post-training body mass index (BMI), content of visceral adipose tissue (VAT), subcutaneous adipose tissue (SCAT), insulin sensitivity (ISIMats), and VO2 peak. The color illustrates the direction and strength of the correlation (red: positive, blue: negative) and the diameter indicates statistical significance; empty circles depict non-significant correlations. Only cytokines with at least one significant correlation are shown.

Figure 4: Lactate levels and correlation with interleukin 6 after acute exercise. (A) Plasma lactate concentrations before (rest) and after (acute) 30 min cycling in untrained subjects and after an 8-week endurance training. (B-C) Correlations between the fold changes in circulating interleukin 6 (IL6) and lactate in (B) untrained and (C) trained subjects. The shaded red area represents the 95% confidence level. AU: arbitrary units, FC: fold change. N = 21-22.

Figure 1

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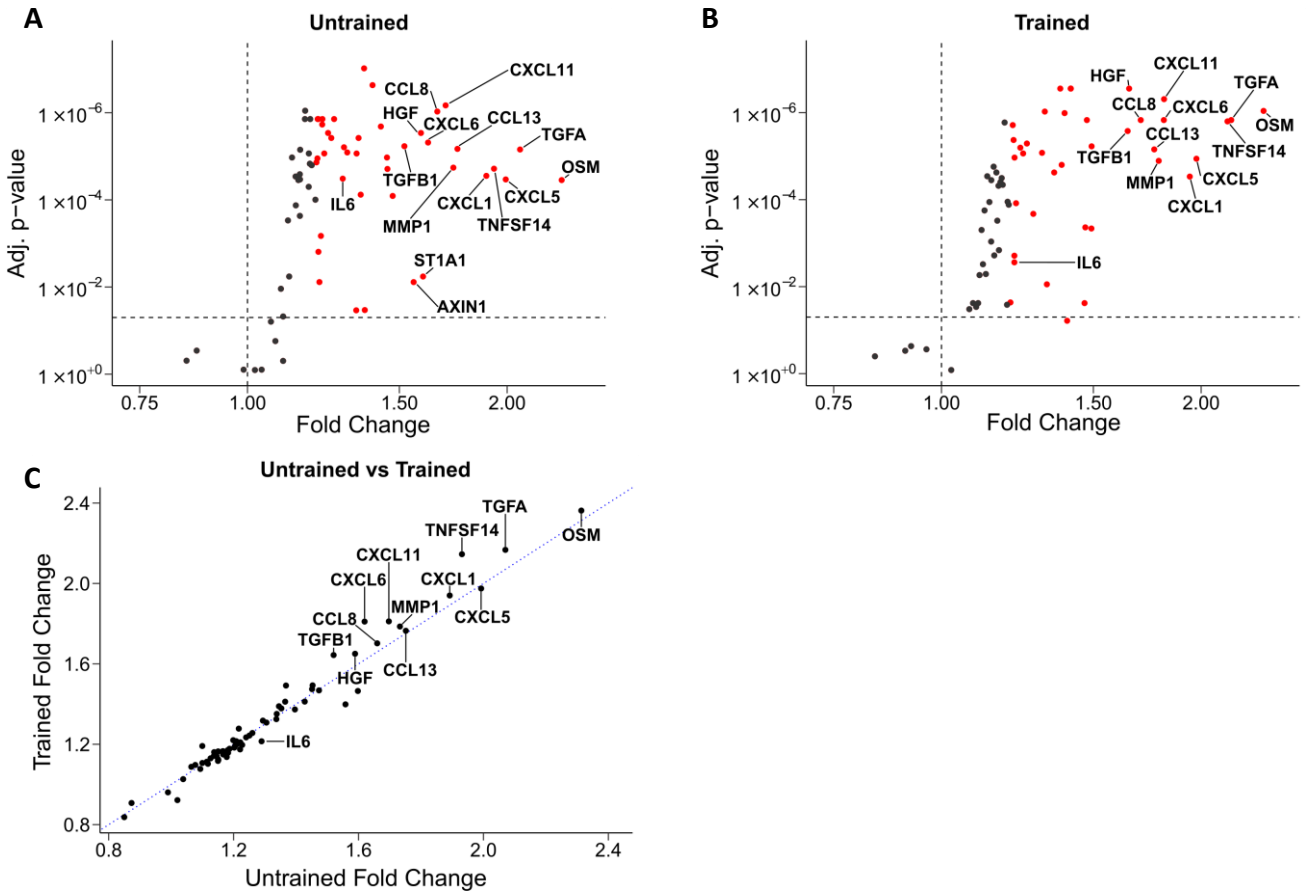

