Accepted: 26 March 2018

DOI: 10.1002/tsm2.23

ORIGINAL ARTICLE

WILEY

Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

R. Terink[1] | C. C. W. G. Bongers[2] | R. F. Witkamp[1] | M. Mensink[1] | T. M. Eijsvogels[2] | J. M. T. Klein Gunnewiek[3] | M. T. E. Hopman[1,2]

[1]Division of Human Nutrition, Wageningen University & Research (WUR), Wageningen, The Netherlands

[2]Department of Physiology, Radboud Institute for Health Sciences, Radboud University Nijmegen, Nijmegen, The Netherlands

[3]Clinical Chemistry and Haematology Laboratory, Gelderse Vallei Hospital (ZGV), Ede, The Netherlands

Correspondence
Rieneke Terink, Division of Human Nutrition, Wageningen University & Research (WUR), Wageningen, The Netherlands.
Email: rieneke.terink@wur.nl

Funding introduction
This study was part of the EAT2MOVE project and supported by a grant from the Province of Gelderland, proposal PS2014-49.

Previous studies have shown that exercise-induced changes in cytokine profiles depend on exercise duration and intensity. Studies are generally limited to a single day, and insight into the time course during multiple days of exercise is lacking. Therefore, this study assessed cytokine responses during multiple days of moderate intensity exercise in men and women. Fifty males (58.9 ± 9.9 years) and fifty females (50.9 ± 11.2 years) were monitored on 4 consecutive days at which they walked on average ~9 h/d at a self-determined pace. Blood samples were collected 1 or 2 days prior to the start of the exercise (baseline) and every walking day immediately post-exercise. Blood samples were analyzed for IL-6, IL-8, IL-10, IL-1β, and TNF-α concentrations. All cytokine concentrations increased from baseline to post-exercise at day 1 ($P < .001$). Thereafter, concentrations decreased from day 1 to day 2 ($P < .01$), remaining rather stable during the next days. IL-1β and TNF-α were higher in men at baseline and during all days. In conclusion, exercise-induced cytokine increases attenuated on subsequent days, although daily workload remained constant. Men and women showed different baseline levels but similar exercise responses. These results suggest that individuals adapt rapidly to this type of repeated exercise.

KEYWORDS
IL-1 beta, interleukins, myokines, repetitive exercise, TNF-alpha

1 | INTRODUCTION

In response to exercise, various signaling proteins are released by the immune and the musculoskeletal systems.[1] Many of these molecules are typically classified as cytokines and mainly known for their immune-regulatory roles. Those which are also, or exclusively, produced by skeletal muscle are often referred to as "myokines".[2] The best-studied cytokine in relation to exercise is interleukin-6 (IL-6), which can act as both anti- and pro-inflammatory cytokine. After exercise, IL-6 concentrations in blood can increase up to 100-fold.[3] IL-6 has a positive effect on glucose uptake and fat oxidation,[4,5] and, when it is considered as an anti-inflammatory cytokine, IL-6 attenuates the production of TNF-α and IL-1β.[5] Pro-inflammatory cytokines that show an increase after long and/or heavy exercise include TNF-α and IL-1β,[3] which are known to be involved in the acute phase reaction and cell proliferation, respectively. Other cytokines that may increase upon exercise are IL-8 and IL-10.[6]

Previous studies demonstrated that cytokine concentrations after a single bout of exercise are influenced by the intensity and duration of exercise.[7] For example, a 6-hour run or a marathon increased IL-6 and IL-8 plasma concentrations,[7-9] while these elevations were not found after a 5-

km run.[10] In addition, high-intensity interval exercise induced significant increases in IL-8 and IL-10 concentrations, whereas moderate intensity interval exercise had no effect on both cytokines.[6]

Almost all previous studies investigated the effect of a single bout of exercise, while health- and performance-enhancing effects of exercise are predominantly apparent after repeated bouts of exercise. Recently, in our laboratory, we showed that repeating exactly the same bout of cycling exercise after 1 week resulted in an attenuated cytokine response in well-trained young men, suggesting an adapted response.[11] Whether such an adaptation also occurs when exercise is performed on consecutive days, or whether cytokines accumulate over time, is not clear from the literature. Suzuki et al[12] showed that 3 consecutive days of 90-minutes bicycling exercise per day increased levels of IL-6 after the first exercise bout, while these levels remained elevated until day 3. A study investigating the effects of an Ironman marathon showed that IL-6 levels remained elevated (+345%; $P < .001$) for more than 24 hours after finishing, before returning to baseline,[13] suggesting that accumulation may occur when a second bout of exercise would have been performed within this time frame.

Furthermore, it is suggested that the cytokine response to exercise may differ according to sex.[14] As contracting skeletal muscles are an important source of IL-6, a higher muscle mass in men could result in higher concentrations of this cytokine compared to women.[15] This effect of muscle mass is clearly illustrated in exercise studies considering small muscle groups, for example, the muscles of the upper extremities, where IL-6 concentrations were not detected immediately after exercise.[16] This would suggest that the immune response to exercise may differ between men and women. However, direct comparisons between men and women in this respect are scarce in the literature.

Therefore, the aim of this study was to assess changes in circulating cytokine levels after long-distance walking (30-50 km) at moderate intensity on 4 consecutive exercise days in middle-aged men and women. In addition, the differences in responses between men and women were explored. We hypothesized that (at least some) cytokines would accumulate over 4 days of repeated prolonged moderate intensity exercise, due to the short recovery period. Secondly, we expected higher cytokine levels in men compared to women.

