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Intensity-dependent gene expression after aerobic exercise in endurance-trained skeletal muscle

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ABSTRACT: We investigated acute exercise-induced gene expression in skeletal muscle adapted to aerobic training. Vastus lateralis muscle samples were taken in ten endurance-trained males prior to, and just after, 4 h, and 8 h after acute cycling sessions with different intensities, 70% and 50% $\dot{V}O_{2max}$. High-throughput RNA sequencing was applied in samples from two subjects to evaluate differentially expressed genes after intensive exercise (70% $\dot{V}O_{2max}$), and then the changes in expression for selected genes were validated by quantitative PCR (qPCR). To define exercise-induced genes, we compared gene expression after acute exercise with different intensities, 70% and 50% $\dot{V}O_{2max}$, by qPCR. The transcriptome is dynamically changed during the first hours of recovery after intensive exercise (70% $\dot{V}O_{2max}$). A computational approach revealed that the changes might be related to up- and down-regulation of the activity of transcription activators and repressors, respectively. The exercise increased expression of many genes encoding protein kinases, while genes encoding transcriptional regulators were both up- and down-regulated. Evaluation of the gene expression after exercise with different intensities revealed that some genes changed expression in an intensity-dependent manner, but others did not: the majority of genes encoding protein kinases, oxidative phosphorylation and activator protein (AP)-1-related genes significantly correlated with markers of exercise stress (power, blood lactate during exercise and post-exercise blood cortisol), while transcriptional repressors and circadian-related genes did not. Some of the changes in gene expression after exercise seemingly may be modulated by circadian rhythm.

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INTRODUCTION

Long-term endurance training induces marked adaptive changes in skeletal muscle: increased mitochondrial volume, capillarization, and intracellular glycogen and fat stores [32]. These changes are associated with improved maximal oxygen consumption rate ($\dot{V}O_{2max}$) and aerobic performance. A lot of metabolites accumulate in blood and working skeletal muscle during acute endurance exercise. These metabolites, as well as mechanical stress, activate different signalling pathways and induce expression of many genes in working muscle during the first hours after a bout of acute exercise [14, 15, 23].

Changes in the transcriptome in human skeletal muscle induced by acute endurance exercise have been investigated recently using DNA microarrays. The transcriptome changes were evaluated during the early (15 min–5 h) [3, 17, 21, 35, 38] and late (24–96 h) [17, 21, 35] stages of the recovery period. The transcriptome changes during the middle stage (8 h) of recovery were investigated only in one study, with a limited number of genes (220) monitored [35]. It has been shown that activation of signalling proteins and expression of genes in response to acute aerobic exercise are less pronounced (more specific) in human skeletal muscle adapted to endurance training in comparison with untrained muscle [22, 27, 35, 43]. Moreover, low substrate availability induces changes in the expression of metabolic genes in the skeletal muscle of humans during recovery from exercise [28]. Most of the transcriptome studies cited above involved untrained subjects and investigated transcriptome response in the fasted state, and only one study involved endurance-trained subjects under normal conditions – in the feeding state [21].

The first part of our study was exploratory; we sought changes in the transcriptome in endurance-trained skeletal muscle during the early (4 h) and middle (8 h) stages of recovery from acute intensive

exercise (70% $\dot{V}O_{2max}$). We investigated the effect of exercise under normal conditions in the feeding state: before and after the exercise our subjects ate a standardized breakfast and lunch, respectively. High-throughput RNA sequencing (RNA-seq) was applied in several samples to evaluate differentially expressed genes. In the second part of our study, the changes in expression for selected genes were validated by quantitative PCR (qPCR). To define exercise-induced genes, we compared gene expression after acute exercise with different intensities, 70% and 50% $\dot{V}O_{2max}$, by qPCR. This approach allowed us to determine genes that demonstrated changed expression in an intensity-dependent manner.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Human Ethics Committee of the Institute of Biomedical Problems (# 295). The study complied with the guidelines set forth in the Declaration of Helsinki. All the participants gave their written consent to participate in this study.

Initial testing

Ten amateur endurance-trained males [runners, cyclists, and cross country skiers with a median age of 23 years (interquartile range 20–27 years), weight 70 kg (66–72 kg), $\dot{V}O_{2max}$ 61 ml/min/kg body weight (58–62 ml/min/kg body weight)] participated in this study. During the first two visits to the laboratory, the participants were familiarized with the test procedures and completed an incremental ramp test on an Ergoselect 200 (Ergoline, Germany) electromagnetic bicycle ergometer. The initial load, load increment, and revolution rate were 0 W, 15 W/min, and 60–70 rpm, respectively. Each participant exercised until exhaustion, as indicated by a revolution rate that slowed to 50 rpm and a respiratory exchange ratio (RER) that increased to more than 1.1. The pulmonary oxygen uptake rate ($\dot{V}O_{2max}$) was measured at consecutive 15 s intervals using an AMIS 2000 (Innovision, Denmark) medical mass spectrometer with a mixing chamber. The highest $\dot{V}O_{2max}$ value for 30 s was taken as $\dot{V}O_{2max}$.

Primary testing

Each subject performed intensive and low-intensity exercise sessions (70% and 50% $\dot{V}O_{2max}$), in a random order, separated by 1 week (Figure 1). All participants were instructed to refrain from strength and vigorous-aerobic exercises for 1 week before the test, and from all exercise for 36 h before. Participants arrived at the laboratory at 09:00 h and ate a standardized breakfast (3624 kJ, 24 g protein, 157 g carbohydrate, and 15 g lipid). A warm-up (5 min, 50% $\dot{V}O_{2max}$) and exercise session (65 min, 70% or 50% $\dot{V}O_{2max}$) started 1 h 45 min after the breakfast. Powers for intensive and low-intensity exercise were 3.2 (2.9–3.3) and 2.4 (2.2–2.5) W/kg body weight, respectively. Two hours after the exercise session, the participants ate a standardized lunch (3714 kJ, 45 g protein, 183 g carbohydrate, 27 g lipid). Capillary blood was drawn from the fingertip prior to, and at 20, 40, and 60 min after, initiation of exercise; lactate concentration was determined using a Super GL easy analyser (Dr. Mül-

