Global mRNA sequencing of human skeletal muscle: Search for novel exercise-regulated myokines

S. Pourteymour 1, K. Eckardt 1, T. Holen 1, T. Langleite 1, Sindre Lee 1, J. Jensen 2, K.I. Birkeland 3, C.A. Drevon 1, M. Hjorth 1,4*

ABSTRACT

Objective: Skeletal muscle is an important secretory organ, producing and releasing numerous myokines, which may be involved in mediating beneficial health effects of physical activity. More than 100 myokines have been identified by different proteomics approaches, but these techniques may not detect all myokines. We used mRNA sequencing as an untargeted approach to study gene expression of secreted proteins in skeletal muscle upon acute as well as long-term exercise.

Methods: Twenty-six middle-aged, sedentary men underwent combined endurance and strength training for 12 weeks. Skeletal muscle biopsies from m. vastus lateralis and blood samples were taken before and after an acute bicycle test, performed at baseline as well as after 12 weeks of training intervention. We identified transcripts encoding secretory proteins that were changed more than 1.5-fold in muscle after exercise. Secretory proteins were defined based on either curated UniProt annotations or predictions made by multiple bioinformatics methods.

Results: This approach led to the identification of 161 candidate secretory transcripts that were up-regulated after acute exercise and 99 that where increased after 12 weeks exercise training. Furthermore, 92 secretory transcripts were decreased after acute and/or long-term physical activity. From these responsive transcripts, we selected 17 candidate myokines sensitive to short- and/or long-term exercise that have not been described as myokines before. The expression of these transcripts was confirmed in primary human skeletal muscle cells during in vitro differentiation and electrical pulse stimulation (EPS). One of the candidates we identified was macrophage colony-stimulating factor-1 (CSF1), which influences macrophage homeostasis. CSF1 mRNA increased in skeletal muscle after acute and long-term exercise, which was accompanied by a rise in circulating CSF1 protein. In cultured muscle cells, EPS promoted a significant increase in the expression and secretion of CSF1.

Conclusion: We identified 17 new, exercise-responsive transcripts encoding secretory proteins. We further identified CSF1 as a novel myokine, which is secreted from cultured muscle cells and up-regulated in muscle and plasma after acute exercise.

Keywords Exercise; Myokine; Colony stimulating factor 1; RNA sequencing; Skeletal muscle secretome

1. INTRODUCTION

Skeletal muscle was recognized as a secretory organ about 15 years ago [1]. Proteins and peptides produced by and released from skeletal muscles are termed myokines, and several myokines play important roles in muscle physiology as well as in tissue cross talk [2–4]. Thus, interest in the secretory function of the skeletal muscle has increased markedly during the last decade. Physical activity alters the secretion of many myokines, several of which may play a role in mediating beneficial health effects of physical activity. Interleukin 6 (IL6) is the most extensively studied myokine, and is secreted from skeletal muscle during acute physical activity [5]. IL6 may act as an energy sensor in skeletal muscle during exercise, promoting increased hepatic glucose output and enhanced glucose uptake in muscle cells [6].

More than 100 myokines have been identified [7], and the skeletal muscle secretome is predicted to include more than 300 proteins [8]. In several proteomics studies, scientists have identified peptides in medium conditioned by cultured human [9–12] or murine myocytes [13–16]. To explain some of the positive effects of physical activity, several studies focused on identifying myokines that are elevated in response to exercise or muscle contraction [11,17–20]. The aim of this study was to identify novel myokines regulated by acute or long-term exercise. Many myokines, such as IL6 and other cytokines, have a low abundance in basal conditions, and are therefore hard to detect with untargeted proteomics. We used global mRNA sequencing to detect all genes expressed in human skeletal muscle.
biopsies. In addition, we used cultures of primary human skeletal muscle cells to monitor the expression of novel myokine candidates during differentiation and after electrical pulse stimulation (EPS).

2. METHODS

2.1. Human trial

A human exercise intervention trial (NCT01803568) was performed as described before [21]. The National Committee for Research Ethics North (Tromsø, Oslo, Norway) approved the trial. This study adhered to the standards set by the Declaration of Helsinki. Briefly, 26 sedentary (≤1 bout of exercise/week during the previous year) men aged 40–65 y with BMI 26 ± 4.0 kg/m², were included in the trial. They were initially recruited to a control group (n = 13) with normal glucose metabolism and a dysglycemic group (n = 13) with fasting serum glucose ≥ 5.6 mmol/L and/or 2 h glucose ≥ 7.8 mmol/L based on an oral glucose tolerance test. Two subjects had normal glucose levels at screening, but both had a glucose infusion rate of 4.4 mg min⁻¹ kg⁻¹ during the euglycemic hyperinsulinemic clamp and were included in the dysglycemic group. Here we did not investigate group differences, and all subjects were therefore included (n = 26).