2 | MATERIALS AND METHODS

2.1 | Study population

We selected 50 male and 50 female walkers who participated in the 2015 edition of the Nijmegen Four Days Marches, a large annual walking event taking place in the Netherlands (http://www.4daagse.nl/en/). Exclusion criteria were known diabetes and/or renal dysfunction. The study was approved by the Medical Ethical Committee of the Radboud university medical center (CMO registration number: 2007/148), and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

2.2 | Study procedure

The study took place in the summer season, that is, July. Actual climatological conditions are specified in the Results section. Measurements were performed before the start of the event ("baseline") and at the 4 consecutive walking days. Baseline measurements, including registering participants' characteristics, collection of a blood sample, and taking questionnaires, were performed in our field laboratory at the event location 1 or 2 days prior to the first walking day, between 09:30 AM and 04:00 PM.

Every walking day, immediately before the start of the march, participants' body weight was determined. Thereafter, participants walked 30, 40, or 50 km, at a self-determined pace, starting between 04:00 and 08:00 AM. The walking track was almost completely flat, typical for the Dutch landscape with only small height differences, for example, for crossing bridges. Details about the routes can be found at https://www.4daagse.nl/en/routes/route-distance.html. Every day, participants registered their fluid intake using a diary. Directly after finishing, post-exercise body weight was determined, a blood sample was taken, and a set of questionnaires was completed. Heart rate was measured during the first walking day every 5 km and at the finish. Mean heart rate during exercise was calculated as the average heart rate, excluding the values derived directly before the start and after the finish. Heart rate was used to estimate exercise intensity as percentage of HRmax = (Measured HR/Expected maximal HR) × 100%, with Expected max HR = 208−(0.7 × Age).[17]

2.3 | Baseline measurements

At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were determined, and body mass index (BMI) was calculated. Thereafter, resting heart rate and blood pressure were measured using an automated sphygmomanometer (M5-1 intellisense, Omron Healthcare, Hoofddorp, The Netherlands) after 5-minute supine rest.

2.4 | Blood samples

Blood samples were taken at baseline and post-exercise at the 4 consecutive walking days. Participants were seated for 5 minutes after which a venous blood sample was taken from the cephalic vein. Blood was collected in a 4 mL EDTA vacutainer (Becton-Dickinson, New Jersey, USA).

The vacutainer was immediately put on melting ice water (0-4°C) and centrifuged at 1200 *g* for 15 minutes at 4°C. Plasma was transferred to polypropylene tubes and stored at −80°C until analysis. We measured IL-6, IL-8, IL-10, IL-1β, and TNF-α concentrations using the ultrasensitive MesoScale Discovery (MSD) QuickPlex SQ 120 Instrument with Multispot assay (Human Pro-inflammatory Panel 1, K15049D, MSD) according to the manufacturers' instructions. The lower detection limit varies per plate and was 0.029-0.159, 0.025-0.051, 0.021-0.042, 0.008-0.061, and 0.034-0.079 pg/mL for IL-6, IL-8, IL-10, IL-1β, and TNF-α, respectively. For cytokine concentrations below the lower detection limit, this lower detection limit was divided by 2, assuming that data below the detection limit were normally distributed.[18] Sixty of 490 samples (<15%) for IL-1β were below the lower detection limit. The other cytokines were all above detection limit.

Precision of these validated kits was as follows: The intrarun % CV for the high-low controls were 3.6%-4.5%, 2.7%-3.0%, 2.6%-3.7%, 3.3%-4.1%, and 2.7%-3.4% for IL-6, IL-8, IL-10, IL-1β, and TNF-α, respectively. The interrun % CV for the high-low controls were 5.2%-7.3%, 5.0%-7.1%, 5.7%-10.1%, 5.5%-7.7%, and 6.1%-10.1% for IL-6, IL-8, IL-10, IL-1β, and TNF-α, respectively. With the high-low concentrations being as follows: 239-18.4 pg/mL, 166-12.5 pg/mL, 107-7.18 pg/mL, 152-11.2 pg/mL, and 75.5-4.45 pg/mL, for IL-6, IL-8, IL-10, IL-1β, and TNF-α, respectively.

Furthermore, an additional blood sample was collected in a 2 mL Lithium Heparin vacutainer (Becton-Dickinson, New Jersey, USA) and used for direct analyses of plasma hemoglobin and hematocrit concentrations (Rapidpoint 400, Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). Relative changes in plasma volume were calculated from blood hematocrit and hemoglobin concentrations using Dill and Costill's equation.[19]

2.5 | Questionnaires

All participants completed an online questionnaire before the event, which included a food intake questionnaire (Food Frequency Questionnaire, FFQ), a validated Short Questionnaire to Assess Health enhancing physical activity (SQUASH) and 4 questionnaires at baseline and the same 4 questionnaires at the end of every walking day. Relevant to this study was a questionnaire about the use of painkilling drugs to check whether measured inflammatory markers were influenced by nonsteroidal anti-inflammatory drugs (NSAID) use. The other 3 questionnaires were related to other ongoing studies and involved questions about mood states, symptoms related to upper respiratory tract infections, and use of supplements. The total amount of physical activity level (PAL) in Metabolic Equivalent (MET) hours per week (MET-h/wk) was calculated by multiplying the exercise time in hours with the accompanying MET score of the activity intensity. We incorporated commuting activities, leisure time activities, and sports to assess activities of daily living (ie, total physical activity).

2.6 | Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), with the level of significance set at $P < .05$. Data are presented as mean ± SD unless indicated otherwise.

The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. As cytokine data were not normally distributed, nonparametric tests were used for analysis. A Friedman's test was used to evaluate the effect of consecutive exercise days on cytokine concentrations. Wilcoxon signed-rank test was used to determine whether data from consecutive days significantly differed from one another. A Mann-Whitney test was used to assess differences in cytokine response between men and women. Participant and exercise characteristics were analyzed with an independent Student's *t* test to examine sex differences.