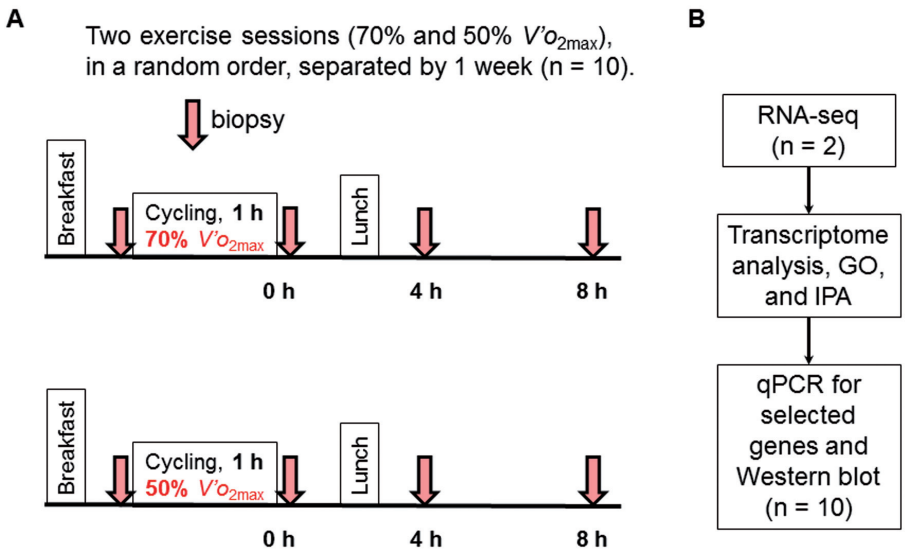

FIG. 1. Design of the study.
(A) Ten subjects performed two exercise sessions (70% and 50% $\dot{V}O_{2max}$), in a random order, separated by 1 week.
(B) RNA sequencing was applied in samples from two typical subjects to evaluate changes in transcriptome, and then the changes in expression for selected genes were validated by quantitative PCR in the samples of ten subjects as well as changes in some phosphoproteins.

ler Gerätebau, Germany). Venous blood was drawn from the vena intermedia cubiti using a catheter prior to, and at 30 min after, the exercise session; the plasma concentration of cortisol was evaluated using an ELISA-Cortisol kit (Immunotek, Russia). Biopsies from the vastus lateralis muscle were taken using a microbiopsy technique [9] under local anaesthesia (2 ml 2% lidocaine) prior to, and at 2 min, 4 h, and 8 h after, exercise. The muscle samples were quickly blotted with gauze to remove superficial blood, and were frozen in

Table 1. Primers used in this study.

Transcript	Strand	Sequence, 5'–3'	Product size, bp
ARNTL	Forward	CTCCAGACATTCCTTCCAGTG	140
	Reverse	ACTTGATCCTTGGTCGTTGTC	
ESRRG	Forward	TGAAAGAAGGGGTGCGTCT	164
	Reverse	CTTCTCCGGTTCAGCCACC	
FOS	Forward	AGACTACGAGGCGTCATCCT	186
	Reverse	CTGGTCGAGATGGCAGTGAC	
GAPDH	Forward	CAAGGTCATCCATGACAACTTTG	496
	Reverse	GTCCACCACCCTGTTGCTGTAG	
HDAC9	Forward	TGATGGACGTGTGGTGTTGG	138
	Reverse	TTCGGGCTTTGGTGGAGAAT	
JUNB	Forward	AAGGGACACGCCTTCTGAAC	189
	Reverse	AAACGTCGAGGTGGAAGGAC	
KLF10	Forward	CTGAAAGGCCAAAAGAGAG	92
	Reverse	GCAGCTCATTGACATAAGTG	
KLF15	Forward	AATGTACTTTCCCTGGCTGC	184
	Reverse	CACTGGTACGGCTTCACACC	
MAFG	Forward	AGCTCAAGGAATCTGTGCCC	93
	Reverse	GTAGCCAAGGCTCAGTGGAG	
MAPK3K14	Forward	CGCAGTTGCAGTACATCGG	107
	Reverse	ATGTTCGAAGGGGCTGATCT	
NFIL3	Forward	GAGCTACACAAGGGAGCCAA	178
	Reverse	TGACTTTCCTACCACACCATCAT	
NFKBIA	Forward	AAGTGATCCGCCAGGTGAAG	188
	Reverse	CTGCTCACAGGCAAGGTGTA	
NR1D1	Forward	TGTATGCAGCCCCAGAAG	154
	Reverse	GTGAAGCTCATGGAGAAATCC	
NR4A3	Forward	CACTACGGCGTGCGAACCT	125
	Reverse	CATCGGTTTCGACGTCTCTTGT	
NT-PPARGC1A	Forward	TCACACCAAACCCACAGAGA	172
	Reverse	CTGGAAGATATGGCACAT	
PDK4	Forward	CTACTCGGATGCTGATGAAC	116
	Reverse	ATCTTGGACCACTGCTACC	
PER1	Forward	CGGGCTCTTGATGTGATGG	82
	Reverse	CTGAGAAGAGTGGGTCATCAG	
PPARGC1A	Forward	CAGCCTCTTTGCCCAGATCTT	101
	Reverse	TCACTGCACCACTTGAGTCCAC	
PPARGC1B	Forward	GCTGGCCCAGATACACTGAC	113
	Reverse	CTGCTGGGCCTCTTTCAGTAA	
PRKAG2	Forward	TTGCCCGTTATTGACCCTATC	130
	Reverse	GCTCATCCAGGTTCTGCTTC	
RARA	Forward	CAGGAAACCTTCCCTGCCC	70
	Reverse	AGACACGTTGTTCTGAGCTG	
RPLP0	Forward	CACTGAGATCAGGGACATGTTG	77
	Reverse	CTTCACATGGGGCAATGG	
SIK1	Forward	AGAGGACGGTGGAGTCACTG	102
	Reverse	CGCACTGGGCATTCCGATA	
TLE1	Forward	GCAATAAGAGCCCTGTCTCC	75
	Reverse	CGGGTAGCAATTTACAGGAAC	

liquid nitrogen and stored at −80°C until needed for analysis. The first biopsy was taken 15 cm proximal to the lateral epicondyle of the femur, and subsequent biopsies were taken 2 cm proximal to the previous one. The biopsy samples were taken on different days (separated by 1 week) from different legs.