The participants underwent 12 weeks of supervised exercise training with two interval sessions (bicycle) and two whole-body strength-training sessions per week [21]. Bicycle tests (45 min at 70% of VO₂max) were conducted before and after the long-term training intervention [21]. Blood samples and biopsies from m. vastus lateralis were collected before, immediately after, and 2 h after the acute bicycle tests (Figure 1A).

2.2. High throughput mRNA sequencing

RNA was isolated from muscle biopsies and reverse-transcribed into cDNA. RNA integrity was determined using Agilent RNA 6000 Nano Chips and a Bioanalyzer 2100. Deep sequencing was performed with the Illumina HiSeq 2000 system with multiplexed design [22]. The cDNA was fragmented, and cDNA fragments with 51 bp nucleotides were selected and amplified. TopHat 2.0.8 with Bowtie 2.1.0 was used (with default settings) to align the RNA-seq reads against the UCSC hg19 annotated transcriptome and genome [23,24]. EdgeR v3.4.2 [25] was used for gene filtering, normalization, and calculation of p-values using a negative binomial generalized linear model in R v3.0.3 (R Core Team 2014). Correction for multiple testing was performed by using Benjamini-Hochberg’s false discovery rate (FDR) control [26], set at FDR < 10%. The dataset generated from RNA-seq has been used in several other publications, including one study where gene expression data for extracellular matrix (ECM) genes were reported [27]. To compare our data on CSF1 with other published data sets on skeletal muscle and exercise, we analyzed two data sets [28,29]. Arrays were analyzed using the R package Oligo v13.6.1 following standard procedures for quality checks and calculation of normalized expression values using robust multi-array average. For differential gene expression analyses we used the LIMMA v3.20.9.

2.3. Identification of exercise-regulated transcripts encoding secretory proteins

We selected all transcripts of single genes that were up- or down-regulated more than 1.5-fold after acute or long-term exercise training. “Fast-responsive transcripts” were up/down-regulated just after the acute bicycle test (A2/A1 and/or B2/B1, Figure 1A–G), whereas “slow-responsive transcripts” were regulated after 2 h (A3/A1 and/or B3/B1, Figure 1A,D,E). The effect of long-term exercise training was assessed as the mRNA expression at B1 vs. A1 (Figure 1A,F).

To identify transcripts encoding secreted proteins, we used the MetaSecKB knowledgebase [30]. MetaSecKB identifies secretory proteins based on either curated evidence of secretion (annotated and reviewed in the UniProtKB/Swiss-Prot database) or being “highly likely” to be secreted based on computationally predicted secretory protein sequences, without containing transmembrane domains or endoplasmic reticulum (ER) retention signals, by several tools (SignalP4, Phobius, TargetP, and WoLF PSORT).

2.4. Cell culture

Biopsies from either m. obliquus internus abdominis or m. vastus lateralis from three male donors (age 33–62 y) were used to isolate primary human satellite cells [31]. Myoblasts were proliferated to passage 4–5 and differentiated [27]. EPS (1 Hz, 2 ms, 11.5 V) was applied to the cells after 5 days of differentiation for 24 h or after 6 days of differentiation for 1–6 h. EPS should mimic muscle contraction similar to in vivo exercise [32]. Supernatants were collected and RNA isolated for further analysis.

2.5. Protein analyses

We measured CSF1 concentration in the supernatant of cultured myotubes and plasma samples using a human CSF1 ELISA Kit (DMC00B; R&D Systems Minneapolis, Minnesota, United States). Total protein concentration of plasma was measured by BC Assay (Protein assay kit, Uptima, Montluçon, France).

2.6. RNA isolation, cDNA synthesis and gene expression analyses

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA was reversely transcribed into cDNA using the High Capacity cDNA Revers Transferrase kit (ThermoFisher, Foster City, California, United States). Quantitative real-time PCR was performed using the CFX96™ Real-Time System (Bio-Rad, Hercules, California, USA). The following pre-designed primers and probe sets were used (TaqMan assays; ThermoFisher, Foster City, California, United States): RPLP0 (Hs00999902_m1), MTRNR2L4 (Hs04276154_s1), SMPLD3A (Hs0037308_m1), FAM20C (Hs00398243_m1), WNT9A (Hs01432312_m1), TEK (Hs00945155_m1), FLT1 (Hs01052961_m1), CSF1 (Hs00171464_m1), CBG (Hs01113922_g1), CHOY (Hs00208704_m1), IL4R (Hs00166237_m1), LGN1 (Hs00159612_g1), STC2 (Hs00175027_m1), THBS4 (Hs00170261_m1), IGBP2 (Hs01407191_m1), KALD1 (Hs00558318_g1), and CTC (Hs00306814_g1). Relative target mRNA expression levels were calculated as 2⁻ⁿ́Ct by normalizing to the expression of RPLP0.