Figure 2

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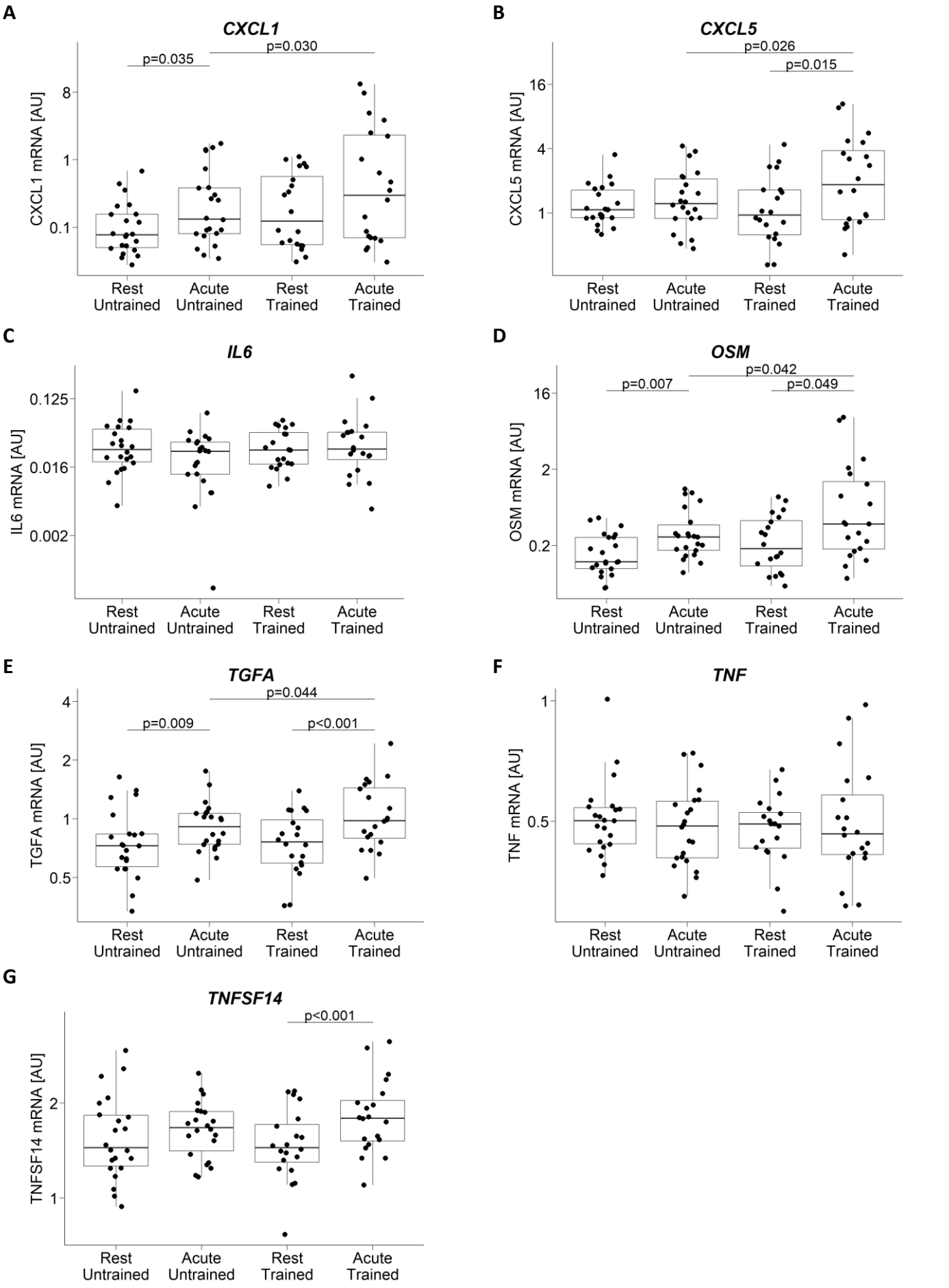

Figure 3

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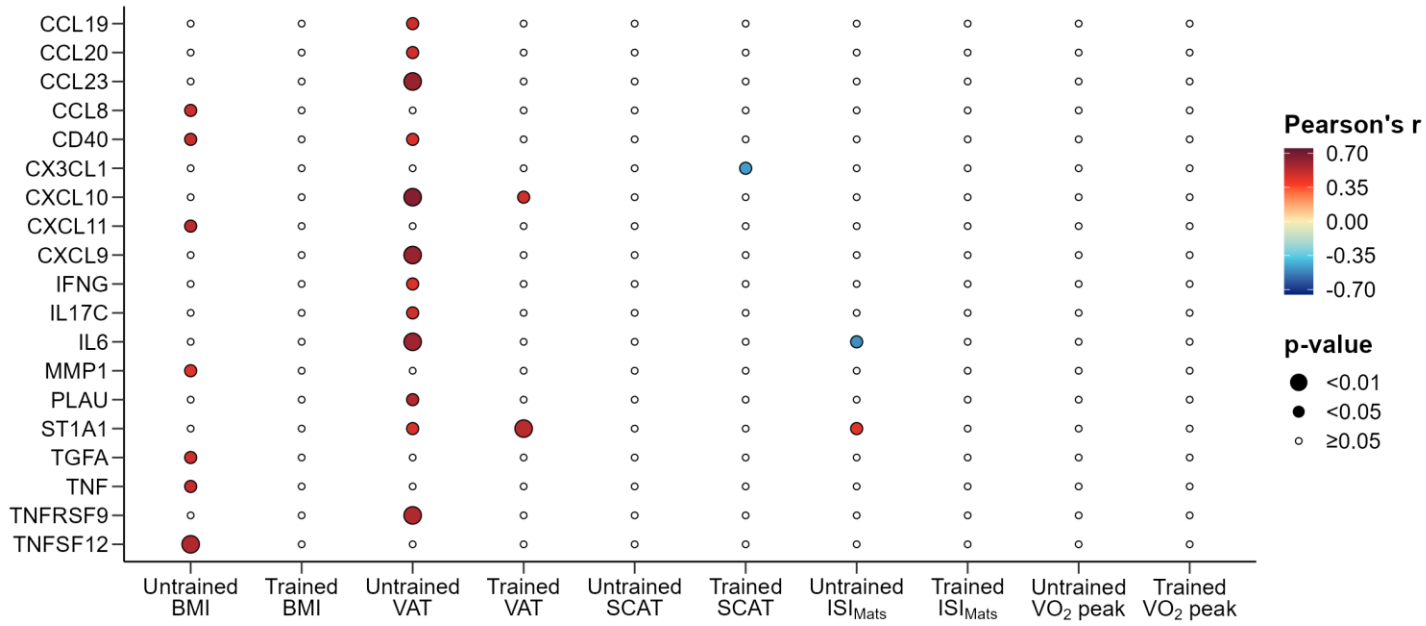