3 | RESULTS

3.1 | Participant characteristics

Two of our female participants did not finish the first day, due to back problems, and were excluded from further analysis. The characteristics of the remaining 98 participants who completed all 4 days are shown in Table 1. Significant differences between men and women were found for age, height, weight, BMI, blood pressure, physical activity level, energy, fiber, and vitamin A and E intake (estimated with FFQ). There were no differences in resting heart rate, macronutrient intake, and average training distance in the year prior to the Marches and vitamin C intake.

3.2 | Exercise characteristics

The Four Days Marches took place under mild ambient conditions, with temperatures varying from 13°C wet bulb globe temperature (WBTG) at the start of the exercise (ie, 04:00 AM) to 24°C at finish time. Exercise intensity determined at the first walking day was found to be 67 ± 9% of the expected maximal heart rate, with an average of 114 ± 17 bpm (Table 2). No difference was found between male and female participants ($P = .679$). Based on these criteria, the exercise was classified as moderate intensity.[20]

Male participants walked 30 km (n = 17), 40 km (n = 20), or 50 km (n = 13). Female participants walked the same distances, but these were differently divided among

TABLE 1 Participants characteristics

	Men (n = 50)	Women (n = 48)	*P* value
Characteristics			
Age (y)	58.9 ± 9.9	50.9 ± 11.2	<.001
Body composition			
Height (cm)	180 ± 6	167 ± 6	<.001
Weight (kg)	88.9 ± 13.3	65.6 ± 8.1	<.001
BMI (kg/m^2)	27.3 ± 3.3	23.45 ± 3.0	<.001
Cardio characteristics			
Resting heart rate (bpm)	63 ± 9.9	64 ± 6.9	.758
Systolic pressure (mm Hg)	142 ± 18	133 ± 21	.036
Diastolic pressure (mm Hg)	88 ± 10	82 ± 11	.014
PAL score (MET-h/wk)	1016 ± 548	1272 ± 609	.033
Dietary intake			
Energy intake (kJ)	10 730 ± 2811	8024 ± 2502	<.001
Protein (En%)	14.9 ± 2.4	15.8 ± 2.5	.090
Fat (En%)	36.2 ± 6.0	35.0 ± 5.5	.308
Carbohydrates (En%)	41.8 ± 6.7	42.3 ± 5.3	.716
Fiber (g)	27 ± 7.8	22.3 ± 8.1	.005
Retinol (μg)	791 ± 437	491 ± 285	<.001
Vitamin E (mg)	16.9 ± 5.4	13.6 ± 5.9	.005
Vitamin C (mg)	112 ± 56	114 ± 54	.871
Walking distances			
Training distance (km)	812 ± 1072	753 ± 921	.770
Walking 30 km	n = 17	n = 5	
Walking 40 km	n = 20	n = 39	
Walking 50 km	n = 13	n = 4	

Means ± SD is shown. BMI, body mass index; kJ, kilojoule; En%: percentage of energy delivered by that macronutrient; PAL score, physical activity level in MET-hours per week; dietary intake values are estimated with a FFQ questionnaire, included in the online questionnaire; training distance: specific walking distance in the year prior to the Four Days Marches; *P* value refers to an unpaired Student's *t* test between male and female participants.

persons (n = 5, n = 39, and n = 4, respectively). Mean exercise duration was longer for women compared to men during the first and last day ($P = .032$ and $P = .017$, respectively). Speed reduced significantly during the 4 days of walking for both men and women ($P = .003$ and $P > .001$, respectively). On average, the highest speed was measured on the first walking day, for both men (4.8 ± 0.8 km/h) and women (4.6 ± 0.6 km/h), followed by the third day and thereafter the second day. The slowest speed was measured at the final walking day for both men (4.5 ± 0.8 km/h) and women (4.2 ± 0.6 km/h). Speed was significantly different between men and women (4.7 ± 0.7 vs 4.5 ± 06, and 4.5 ± 0.8 vs 4.2 ± 0.6 km/h) at days 3 and 4 ($P = .046$ and $P = .009$, respectively, Table 2).

3.3 | Cytokines

3.3.1 | Baseline

Baseline IL-1β and TNF-α concentrations were significantly higher in men compared to women ($P < .01$, Table 3). Baseline IL-6, IL-8, and IL-10 concentrations were not different between men and women ($P = .146$, .963 and .134, respectively, Table 3).

3.3.2 | Consecutive exercise days

A significant change was seen for all cytokines during the 4 consecutive walking days (all *P*-values < .001). After the first day of exercise, IL-6, IL-8, IL-10, IL-1β, and TNF-α were all increased compared to baseline cytokine concentrations (all *P*-values < .001). A 13-fold increase was observed for IL-6, and a 1.4-fold increase for IL8, 2.3-fold for IL-10, 1.3-fold for IL-1β, and 1.1-fold for TNF-α (Figure 1). After the second walking day, IL-6, IL-8, IL-10, IL-1β, and TNF-α concentrations were significantly lower compared to post-exercise concentrations at day 1 ($P < .01$). However, these values were still significantly higher compared to baseline ($P < .05$). Compared to the second walking day, IL-6 showed a further decrease at day 3 ($P < .001$), with again a slight increase at day 4 ($P < .01$) (Figure 1A). IL-8 decreased further until day 3 ($P < .001$) and did not change at day 4 compared to day 3 ($P = .682$) (Figure 1B). IL-10 did not change significantly between day 2 and day 3 ($P = .319$) and between day 3 and day 4 ($P = .829$) (Figure 1C). IL-1β did not change from day 2 till day 3 ($P = .166$), but declined significantly from day 3 until day 4 ($P < .001$) (Figure 1D). TNF-α slightly decreased on the third walking day compared to the second walking day ($P < .001$) and thereafter increased at the last walking day ($P < .05$) (Figure 1E).