RNA extraction

RNA was extracted from the frozen samples (~20 mg) using an RNeasy mini kit (Qiagen). After DNase (Fermentas) treatment, an MMLV RT kit (Evrogen) was used to obtain cDNAs, with 1 μg of total RNA, as described in our previous study [29].

RNA-seq and data analysis

RNA-seq was used to identify targets for validation by qPCR. Total RNA was taken from the samples of two typical subjects ($\dot{V}O_{2max}$ 54 and 61 ml/min/kg) for RNA-seq analysis prior to, and at 4 h and 8 h after, exercise at 70% $\dot{V}O_{2max}$. RNA-seq was performed by an Illumina HiSeq 2000 instrument with a read length of 50 base pairs as described elsewhere [29]. To analyse differential gene expression, the DESeq2 method was used; the false discovery rate-corrected *P* level (P_{adj}) was 0.05. The RNA-seq data can be found at the Gene Expression Omnibus under accession number GSE86931.

The gene ontology (GO) analysis for the biological process (GOTERM_BP_ALL) was carried out using DAVID 6.7 [12]. Then, the results of GO analysis were summarized by removing redundant GO terms and visualized using REViGO [37]. To evaluate genes related to different regulatory gene families, such as 'transcriptional regulators', 'protein kinases and phosphatases', and 'cytokines and growth factors', the Molecular Signatures Database GSEA (The Broad Institute) was used. Importantly, only genes for which the proteins are detectable in human skeletal muscle (according to the Human Protein Atlas, http://www.proteinatlas.org/) were included in the analysis.

To predict the upstream transcriptional regulators playing a role in the activation and inhibition of exercise-induced differentially expressed genes, the Upstream Regulator Analysis tool [Ingenuity Pathway Analysis (IPA); Qiagen] was applied. Activation/inhibition of transcription regulators was evaluated using the *z*-score. Transcription factors with an overlap *P* value under 0.05 and a *z*-score above 1.5 (or under −1.5) were considered as biologically relevant.

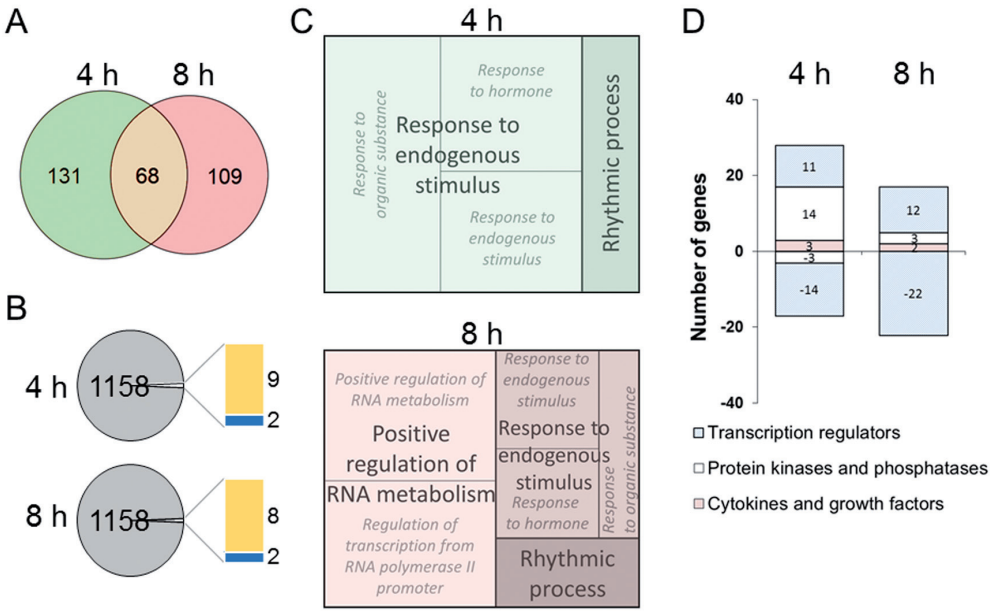

FIG. 2. Acute cycling exercise (70% $\dot{V}O_{2max}$) changed the expression of genes in trained human vastus lateralis muscle at 4 h and 8 h recovery.
(A) Venn diagram representing the number of genes that had changed expression ($P_{adj} < 0.05$) after the exercise.
(B) More than 1000 genes encode human mitochondrial proteins, while in response to the exercise only 8–9 genes that encode mitochondrial proteins had increased expression and 2 genes decreased ($P_{adj} < 0.05$).
(C) Biological processes related to exercise-induced genes. The most important groups of GO terms are shown. The significance level ($-log_{10}\ P_{adj}$) of each group of GO terms is proportional to the area of the corresponding rectangle.
(D) The number of up- and down-regulated genes related to different gene families (positive and negative values, respectively). The genes with detectable proteins in human skeletal muscle (according to The Human Protein Atlas, http://www.proteinatlas.org/) were included.

Real-time PCR

Real-time PCR was performed using a Rotor-Gene Q cycler (Qiagen) for the samples of all the subjects (n = 10) using EvaGreen master mix (Syntol) as described elsewhere [29]. The specificity of the amplification was monitored by analysis of melting curves and agarose (1%) gel electrophoresis. Each sample was run in triplicate, and a non-template control was included in each run. Target gene mRNA expression levels were calculated by the efficiency-corrected ΔCt method [16] as

$$\frac{\sqrt[2]{(1+E_{ref1})^{Ct_{ref1}} \times (1+E_{ref2})^{Ct_{ref2}}}}{(1+E_{tar})^{Ct_{tar}}}.$$

PCR efficiency (*E*) was calculated using standard curves corresponding to the target and two reference genes (*RPLP0*, *GAPDH*). The primer sequences are shown in Table 1.

Western blot

Expression of phosphoproteins for the samples of all the subjects (n = 10) were evaluated using anti-phospho-Forkhead box protein O1 (FOXO1^{Ser256}, 1:1000, sc-101681), anti-phospho-AMP-activated protein kinase (AMPKα1/2^{Thr172}, 1:200; sc-33524, all from Santa Cruz Biotechnology, USA), anti-phospho-p38 mitogen-activated protein kinase (p38 MAPK$^{Thr180/Tyr182}$, 1:500; ab4822), anti-phospho-Ca^{2+}/calmodulin-dependent protein kinase (CaMKII^{Thr286}, 1:2500; ab32678) or anti-GAPDH (1:2500; ab9485) antibodies (the latter three were all from Abcam, UK), and anti-rabbit secondary antibody (Cell Signaling, USA) as described elsewhere [29]. Luminescent signals were captured using a ChemiDoc imaging system (Bio-Rad, USA). All data are expressed as the ratio of target protein to GAPDH.