3. RESULTS

3.1. Fast exercise-responsive transcripts

To identify myokines up-regulated by acute exercise, we evaluated gene transcription in skeletal muscle biopsies immediately after two acute, 45 min bicycle tests (Figure 1A). After the first bicycle test (A2/A1), 551 transcripts were more than 1.5-fold up-regulated, and 97 of these were classified as secretory (Figure 1B, Supplementary Table 1). After the second bicycle test (after the exercise intervention; B2/B1), 587 transcripts were enhanced (≥1.5-fold), and 108 of these encode secretory proteins (Figure 1C, Supplementary Table 2). In total, we identified 117 secretory transcripts that were increased immediately after acute exercise (≥1.5-fold at A2/A1 and/or B2/B1, Figure 2A, Table 1). There was extensive overlap in expression pattern between...
Figure 1: A) Overview of the study design. Skeletal muscle biopsies and blood samples were harvested before (A1, B1), immediately after (A2, B2) and 2 h after (A3, B3) the end of the bicycle sessions. B–F) Secretory genes up- or down-regulated >1.5-fold at one or several time-points after acute or long-term exercise. Log2 (FC) from baseline (A1 or B1). Blue dots represent up-regulated genes, purple triangles represent down-regulated genes. B) Genes up- or down-regulated >1.5-fold at A2/A1. C) Genes up- or down-regulated >1.5-fold at B2/B1. D) Genes up- or down-regulated >1.5-fold at A3/A1. E) Genes up- or down-regulated >1.5-fold at B3/B1. F) Genes up- or down-regulated >1.5-fold after 12 weeks exercise training (B1/A1).
Genes up-regulated >1.5 fold

|   | Immediately after acute exercise | 2 h after acute exercise | Immediately or 2 h after acute exercise | Acute or 12 w exercise |
|---|----------------------------------|--------------------------|-----------------------------------------|------------------------|
| A | A2/A1: 88, B2/B1: 20             | A3/A1: 41, B3/B1: 8      | A2/A1: 42, B2/B1: 70                    | Acute: 149, B1/A1: 87  |
|   | Total: 117 genes                 | Total: 91 genes          | Total: 161 genes                        | Total: 248 genes       |
| B | A2/A1: 7, B2/B1: 10              | A3/A1: 35, B3/B1: 10     | A2/A1: 17, B2/B1: 7                     | Acute: 83, B1/A1: 4    |
|   | Total: 24 genes                  | Total: 70 genes          | Total: 87 genes                        | Total: 92 genes        |

Genes down-regulated >1.5 fold

|   | Immediately after acute exercise | 2 h after acute exercise | Immediately or 2 h after acute exercise | Acute or 12 w exercise |
|---|----------------------------------|--------------------------|-----------------------------------------|------------------------|
| E | A2/A1: 7, B2/B1: 10              | A3/A1: 35, B3/B1: 10     | A2/A1: 17, B2/B1: 7                     | Acute: 83, B1/A1: 4    |
|   | Total: 24 genes                  | Total: 70 genes          | Total: 87 genes                        | Total: 92 genes        |

Figure 2: Venn diagrams showing the number of secretory genes that were up- or down-regulated >1.5-fold at different time points after acute and/or long-term exercise.

the two bicycle tests; 88 transcripts were detected after both tests (Figure 2A). Furthermore, 95 of the 97 genes detected after the first test were also up-regulated after the second test (FC > 1, p-value < 0.05), although not necessarily to above the 1.5-fold cut-off (Supplementary Table 1).

Many of these fast-responding transcripts encode cytokines or chemokines, with IL6 being the most up-regulated gene. Other examples were IL1B, CXCL1, CXCL2, CXCL3 and CXCL8, CCL2, and CCL8. These cytokines and chemokines were typically expressed at very low levels at baseline, but were markedly increased after exercise (Table 1). Furthermore, most of them returned to basal levels after 2 h rest.

Other fast-responding transcripts encode growth factors (CTGF, FGF6, FGF18, PDGF, PDGFB, TGFbeta, VEGFA, and VEGFC) or proteases and protease inhibitors involved in ECM remodeling (ADAMTS4, ADAMTS1, PLAU, MMP19, ADAM8, SERPINE1, SERPINH1, SERPINA3, and TIMP1). We have previously published and discussed results related to ECM [27]. Only a few secretory transcripts were down-regulated after acute exercise; 14 and 17 transcripts were identified after the first (A2/A1) and second (B2/B1) bicycle test, respectively (Figure 2E, Supplementary Tables 3 and 4).