Cytokine IL-6 concentrations were significantly higher in men compared to women only after the second day of exercise (3.94 ± 1.98 vs 3.16 ± 2.07 pg/mL; $P = .015$), while there were no differences in IL-6 on other days. Cytokine IL-10 concentrations were significantly higher in men compared to women after the first day of exercise (0.66 ± 0.84 vs 0.38 ± 0.40 pg/mL; $P = .032$), while there were no differences on the subsequent days. Just as at baseline, IL-1β and TNF-α concentrations were higher in men compared to women, during all days of exercise ($P < .01$). IL-8 concentrations were not different between men and women ($P > .05$) (Table 3).

In total, 29 participants used NSAIDs at 1 or more days during the Four Days Marches (baseline included).

TABLE 2 Exercise characteristics presented for men and women at day 1 to day 4, for men and women separately

	Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Walking					
Exercise duration (h:min)					
Men	7:52 ± 2:32	8:32 ± 1:31	8:23 ± 1:45	8:51 ± 2:03	.001
Women	8:43 ± 1:04	9:05 ± 1:16	8:41 ± 2:15	9:45 ± 1:32	<.001
P value	.032	.054	.459	.017	
Speed (km/h)					
Men	4.8 ± 0.8	4.6 ± 0.8	4.7 ± 0.7	4.5 ± 0.8	.003
Women	4.6 ± 0.6	4.4 ± 0.7	4.5 ± 0.6	4.2 ± 0.6	<.001
P value	.235	.186	.046	.009	
Luid intake (L)					
Men	4.4 ± 1.6	4.4 ± 1.5	4.3 ± 1.3	2.8 ± 1.1	<.001
Women	4.1 ± 1.7	3.8 ± 1.5	3.6 ± 1.6	2.6 ± 1.0	<.001
P value	.367	.034	.015	.397	
Physical parameters					
Weight change (kg)					
Men	−1.4 ± 1.0	−0.8 ± 0.7	−0.5 ± 0.6	−0.9 ± 0.8	<.001
Women	−0.3 ± 0.7	−0.3 ± 0.5	−0.3 ± 0.5	−0.5 ± 0.5	.009
P value	<.001	<.001	.158	.016	
Plasma volume change from baseline (%)					
Men	−4.29 ± 12.21	−1.93 ± 8.34	3.62 ± 10.37	6.05 ± 8.98	<.001
Women	0.001 ± 5.91	3.40 ± 6.25	7.82 ± 7.45	8.67 ± 7.08	<.001
P value	.030	.001	.024	.113	
Mean heart rate (bpm)					
Men	113 ± 18				
Women	115 ± 15				
P value	.512				
Max heart rate (bpm)					
Men	124 ± 27				
Women	129 ± 18				
P value	.260				
Exercise intensity (%)					
Men	67 ± 11				
Women	67 ± 8				
P value	.679				

Values are mean values for the 4 walking days. *P* values refer to an unpaired Student's *t* test between male and female participants. Asymp. Sign *P* values refer to an intragroup Friedman's ANOVA test for the effect of days. Weight difference is calculated as post-exercise−pre-exercise, and a negative value means weight loss. Plasma volume change is calculated as day # - baseline, and plasma volume is calculated with Dill and Costill calculation 1974.

NSAIDs that were used by our participants included (name [dosage]): ibuprofen (200 mg, 400 mg, and 600 mg), diclofenac (50 mg), advil/naproxen (200 mg), and maxalt (5 mg). NSAID use was higher in women (NSAIDs were used 41 times spread over all days) compared to men (20 times in total). Re-evaluating cytokine responses by excluding those participants who used NSAIDs at baseline and/or during the exercise days, resulted in comparable results. Trends in cytokine responses during the walking days did not differ between users and nonusers.

3.4 | Plasma volume

Plasma volume changed significantly over time, be it with a different pattern in men and women ($P > .001$). Male participants showed a decline in plasma volume

TABLE 3 Mean cytokine concentrations (pg/mL) at baseline and day 1 to day 4, for men and women separately

	Baseline	Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
IL-6						
Men	0.60 ± 0.34	7.42 ± 4.74	3.94 ± 1.98	2.94 ± 1.75	3.54 ± 2.93	<.001
Women	0.51 ± 0.32	7.58 ± 7.62	3.16 ± 2.07	2.88 ± 2.33	3.97 ± 4.19	<.001
P value	.146	.268	.015	.227	.877	
IL-8						
Men	8.00 ± 2.81	12.14 ± 4.38	9.55 ± 3.56	8.19 ± 3.70	7.85 ± 3.31	<.001
Women	7.78 ± 2.50	10.66 ± 3.18	8.28 ± 2.25	6.92 ± 2.01	7.18 ± 1.74	<.001
P value	.963	.064	.105	.121	.729	
IL-10						
Men	0.23 ± 0.22	0.66 ± 0.84	0.29 ± 0.26	0.28 ± 0.27	0.33 ± 0.44	<.001
Women	0.23 ± 0.38	0.38 ± 0.40	0.23 ± 0.14	0.30 ± 0.48	0.24 ± 0.21	<.001
P value	.134	.032	.117	.771	.486	
IL-1B						
Men	0.24 ± 0.37	0.30 ± 0.39	0.32 ± 0.39	0.37 ± 0.74	0.18 ± 0.08	<.001
Women	0.14 ± 0.13	0.19 ± 0.20	0.15 ± 0.16	0.18 ± 0.31	0.11 ± 0.12	<.001
P value	.002	<.001	<.001	<.001	<.001	
TNF-a						
Men	1.76 ± 0.49	1.92 ± 0.65	1.80 ± 0.48	1.78 ± 0.63	1.81 ± 0.61	.001
Women	1.51 ± 0.34	1.58 ± 0.30	1.51 ± 0.30	1.46 ± 0.31	1.51 ± 0.31	<.001
P value	.006	.002	.001	.001	.004	