Statistics

Sample volumes were small with non-normal data distributions; therefore, the data have been expressed as medians and interquartile ranges. The Wilcoxon matched pairs signed-rank test with Holm-Bonferroni correction was used to compare repeated measurements in intensive and low-intensity exercise sessions. The relation between samples was evaluated by the Spearman rank correlation test at each time point. The level of significance was set at $P \leq 0.05$.

RESULTS

Physiological data

The physiological effects of intensive and low-intensity exercise sessions (70% and 50% $\dot{V}O_{2max}$, respectively) have been described in our previous study [29]. Briefly, intensive exercise increased the blood lactate level from 2.3 (1.8–2.6) mM to 4.3 (3.9–5.4) mM ($P<0.01$), and the blood cortisol level from 295 (245–351) nM to 417 (364–567) nM, $P<0.05$. In contrast, both the lactate level during low-intensity exercise and the post-exercise cortisol level demonstrated small decreases [from 1.9 (1.7–2.2) nM to 1.2 (1.1–1.6) nM, $P<0.01$, and from 335 (303–378) nM to 302 (208–327) nM, $P<0.01$, for lactate and cortisol, respectively]. The differences between intensive and low-intensity sessions were significant ($P<0.01$). The markers of metabolic and mechanical stress, blood lactate and cortisol levels, and intensity of exercise expressed as a percentage of $\dot{V}O_{2max}$ and W/kg body weight, closely correlated with each other ($r=0.74$–0.84, $P<0.001$ for all).

Changes in the transcriptome after intensive exercise (RNA-seq study)

We found that 199 and 177 genes changed their expression level ($P_{adj}<0.05$) at 4 h and 8 h after intensive exercise (70% $\dot{V}O_{2max}$), respectively; 68 genes displayed overlap (Figure 2A). The exercise-induced gene expression was associated with several main groups of GO terms related to 'response to endogenous stimulus', 'positive regulation of RNA metabolism', and 'rhythmic process' (Figure 2C). Using the Molecular Signatures Database GSEA, we revealed that the most pronounced changes in gene expression were found for 'protein kinases and phosphatases', and 'transcriptional regulators' gene families. The genes encoding 'protein kinases and phosphatases' were up-regulated mainly at 4 h of recovery, while the 'transcriptional regulators' family included both up- and down-regulated genes at 4 h and 8 h of recovery (Figure 2D, Table 3).

Intensity-dependent gene expression (qPCR study)

Based on the results of the RNA-seq study, some regulatory genes were investigated by qPCR in samples from all the subjects. We confirmed the results of the RNA-seq study showing that genes encoding protein kinases (*PRKAG2*, *MAP3K14*, *PDK4*, *SIK1*), oxidative phosphorylation (OXPHOS)-related regulators (*NR4A3*, *PPARGC1A* and its N-truncated isoform *NT-PPARGC1A*, *ESRRG*, *PPARGC1B*), and the AP-1-related gene *JUNB* were up-regulated after intensive exercise (70% $\dot{V}O_{2max}$), while genes encoding transcriptional repressors (*TLE1*, *HDAC9*, *MAFG*, *NFKBIA*), circadian-related genes (*ARNTL*, *PER1*, *RARA*, *KLF15*, *NR1D1*, *NFIL3*), and the AP-1-related gene *FOS* were down-regulated (data not shown).

For defined exercise-induced genes, correlations between the gene expression after exercise of different intensities and markers of exercise stress were investigated. Genes with post-exercise changes in expression that were significantly ($P<0.05$) correlated with either of these markers were considered as exercise-induced genes. We found that the genes encoding protein kinases, OXPHOS, and AP-1-related genes significantly correlated with markers of exercise stress, while transcriptional repressors and circadian-related genes did not (Figure 3B).

Intensity-dependent regulation of signalling

We used RNA-seq data and a computational approach (IPA) to predict the upstream transcriptional regulators playing a role in the activation and inhibition of differentially expressed genes after exer-

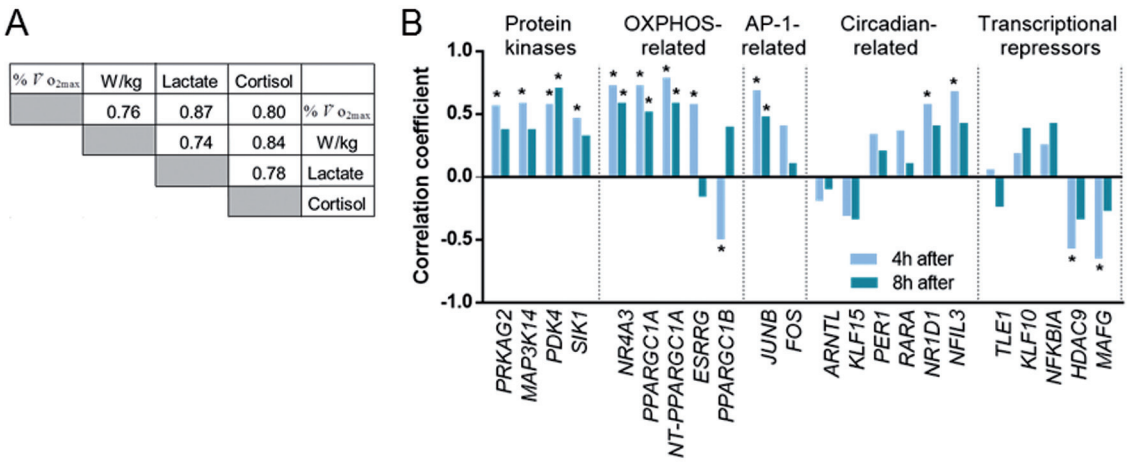

FIG. 3. Exercise-induced and intensity-dependent expression of regulatory genes.
(A) The markers of metabolic and mechanical stress, blood cortisol and lactate levels, and intensity of exercise, expressed as a percentage of $\dot{V}O_{2max}$ and W/kg body weight, closely correlated with each other after the exercise with different intensities (70% and 50% $\dot{V}O_{2max}$; n=10).
(B) Spearman correlations between the markers of exercise stress and the changes in expression of regulatory genes in trained human vastus lateralis 4 h and 8 h after exercise with different intensities (70% and 50% $\dot{V}O_{2max}$; n=10). * indicates significant ($P < 0.05$) correlation.

cise at 70% $\dot{V}O_{2max}$ We predicted that intensive exercise activates some transcriptional regulators and simultaneously deactivates others. Several metabolic-related (PPARGC1A, SP1, NR1H, FOXO1, -4), AP-1-related (ATF4), and lipid-metabolism-related (PPARA, -G, SREBF1, -2, NR1H, CEBPA) regulators were activated at 4 h and 8 h of recovery, while several transcriptional repressors (RB1, HDAC1, NCOR1, EHF), and inflammatory-related factors (STAT3 and PIAS1) were deactivated mainly at 8 h of recovery. The co-activator PPARGC1A was the most significant exercise-induced transcriptional regulator according to IPA (Figure 4A).