Figure 4

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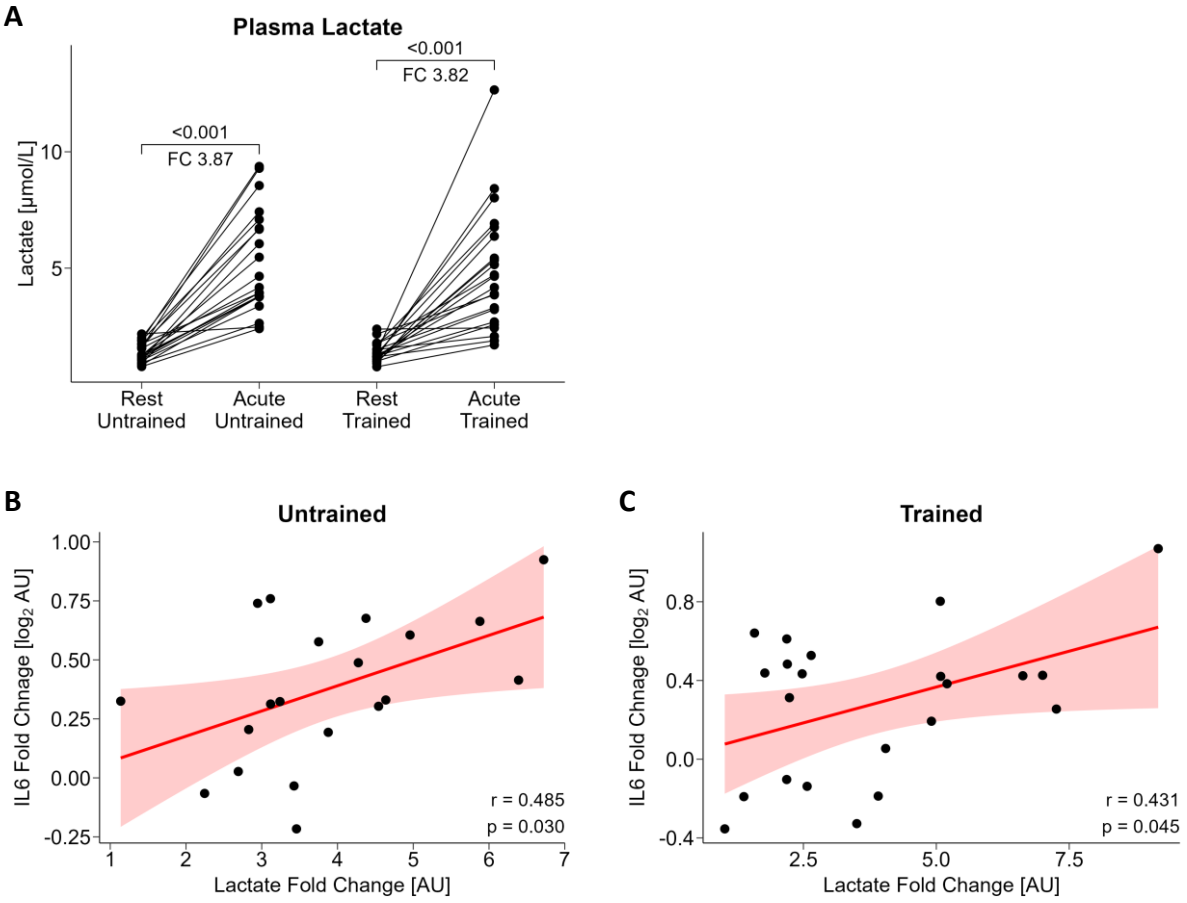

Supplemental Figure

Supplemental Table