P-value represents Mann-Whitney test for differences between men and women. Asymp. Sign *P* values refer to an intragroup Friedman's ANOVA test for the effect of days.

during the first (−4.3 ± 12.2%) and second walking day and an increase during the third and last walking day, while female participants showed no change during the first walking day (0.0 ± 5.9%), and an increase on the successive walking days (see Table 2). Change in body mass was significantly larger for men (−1.4 ± 1.0 kg) after the first day of exercise compared to women (−0.3 ± 0.7 kg) ($P < .001$), while no difference between sexes was observed in mean fluid intake at day 1 ($P = .367$, Table 2). Change in body mass and fluid intake was both significantly different between men and women at day 2 ($P < .001$ and $P = .034$, respectively), weight change was higher in men, while fluid intake was higher in men as well. Finally, weight change was significantly higher in men (−0.9 ± 0.8 kg) compared to women (−0.5 ± 0.5 kg) at day 4 ($P = .016$), while there was no significant difference in fluid intake that day ($P = .397$).

4 | DISCUSSION

The aim of this study was to assess changes in plasma cytokine levels during 4 consecutive days of long-distance walking (~9 hours each day) at moderate intensity in a group of middle-aged men and women. Our main finding was that most of the measured cytokines peaked after the first day, while showing no further increase, and even a decline during the subsequent days. This was in contrast to our hypothesis that cytokines would be likely to accumulate considering the duration, intensity as well as the relatively short recovery periods between exercise bouts. These results suggest a rapid adaptation to this type of exercise. We also observed that men showed higher baseline IL-1β and TNF-α concentrations and that these levels remained higher in men compared to women during all 4 exercise days. This was in line with our hypothesis, based on previous studies.[15] To the best of our knowledge, studies comparable to the present study are scarce if not absent in the literature.

4.1 | Cytokine concentrations after 1 day of exercise

Our data showed a clear increase in IL-6, IL-8, IL-10, IL-1β, and TNF-α plasma levels after the first walking day, which is in accordance with previous studies considering different types of exercise.[2,5,7-9,15,21] This indicates that in

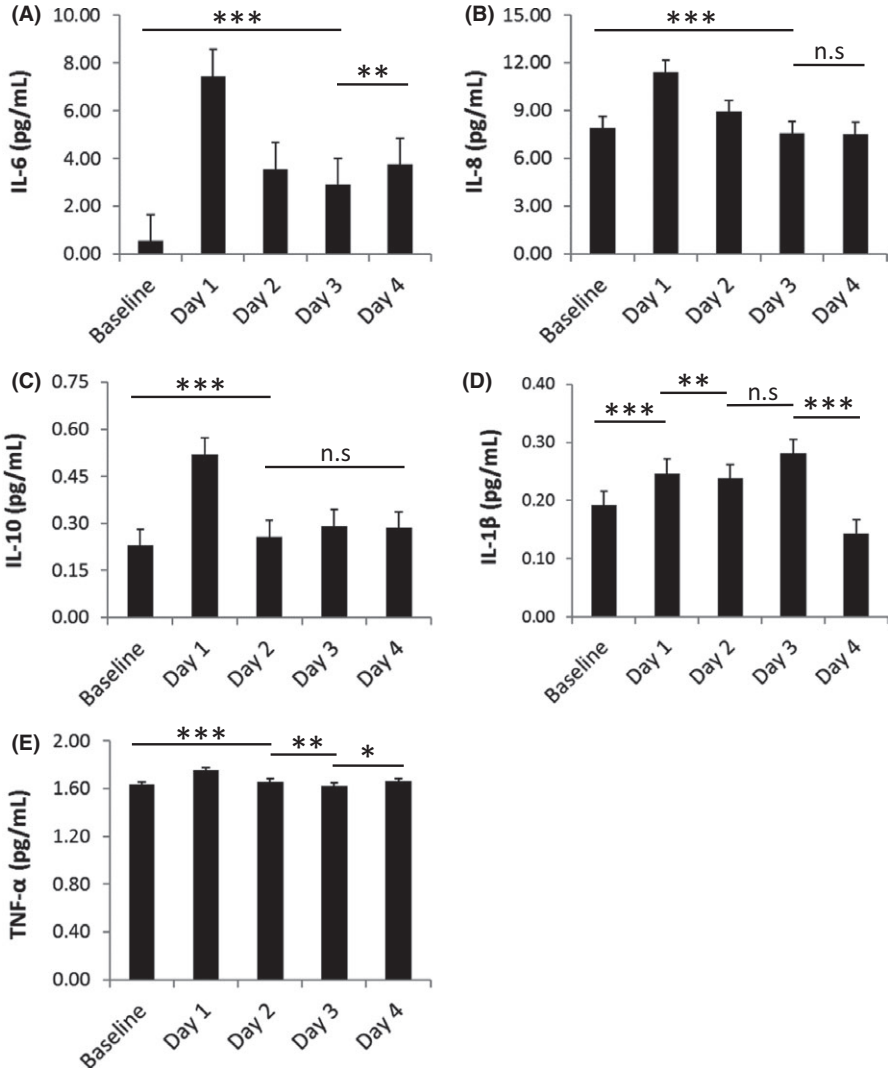

FIGURE 1 Cytokine concentrations for all participants together. Means ± SE are shown. Significant differences between days are presented with horizontal lines, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

our participants, a walking exercise with an average duration of 8 h 44 minutes (range 4 h 45 min-12 h 50 min) at 67 ± 9% of HRmax (range 40%-88%) induces a cytokine response more or less comparable to responses seen after a marathon,[9] or an ultramarathon.[21] In comparison, a moderate intensity walk of 30 minutes at 50% of maximal oxygen uptake does not cause an increase in circulating cytokines.[22]