Therefore, signalling pathways related to activation of PPARGC1A were evaluated by immunoblotting before and just after exercise in both intensive and low-intensity exercise sessions. No changes in the phosphorylation level of p38 MAPK$^{Thr180/Tyr182}$ and CaMKII^{Thr286} were found after either exercise session, while the phosphorylation level of AMPKα1/2^{Thr172} was increased (1.44-fold, $P<0.05$) just after intensive exercise only (Figure 4B-C). Exercise-induced changes in the AMPK1/2^{Thr172} phosphorylation level correlated with power ($r=0.48$, $P<0.05$).

The post-exercise phosphorylation level of another predicted exercise-induced transcriptional regulator, FOXO1^{Ser256}, also changed in an intensity-dependent manner: it increased (a marker of deactivation) just after the low-intensity exercise (1.12-fold, $P<0.01$), while after the intensive exercise it was decreased (1.3-fold, $P<0.05$) at 4 h of recovery (Figure 4B-C). The exercise-induced changes in the FOXO1^{Ser256} phosphorylation level negatively correlated with both exercise intensity (percentage of $\dot{V}O_{2max}$) and lactate level during exercise ($r=-0.55$ and -0.61, respectively, $P<0.05$ for both).

DISCUSSION

Exercise-induced transcriptome response

The present data showed that about 200 genes changed expression at 4 h and 8 h after intensive aerobic exercise, but only 68 genes showed overlap (Figure 2A). It means that the transcriptome in endurance-trained skeletal muscle dynamically changed from the early to the middle stage of recovery.

It is well established that endurance training and acute endurance exercise induce mitochondrial biogenesis in skeletal muscle. More than 1000 genes have been shown to encode human mitochondrial proteins [2]. Surprisingly, we found no groups of GO terms related to mitochondrial biogenesis, oxidative reactions, or carbohydrate metabolism. Additionally, in our study only about 10 genes that encode mitochondrial proteins showed increased expression in response to the intensive exercise (Figure 2B and 2C, Table 2). This weak response may be explained by the fitness level of our subjects. Indeed, it has been shown that after 8 weeks of aerobic training the response of metabolic genes in skeletal muscle to acute exercise is substantially lower compared to the untrained state [35]. Therefore, we suggest that the response to intensive exercise in trained muscle consists mainly of the expression of regulatory genes. This notion was supported by GO analysis: one of the most significant groups of GO terms was related to regulation of transcription (Figure 2C). Subsequently, we evaluated the number of genes related to different regulatory gene families. The most pronounced changes were found

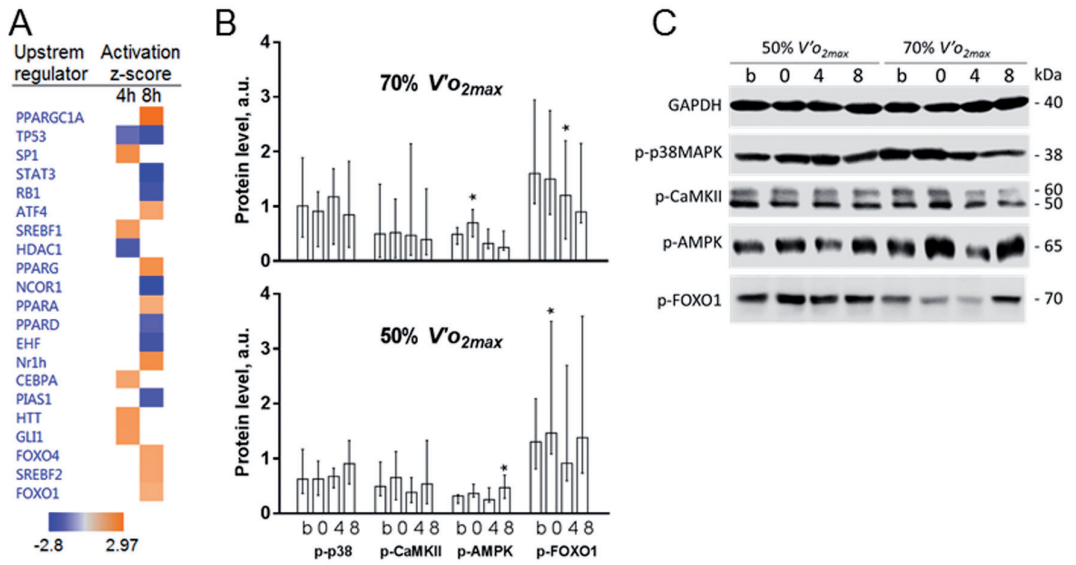

FIG. 4. Acute cycling exercise changed activation of signalling pathways in trained human vastus lateralis muscle.
(A) The heatmap shows upstream transcriptional regulators playing a potential role in the activation and inhibition of gene expression in trained human vastus lateralis at 4 h and 8 h of recovery after intensive exercise (70% $\dot{V}O_{2max}$; n=2). For prediction of upstream regulators IPA (Qiagen) was applied. Activation/inhibition of a transcription factor was evaluated using *z*-score; transcription factors with an overlap $P<0.05$ and a *z*-score above 1.5 (or under −1.5) are shown.
(B) The phosphorylated p38 MAPK$^{Thr180/Tyr182}$, CaMKII^{Thr286}, and AMPKα1/2^{Thr172} kinases, which lie upstream of PPARGC1A, and the phosphorylated FOXO1^{Ser256} before (b), just after (0), and 4 h and 8 h after both intensive and low-intensity exercise sessions (70% and 50% $\dot{V}O_{2max}$, respectively; n=10). * and ** indicate differences from the initial level at $P<0.05$ and $P<0.01$, respectively.
(C) The representative immunoblotting of GAPDH, p38 MAPK$^{Thr180/Tyr182}$, CaMKII^{Thr286}, AMPKα1/2^{Thr172}, and FOXO1^{Ser256}.