The exercise-induced increase in IL-6 is known to be related to the duration and the intensity of the exercise.[15] Based on previous studies, it is plausible that the increase in plasma IL-6 after the first day of exercise is triggered by a reduced glucose availability, that is, a decrease in muscle glycogen concentration during exercise.[5] Indeed, it has been shown that pre-exercise carbohydrate (CHO) status can influence the plasma IL-6 response, with higher responses after low CHO intake and lower responses after high CHO intake.[23] Whether this solely explains our observations is unlikely. For example, an increase in cytokine levels can also be related to physical tissue stress and microdamage,[3,24] which is most likely present during multiple hours of walking exercise. Moreover, participants were able to eat at any moment during the study including during the exercise. The increased levels of IL-6 may contribute to the increased levels of IL-10, as IL-6 activates monocytes to secrete IL-10. However, it is questionable whether the increases in IL-6 are high enough to induce this IL-10 increase.[25] An increase in IL-10 in turn serves as a feedback to inhibit the synthesis of pro-inflammatory cytokine TNF- α.[26]

Mild hypoxia might play a role in the increase in IL-8. Such an increase has been associated with beneficial training adaptations, as IL-8 promotes angiogenesis.[27] Like IL-6, IL-8 can be released by skeletal muscle, which makes it a "myokine." It is suggested that reactive oxygen species (ROS) stimulate the production of myokines in skeletal

muscle in response to exercise.[28] Some human intervention studies support this idea.[29,30]

Although the interpretation of levels of individual cytokines in terms of their effects remains difficult, IL-6 and IL-8 are at least partly associated with an anti-inflammatory component. By contrast, the increase in IL-1β and TNF-α might indicate a more pro-inflammatory stimulus. These cytokines are known to be induced by endotoxemia. A study with athletes performing an 89.4 km race showed that 81% of the participants had plasma endotoxin concentrations above 0.1 ng/mL.[31] In addition, increased plasma levels of lipopolysaccharides (LPS), indicative of endotoxemia, in athletes who took part in an ultra-distance triathlon have been recorded.[32] When endotoxins cross the gut epithelial barrier and enter into the circulation, this triggers a cascade involving TNF-α, IL-1β, and IL-6.

4.2 | Consecutive exercise days

Our observation that cytokine concentrations did not further increase after the first day of walking is remarkable and might suggest rapid adaptation. Unfortunately, other studies concerning repeated exercise bouts mainly focussed on eccentric exercise[33,34] and not on prolonged repeated moderate intensity exercise. In a previous study in our laboratory,[11] be it with a different design (bicycle exercise tests), we also found marked attenuated cytokine responses after repeating the same exercise with 1 week in between. In contrast, Suzuki et al[12] studied the effect of 3 consecutive days of 90-minutes (at 90 W) bicycling exercise on cytokine concentrations and found increased levels of IL-6 after the first exercise bout, but these levels remained elevated until day 3. Why they found no attenuated response is not clear, as their exercise protocol seems less challenging compared to 4 days of prolonged walking.

If some form of muscle damage occurred, an explanation could also be found in the degree of muscle injury and the potential adaptations in muscle to become more resistant to subsequent injury. Studies with repeated bouts of eccentric exercise, applying intervals between subsequent exercise bouts varying between 5 days until 10 weeks,[33,34] show that changes in variables like muscle soreness and serum creatine kinase[33] were significantly smaller after a second bout of exercise. These results suggest that an adaptation response took place following the initial eccentric exercise bout. It is plausible that similar adaptation mechanisms are induced following endurance exercise in the current study.

Additionally, the decrease in plasma volume was highest after the first day of exercise, which could result in a high peak of cytokines. However, the increase in plasma cytokine levels ranged between 1.1-fold and 13-fold. Therefore, the change in plasma volume could not solely be responsible for the increase in cytokines.

Finally, a decrease in exercise intensity on day 2 till 4 could also have played a role in the attenuated cytokine responses. Exercise intensity as percentage of maximal heart rate was only measured on the first day of walking due to practical reasons. However, speed was highest on the first day of exercise, which might suggest that exercise intensity was also the highest on the first day of exercise. Minor muscle damage after the first walking day might cause speed to drop on successive days, which could result in lower intensity. It has been shown that exercise intensity determines the cytokine change after exercise.[7] Therefore, this change in speed during the 4 walking days could attribute to the attenuated cytokine response on the second, third, and final walking day.