Table 2. Expression level of genes encoding mitochondrial proteins in m.vastus lateralis prior to, 4 h and 8 h after intensive cycling exercise (70 min, 70% $\dot{V}O_{2max}$; n=2). Fold change was shown only for significantly ($P_{adj} < 0.05$) differentially expressed genes. PGC-1α-inducible genes, according to The Human MitoCarta 2.0, were marked as fold change in transcript induction following overexpression of PGC-1α protein.

Official symbol	Expression level at rest, FPKM	4 h recovery		8 h recovery		PGC-1α induction score according to The Human MitoCarta 2.0, fold change
		Fold change, log_2	P_{adj}	Fold change, log_2	P_{adj}	
C10orf10	7.6	-0.8	2.98E-03			0
FASN	0.5			1.7	7.13E-03	-0.59
GPD2	4.4	-0.5	4.44E-02	-0.5	3.22E-02	0
HK2	4.0	1.6	4.61E-02			0
IDH1	3.7			1.0	1.84E-02	0.23
IDI1	25.0	0.9	4.55E-04	0.8	1.07E-02	0
MPV17L2	2.3			1.5	3.54E-02	0
NT5DC2	0.5	1.3	4.33E-02			0
PACSIN2	5.7	0.9	2.41E-02			0
PPTC7	17.4	1.0	1.05E-03	1.0	1.32E-04	3.53
PRODH	0.4	2.1	3.04E-05	2.0	1.31E-04	0
SFXN1	1.1			1.1	9.14E-03	0
SLC16A1	16.7	0.9	3.38E-02	1.3	4.87E-07	1.79
SLC25A33	2.5			-1.5	4.04E-04	0.47
TKT	0.4	1.3	8.84E-03			0.71
WDR81	1.0	1.2	1.93E-03			0

Table 3. Expression level of genes encoding transcription regulators, kinases and phosphatases, and cytokines and growth factors in m.vastus lateralis prior to, 4 h and 8 h after intensive cycling session (70 min, 70% $\dot{V}O_{2max}$; n=2). Fold change was shown only for significantly ($P_{adj} < 0.05$) differentially expressed genes. Genes with supportive protein reliability in human skeletal muscle according to The Human Protein Atlas were marked: + detectable, **n/d** not detectable, **n/a** not available.

Official symbol	Expression level at rest, FPKM	4 h recovery		8 h recovery		Evidence at protein level according to The Human Protein Atlas
		Fold change, log_2	P_{adj}	Fold change, log_2	P_{adj}	
Transcription regulators						
ARNTL	0.34	1.6	3.68E-002	3.5	1.18E-017	+
ATOH8	1.48	1.2	2.98E-003			n/a
BHLHE41	18.66			-1.2	8.54E-004	+
CEBPB	34.76			-0.7	8.54E-004	+
CIART	6.86	-1.3	7.52E-005	-2.9	3.49E-018	n/d
CITED2	5.17			2.0	2.32E-008	+
DBP	2.56			-1.3	1.29E-003	+
ESRRG	0.98	3.4	9.78E-017	1.6	2.78E-002	+
FOS	1.11			-3.3	1.28E-009	+
FOXK1	1.19			1.0	2.50E-002	+
FOXO3	8.53			-1.2	9.74E-005	+
HDAC4	2.00			1.2	7.65E-004	+
HDAC9	1.18	-1.4	8.28E-006	-1.5	1.16E-006	+
HES1	11.25	-1.4	8.84E-003	-1.6	6.18E-003	+
HES4	4.56	1.8	2.13E-002			n/a
HSF2	16.48			-0.8	2.49E-003	n/d
JUNB	3.58	2.0	4.84E-006			+
JUND	19.28			-0.7	2.06E-002	n/d
KLF10	15.55			-1.3	2.15E-002	+
KLF13	6.89			-0.7	1.19E-002	+
KLF15	11.73	-0.8	2.64E-003	-0.8	3.53E-003	+
LDB2	2.11	-0.7	4.46E-002			+
MAFF	0.65	4.4	8.55E-026			n/d
MAFG	4.32			-1.0	1.41E-003	+
MNT	0.36	1.4	2.96E-002			n/a
NCOA3	3.38	-0.5	3.13E-002	-0.8	4.57E-004	+
NFIL3	3.44	1.3	4.19E-003	1.7	4.56E-006	n/d
NFKBIA	12.03	-0.5	2.14E-002			+
NR1D1	9.33	-1.4	3.02E-002			+
NR1D2	17.08	-0.9	2.06E-002	-1.3	7.69E-004	n/d
NR4A3	0.73	5.5	2.98E-003			+
PER1	14.15	-1.7	5.48E-015	-2.2	1.02E-024	+
PER2	1.95	-1.3	7.28E-004	-2.7	1.48E-012	+
PER3	3.23	-0.8	5.19E-003	-1.9	4.56E-012	+
PITX2	6.55	-0.5	3.38E-002			n/a
PLAGL2	2.02	-0.7	1.02E-002	-0.7	1.05E-002	+
PPARA	4.35			1.0	3.81E-002	+
PPARGC1A	5.73	2.8	1.39E-022			+
PPARGC1B	0.41	1.7	1.47E-006	1.5	6.00E-005	+
RAI1	0.49			1.5	1.25E-003	n/d
RARA	1.83	1.6	1.37E-003			+
RBM20	2.30	1.5	3.83E-005			+
RRN3	6.98			1.0	1.48E-002	+
SERTAD1	3.15	1.3	6.90E-003			n/a