4.3 | Differences in cytokine between men and women

Prior to the study, we expected higher cytokine levels in men compared to women for all cytokines, during all days. As contracting skeletal muscles are an important source of IL-6, a higher average muscle mass in men may result in higher IL-6 concentrations in men compared to women.[15] Remarkably, we found only higher baseline IL-1β and TNF-α concentrations in men compared to women. This has not been reported before. At the same time, IL-6, IL-8, and IL-10 baseline concentrations were comparable between men and women. This was in agreement with a study from Larsson et al[35] who found no differences in IL-6, IL-8, and IL-10 levels between men and women and with Edwards et al[14] (2006) who found comparable baseline IL-6 levels in men and women. Notwithstanding some differences at baseline, we did not find significant sex differences in exercise-induced responses in cytokines. An exception to this appeared to be IL-10 showing a significantly higher peak after the first day of walking in men compared to women. Opposite findings regarding IL-10 response between men and women were reported immediately and 1.5 hours after completing a marathon, where levels were comparable between men and women.[36] Unfortunately, we did not ask our female participants at which point in their menstrual cycle they were. It has been shown that the luteal phase of the menstrual cycle is associated with a greater capacity of immune cells to produce cytokines compared to the follicular phase.[37,38]

4.4 | Use of Nonsteroidal anti-inflammatory drug (NSAID)

NSAIDs are able to suppress cytokine production.[39] As NSAID use was quite common in our population, this

could have affected the responses. However, we found no differences when NSAID users were excluded from statistical analysis. This might suggest that the cytokine production induced by this type of exercise outweighs the impact of anti-inflammatory medication on cytokine responses.

Other use of medicines (corticosteroids, beta-blockers, ACE inhibitors, and statins) with potential (mild-strong) anti-inflammatory effects was not investigated.

4.5 | Limitations, strengths, and conclusion

The present study had some practical limitations. First, we did not include a pre-exercise blood withdrawal every day. Therefore, we do not know whether cytokine concentrations were already lowered in the morning of the second walking day or whether cytokine concentrations decreased during exercise that day. Secondly, we did not investigate the phase of the menstrual cycle of our female participants, which could have influenced cytokine levels. Thirdly, baseline blood draws were not collected at the same time of day across participants, which introduces some variability into the measurement. This was for practical reasons, as participants arrived in the city of Nijmegen at a different time of the day prior to the event. And finally, we did not measure markers for muscle damage, which could be related to cytokine changes.

A strength of the present study was the inclusion of a large group of participants, with only 2 drop-outs. This large population not only enabled to determine baseline differences in cytokine levels between men and women, but also to establish rapid adaptation occurring to this type of exercise. Furthermore, our study population, characterized as generally healthy, regularly exercising middle-aged persons is quite unique and apparently underrepresented in the field of exercise physiology.

In conclusion, these results indicate that in this population, prolonged exercise at moderate intensity causes acute effects on cytokine levels in a degree comparable to that seen after running a marathon. However, this effect is attenuated when performing the same exercise on consecutive days, suggesting rapid adaptation. Furthermore, gender differences resulted in higher baseline IL-1β and TNF-α cytokine concentrations in men, and higher IL-10 and IL-6 concentrations in men compared to women after the first and second day of walking, respectively.

5 | PERSPECTIVES

The finding that this form of exercise causes a profound cytokine response which gradually phases out during consecutive exercise days, suggests an adaptive response to prolonged repeated exercise. The exercise load is rather heavy for this group of adults. Nowadays, regular exercise such as walking and cycling is practiced by many middle-aged adults with the aim to promote health. More insight into the associations between exercise load, repeats, and health effects for this specific group is desirable. In further studies, cytokine levels could be used as biomarkers or to increase our understanding of underlying mechanisms.

ACKNOWLEDGEMENTS

We would like to acknowledge Nhien Ly for analyzing the cytokine concentrations.

CONFLICT OF INTEREST

The results of this study do not constitute endorsement by ACSM. We declare that the results of this study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

AUTHORS' CONTRIBUTION

The study was designed by R. Terink, C.C.W.G. Bongers, M.T.E. Hopman, T.M. Eijsvogels and M.R. Mensink; data were collected and analyzed by R. Terink and C.C.W.G. Bongers; data interpretation and manuscript preparation were undertaken by R. Terink, C.C.W.G. Bongers, M.T.E. Hopman, R.F. Witkamp, M.R. Mensink, T.M. Eijsvogels, and J.M.T. Klein Gunnewiek. All authors approved the final version of the manuscript.

ORCID

R. Terink http://orcid.org/0000-0003-2608-7522

REFERENCES

1. Ball D. Metabolic and endocrine response to exercise: sympathoadrenal integration with skeletal muscle. *J Endocrinol*. 2015;224:R79-R95.
2. Pedersen BK, Steensberg A, Fischer C, et al. Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil*. 2003;24:113-119.
3. Ostrowski K, Rohde T, Zacho M, Asp S, Pedersen BK. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol*. 1998;508(Pt 3):949-953.
4. Steinbacher P, Eckl P. Impact of oxidative stress on exercising skeletal muscle. *Biomolecules*. 2015;5:356-377.
5. Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev*. 2008;88:1379-1406.
6. Dorneles GP, Haddad DO, Fagundes VO, et al. High intensity interval exercise decreases IL-8 and enhances the immunomodulatory cytokine interleukin-10 in lean and overweight-obese individuals. *Cytokine*. 2016;77:1-9.