Official symbol	Expression level at rest, FPKM	4 h recovery		8 h recovery		Evidence at protein level according to The Human Protein Atlas
		Fold change, log_2	P_{adj}	Fold change, log_2	P_{adj}	
SERTAD3	5.98			-0.6	2.71E-002	n/a
SOX6	1.07			-0.8	7.82E-005	+
SREBF1	1.77			1.2	3.70E-003	n/d
SS18L1	2.70			0.9	1.71E-002	+
TCF15	5.30	-1.1	2.55E-003			n/d
TEF	12.22			-0.8	6.73E-005	n/d
TLE1	10.73	-0.7	2.99E-004	-1.0	9.36E-008	+
TLE4	2.42			-0.7	1.05E-002	n/a
TRIM27	1.24			0.9	4.27E-002	+
VGLL2	34.22	1.4	2.97E-006			+
ZBTB16	4.25			-1.1	3.10E-003	n/d
ZC3H6	1.52	-0.6	1.41E-002	-0.6	1.58E-002	+
ZFP36	6.61			-1.1	1.11E-003	+
ZMIZ1	1.58	1.1	2.79E-004			n/d
ZNF493	0.23			1.4	2.95E-002	n/a
ZNF629	1.35			1.3	7.66E-005	n/d
ZNF827	0.70			1.3	1.32E-004	+
Kinases and phosphatases						
ALPK3	23.22	1.2	1.17E-005	0.8	4.24E-002	+
CTDSP2	18.16	-0.4	2.00E-002			+
DUSP1	20.47	-0.9	9.60E-004	-1.2	2.03E-005	+
DUSP10	4.91	-0.6	4.86E-002			n/a
DUSP7	3.68			-0.9	8.28E-003	+
FAM20C	3.08	1.1	1.64E-003			+
FN3K	2.06	1.1	4.83E-002			+
HK2	4	1.6	4.61E-002			+
MAP3K14	1.33	1.4	5.82E-004			+
MKNK2	73.71	0.9	1.83E-002			+
PACSIN2	5.69	0.9	2.41E-002			n/d
PIK3R3	1.57	-0.6	4.23E-002			n/d
PPP1R16B	1.09	1.2	2.75E-002			n/d
PPP1R9B	2	1.2	1.67E-002			n/a
PPP2R3A	25.62			-0.4	1.71E-002	+
PPTC7	17.36	1	1.05E-003	1	1.32E-004	+
PRKAG2	1.1	5.1	4.33E-152	4.8	7.77E-135	+
PRKG1	9.9			-0.5	1.50E-002	+
SGK1	2.84			1.2	1.81E-002	+
SIK1	0.39	4.1	4.96E-004			+
SIK2	1.55	1.1	5.19E-003			+
SMG1	2.79	0.8	3.13E-002			+
TEK	2.9	1	1.54E-002			+
TRPM7	1.21	1.2	4.13E-003			+
Cytokines and growth factors						
ANGPT2	0.29	2.3	1.55E-003			+
APLN	1.18	1.9	4.62E-003	2.1	4.00E-004	n/d
STC2	0.12	3.3	2.68E-007	2.3	9.14E-003	n/d
VEGFA	6.25	1.3	5.69E-003			+
WNT11	2.84			1.7	1.64E-004	+
WNT9A	1.47	2	4.54E-006			n/a

for 'protein kinases and phosphatases', and 'transcriptional regulators' families (Figure 2D). The genes encoding 'protein kinases and phosphatases' were up-regulated mainly at 4 h recovery, while the 'transcriptional regulators' family included both up- and down-regulated genes at both time points (Figure 2D). There were several OXPHOS-related genes (*NR4A3*, *PPARGC1A* and its truncated isoform *NT-PPARGC1A*, *PPARGC1B*, *ESRRG*), metabolic- and circadian-related genes (*ARNTL*, *RARA*), and Activator Protein 1 (AP-1)-related gene (*JUNB*) among the up-regulated genes in the 'transcriptional regulators' family (Table 3). Interestingly, *NR4A3* was the most up-regulated (~45-fold) among all genes. Expression of *NR4A3* in skeletal muscle is regulated by a CREB-dependent mechanism; NR4A3 protein up-regulates genes encoding many proteins involved in glycolysis, OXPHOS, and fat and carbohydrate metabolism, as well as expression of *PPARGC1A* via a canonical promoter [24, 25]. The well-described co-activators PPARGC1A (also known as PGC-1α), PPARGC1B [15, 34], and nuclear receptor ESRRG [20, 30] play an important role in the regulation of mitochondrial biogenesis and angiogenesis in skeletal muscle. Notably, ESRRG is a key partner of PPARGC1A in skeletal muscle [1, 11]. Another important partner of PPARGC1A is the heterodimeric complex AP-1 [1], which may include JUNB. The potent circadian regulator ARNTL regulates many circadian-related genes in skeletal muscle, as well as muscle-specific genes [36].

Half of the genes related to the 'transcriptional regulators' family were down-regulated (Figure 2D). In contrast to the early stage of recovery, AR-1-related genes (*FOS* and *MAFG*) were down-regulated at 8 h. There were many transcription repressors (*HES1*, *KLF10*, *-13*, *TLE1*, *NFKBIA, MAFG*, *HDAC9*), circadian-related regulators (*PER1*, *-2*, *-3*, *NR1D1*, *BHLHE41, KLF15*), metabolic-related genes (*NR1D1*, *KLF15, FOXO3*, *NCOA3*), and inflammatory-related genes (*NFKBIA*, *CEBPB, NCOA3*) among the down-regulated genes encoding transcriptional regulators at both time points (Table 3). It is logical to assume that the decreased expression of the transcription repressors is favourable to exercise-induced adaptation. However, it was surprising to find the decreased expression of several genes (*NR1D1*, *KLF15*, *NCOA3*) that have been described as positive regulators of muscle oxidative capacity [8, 39, 42]. For example, knock-down of *NR1D1* in mouse resulted in deactivation of the AMPK-PPARGC1A axis, reduced mitochondrial content and muscle oxidative capacity, and a substantial decrease of aerobic performance [39]. We suggested that decreased expression of the genes is not related to exercise per se and is regulated via other stimuli.