7. Pedersen BK. Special feature for the Olympics: effects of exercise on the immune system: exercise and cytokines. *Immunol Cell Biol*. 2000;78:532-535.
8. Drenth JP, Van Uum SH, Van Deuren M, Pesman GJ, Van der Ven-Jongekrijg J, Van der Meer JW. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF-alpha and IL-1 beta production. *J Appl Physiol (1985)*. 1995;79:1497-1503.
9. Suzuki K, Yamada M, Kurakake S, et al. Circulating cytokines and hormones with immunosuppressive but neutrophil-priming potentials rise after endurance exercise in humans. *Eur J Appl Physiol*. 2000;81:281-287.
10. Drenth JP, Krebbers RJ, Bijzet J, van der Meer JW. Increased circulating cytokine receptors and ex vivo interleukin-1 receptor antagonist and interleukin-1beta production but decreased tumour necrosis factor-alpha production after a 5-km run. *Eur J Clin Invest*. 1998;28:866-872.
11. JanssenDuijghuijsen LM, Keijer J, Mensink M, et al. Adaptation of exercise-induced stress in well-trained healthy young men. *Exp Physiol*. 2017;102:86-99.
12. Suzuki K, Totsuka M, Nakaji S, et al. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *J Appl Physiol (1985)*. 1999;87:1360-1367.
13. Neubauer O, Konig D, Wagner KH. Recovery after an Ironman triathlon: sustained inflammatory responses and muscular stress. *Eur J Appl Physiol*. 2008;104:417-426.
14. Edwards KM, Burns VE, Ring C, Carroll D. Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. *J Sports Sci*. 2006;24:855-862.
15. Fischer CP. Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev*. 2006;12:6-33.
16. Nosaka K, Clarkson PM. Changes in indicators of inflammation after eccentric exercise of the elbow flexors. *Med Sci Sports Exerc*. 1996;28:953-961.
17. Tanaka H, Monahan KD, Seals DR. Age-predicted maximal heart rate revisited. *J Am Coll Cardiol*. 2001;37:153-156.
18. Staff USOoEIQ. Guidance for Data Quality Assessment: Practical Methods for Data Analysis EPA QA/G-9, (QA00 Update). Washington, DC: U.S. Environmental Protection Agency; 2000.
19. Dill DB, Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol*. 1974;37:247-248.
20. Medicine ACoS. ACSM's Guidelines for Exercise Testing and Prescription. Baltimore, MD: Lippincott Williams & Wilkins; 2013.
21. Nieman DC, Peters EM, Henson DA, Nevines EI, Thompson MM. Influence of vitamin C supplementation on cytokine changes following an ultramarathon. *J Interferon Cytokine Res*. 2000;20:1029-1035.
22. Markovitch D, Tyrrell RM, Thompson D. Acute moderate-intensity exercise in middle-aged men has neither an anti- nor proinflammatory effect. *J Appl Physiol (1985)*. 2008;105:260-265.
23. Bishop NC, Walsh NP, Haines DL, Richards EE, Gleeson M. Pre-exercise carbohydrate status and immune responses to prolonged cycling: II. Effect on plasma cytokine concentration. *Int J Sport Nutr Exerc Metab*. 2001;11:503-512.
24. Bruunsgaard H, Galbo H, Halkjaer-Kristensen J, Johansen TL, MacLean DA, Pedersen BK. Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. *J Physiol*. 1997;499(Pt 3):833-841.
25. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol*. 2007;8:1363-1371.
26. Dagvadorj J, Naiki Y, Tumurkhuu G, et al. Interleukin-10 inhibits tumor necrosis factor-alpha production in lipopolysaccharide-stimulated RAW 264.7 cells through reduced MyD88 expression. *Innate Immun*. 2008;14:109-115.
27. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol*. 2005;7:122-133.
28. Scheele C, Nielsen S, Pedersen BK. ROS and myokines promote muscle adaptation to exercise. *Trends Endocrinol Metab*. 2009;20:95-99.
29. Vassilakopoulos T, Karatza MH, Katsaounou P, Kollintza A, Zakynthinos S, Roussos C. Antioxidants attenuate the plasma cytokine response to exercise in humans. *J Appl Physiol (1985)*. 2003;94:1025-1032.
30. Fischer CP, Hiscock NJ, Penkowa M, et al. Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. *J Physiol*. 2004;558(Pt 2):633-645.
31. Brock-Utne JG, Gaffin SL, Wells MT, et al. Endotoxaemia in exhausted runners after a long-distance race. *S Afr Med J*. 1988;73:533-536.
32. Bosenberg AT, Brock-Utne JG, Gaffin SL, Wells MT, Blake GT. Strenuous exercise causes systemic endotoxemia. *J Appl Physiol (1985)*. 1988;65:106-108.
33. Ebbeling CB, Clarkson PM. Muscle adaptation prior to recovery following eccentric exercise. *Eur J Appl Physiol Occup Physiol*. 1990;60:26-31.
34. Nosaka K, Clarkson PM, McGuiggin ME, Byrne JM. Time course of muscle adaptation after high force eccentric exercise. *Eur J Appl Physiol*. 1991;63:70-76.
35. Larsson A, Carlsson L, Gordh T, Lind AL, Thulin M, Kamali-Moghaddam M. The effects of age and gender on plasma levels of 63 cytokines. *J Immunol Methods*. 2015;425:58-61.
36. Nieman DC, Henson DA, Smith LL, et al. Cytokine changes after a marathon race. *J Appl Physiol (1985)*. 2001;91:109-114.
37. Faas M, Bouman A, Moesa H, Heineman MJ, de Leij L, Schuiling G. The immune response during the luteal phase of the ovarian cycle: a Th2-type response? *Fertil Steril*. 2000;74:1008-1013.
38. Konecna L, Yan MS, Miller LE, Scholmerich J, Falk W, Straub RH. Modulation of IL-6 production during the menstrual cycle in vivo and in vitro. *Brain Behav Immun*. 2000;14:49-61.
39. Housby JN, Cahill CM, Chu B, et al. Non-steroidal anti-inflammatory drugs inhibit the expression of cytokines and induce HSP70 in human monocytes. *Cytokine*. 1999;11:347-358.

How to cite this article: Terink R, Bongers CCWG, Witkamp RF, et al. Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women. *Transl Sports Med*. 2018;1:110-119. https://doi.org/10.1002/tsm2.23