Intensity-dependent expression of genes

Studies in mice have revealed that in non-exercised skeletal muscle, daily oscillation of many metabolic-related and muscle-specific genes occurs in a circadian-dependent manner, and depends on the expression of the circadian regulators *Clock* and *Arntl* [18, 19, 36]. In our study, the GO analysis showed that the expression of several genes after intensive exercise was connected with the 'rhythmic process' (Figure 2C). It is quite possible that some of the changes in gene expression during 8 h after the exercise might be affected by circadian rhythm, but not by exercise *per se*. To reveal exercise-related genes, we evaluated changes in gene expression by qPCR after both intensive and low-intensity exercise sessions for all subjects. We found that some genes encoding transcriptional regulators changed their expression level in an intensity-dependent manner but others did not. In fact, all examined genes encoding protein kinases, OXPHOS, and AP-1-related genes increased expression in an intensity-dependent manner, while genes encoding repressors (*HDAC9* and *MAFG*) decreased expression in an intensity-dependent manner (Figure 3B). In contrast, other transcriptional repressors (*TLE1*, *KLF10*, *NFKBIA*) and some circadian-related genes (*ARNTL*, *PER1, KLF15*) changed expression in an intensity-independent manner. Expression of some genes seemingly may be modulated by both exercise and circadian oscillation. Thus, the expression of *NR1D1* was decreased and positively associated with the intensity of exercise. This finding is in accordance with data showing that *NR1D1* plays a role in the regulation of both circadian rhythm [5] and muscle oxidative capacity [39]. It means that, theoretically, an optimal period of day may be found to maximize the molecular response to acute exercise.

Intensity-dependent regulation of signalling

To predict upstream transcriptional regulators that are potentially involved in intensive-exercise-induced gene expression, the RNA-seq data and IPA were used. We found that the co-activator PPARGC1A was the most significant exercise-induced transcriptional regulator (Figure 4A). To investigate the signalling involved in the regulation of PPARGC1A, we evaluated the phosphorylation levels of well-established kinases that phosphorylate PPARGC1A after both intensive and low-intensity exercise (70% and 50%$\dot{V}O_{2max}$, respectively). There were no changes in the post-exercise phosphorylation level of p38 MAPK$^{Thr180/Tyr182}$ and CaMKII^{Thr286} in both exercise sessions, while the phosphorylation level of AMPKα1/2^{Thr172} was increased just after exercise in an intensity-dependent manner (Figure 4B-C). This finding confirms that in trained skeletal muscle AMPK plays an important role in adaptation to acute exercise.

IPA also revealed several other transcriptional regulators with increased activity after intensive exercise: lipid metabolism-related (PPARA, -G, SREBF1, -2, NR1H, CEBPA), AP-1-related (ATF4), and metabolic-related (SP1, NR1H, FOXO1, FOXO4) regulators. PPARA and -G are well-described partners of PPARGC1A [34]. Recently SP1 and AP-1 have been shown to play a role in the PPARGC1A-controlled gene programme [1, 31]. Moreover, a role of SP1, as regulator of cytochrome c expression under conditions of increased contractile activity, has been confirmed previously in murine skeletal muscle cells [4]. ATF4 is a member of the ATF/CREB family, which may form the heterodimer AP-1 with proteins of the FOS/JUN family [7] and, therefore, may be involved in regulation of the PPARGC1A-controlled gene programme together with other AP-1-related proteins.

Other predicted transcriptional regulators that were up-regulated

after the intensive exercise were FOXO1 and FOXO4. We confirmed that FOXO1^{Ser256} was dephosphorylated (a marker of activation) in an intensity-dependent manner (Figure 4B-C). A role of FOXO family transcription factors in adaptation to exercise has been extensively discussed [33], but is not fully clear. Transgenic mice specifically overexpressing FOXO1 in skeletal muscle have been shown to have a reduced skeletal muscle mass, size of both type I and II fibres, number of type I fibres, and spontaneous locomotive activity. These changes may be caused by enhanced expression of autophagy and lysosomal-related genes and proteins, decreased expression of genes related to the structural proteins of type I muscles, and a decreased level of OXPHOS proteins [13]. Surprisingly, transgenic mice showed an increased PPARGC1A protein level. It has been revealed that FOXO1 may interact with insulin response sequences on the canonical promoter of the *PPARGC1A* gene in HepG2 cells, rat kidney mesangial cells, and human skeletal muscle, and thereby up-regulate *PPARGC1A* expression [6, 10, 40]. These findings may partially explain the physiological role of the intensity-dependent activation of FOXO1 in our study.

However, the upstream analysis predicted that the activity of several transcriptional repressors (RB1, HDAC1, NCOR1, EHF) and inflammatory-related factors (STAT3, PIAS1) was reduced mainly at 8 h of recovery after the intensive exercise. Interestingly, muscle-specific NCOR1$^{-/-}$ mice have been shown to display increased PPARGC1A-mediated co-activation of ESRRs and expression of genes involved in mitochondrial biogenesis, muscle fibre type shift towards oxidative fibres, and remarkably enhanced $\dot{V}O_{2max}$ and aerobic performance [26, 41]. Therefore, the post-exercise suppression of NCOR1 activity may play an important role in the adaptation of human skeletal muscle to exercise.

The main limiting factor of the study is the sample size for RNA-seq. We used RNA-seq to identify exercise-induced genes, and to predict exercise-induced biological processes and transcriptional regulators. The results of the RNA-seq study were confirmed for some genes in samples from all the subjects (n=10) by qPCR. However, future investigations are needed to confirm other results of the RNA-seq study (GO analysis and IPA). Importantly, molecular responses to aerobic exercise in endurance-trained muscle may significantly differ from those in untrained skeletal muscle and/or from responses to other exercises (e.g. strength exercise, sprint exercise).

CONCLUSIONS

In this exploratory study we sought to examine exercise-induced overall changes in gene expression in endurance-trained skeletal muscle under normal conditions (in the feeding state) using RNA-seq. After aerobic exercise at 70% $\dot{V}O_{2max}$, the transcriptome was dynamically changed during the first hours of recovery. A computational approach revealed that the changes might be related to up- and down-regulation of the activity of transcription activators and repressors, respectively. The exercise increased expression of many genes encoding protein kinases, while genes encoding transcriptional regulators were both up- and down-regulated. Evaluation of the gene expression after both intensive and low-intensity exercise revealed that some genes changed expression in an intensity-dependent manner, but others did not: the majority of genes encoding protein kinases, OXPHOS and AP-1-related genes significantly correlated with markers of exercise stress, while transcriptional repressors and circadian-related genes did not. Some of the changes in gene expression after exercise seemingly may be affected by circadian rhythm or by both exercise and circadian oscillation. Our results may provide better understanding of molecular mechanisms involved in adaptation of endurance-trained skeletal muscle to acute aerobic exercise and aerobic training.

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Conflict of interest declaration

The authors declare no competing financial interests.

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