3.2. Slow exercise-responsive transcripts

Two hours after the first bicycle test (A3/A1), 501 transcripts were increased >1.5-fold, and of these, 83 were classified as secretory (Figure 1D, Supplementary Table 5). Furthermore, 274 transcripts were up-regulated 2 h after the second bicycle test (B3/B1), and 49 of these were secretory (Figure 1E, Supplementary Table 6). In total, we detected 91 secretory transcripts that were increased 2 h after either of the two tests (Table 2, Figure 2B). Some of these slow-responsive transcripts encode cytokines and growth factors (INHBE, TGFbeta, CLCF1, VEGFA and CCL2) and proteases and protease inhibitors (PLG, SERPINA1, SERPINA3, SERPINF2, ADAMTS8, ADAMTS9 and ADAM9).

The gene expression response was generally stronger after the first bicycle test as compared with the second; a higher number of transcripts were identified after the first bicycle test (Figure 2B). Furthermore, of the 91 secretory transcripts detected, 77 increased more after the first test as compared with the second test (Table 2). Still, there was a substantial overlap between the results; 71 of the 83 transcripts detected after the first bicycle test were also significantly increased after the second test (FC > 1, p < 0.05, Supplementary Table 5). Several secretory transcripts were down-regulated 2 h after bicycling; 60 and 35 secretory transcripts were decreased (>1.5-fold) after the first (A3/A1) and second (B3/B1) bicycle test, respectively (Figures 1D,E,2F, Supplementary Tables 7 and 8).

3.3. Transcripts regulated after long-term training

After 12 weeks exercise intervention, 289 transcripts were increased >1.5-fold in skeletal muscle (B1/A1). Of these, 99 were classified as secretory (Figure 1F, Table 3, Supplementary Table 1). The transcript encoding matrix-remodeling associated protein 5 (MXRA5) had the highest relative increase (2.8-fold). SPARC exhibited the highest expression level at baseline (71.5 FPKM) and was increased 1.8-fold after the intervention.

A large proportion of the up-regulated transcripts after long-term training were related to ECM (Table 3). We identified 10 collagens (e.g. collagen type I, III, IV), proteoglycans (e.g. AGRN, LUM and BGN) and a variety of ECM glycoproteins (e.g. LAMB1, SPARC, ND1 and ELM). Some of these data were recently reported and discussed in another publication [27].

Only 9 transcripts encoding secretory proteins were decreased >1.5-fold in skeletal muscle after 12 weeks of intervention (B1/A1) (Figure 1F, Supplementary Table 10). One of these transcripts encodes MSTN (FC = −1.7), which is a negative regulator of muscle growth. This result was recently reported and discussed in another publication [33].

3.4. Transcripts up-regulated after both acute and long-term exercise

In total, we detected 161 unique secretory transcripts that were up-regulated after acute exercise (post-immediate and/or after 2 h; Figure 2C) and 99 transcripts that were increased after 12 weeks exercise intervention. We detected 12 genes that were up-regulated >1.5-fold after both acute and long-term exercise (Figure 2D). However, a large number of acute genes were also significantly increased...
| Symbol | Gene name | FPKM A1 | FPKM B2 | A1/A2 | B2/B1 | Metaz-SecKB | Detected |
|--------|-----------|---------|---------|-------|-------|------------|----------|
| IL6    | Interleukin 6 | 0.09 | 0.12 | 65.4 | 9E-28 | 50.8 | 2E-20 | Curated | Antibody array |
| CXCL8  | Interleukin 8 | 0.03 | 0.04 | 29.1 | 3E-21 | 34.4 | 7E-12 | Curated | Antibody array |
| CXCL1  | Chemokine (C-X-C motif) ligand 1 | 0.05 | 0.08 | 26.2 | 3E-39 | 18.9 | 1E-34 | Curated | Antibody array |
| CCL8   | Chemokine (C-C motif) ligand 8 | 0.07 | 0.13 | 24 | 5E-33 | 18.1 | 6E-40 | Curated | Antibody array |
| ADAMTS4 | ADAM metallopeptidase with thrombospondin type 1 motif 4 | 0.38 | 0.58 | 18.9 | 3E-64 | 19.2 | 1E-32 | Curated | MS Murine |
| PTGDS  | Prostaglandin-endoperoxide synthase 2 | 0.02 | 0.03 | 17.3 | 2E-32 | 18.5 | 2E-25 | Highly likely | — |
| CXCL2  | Chemokine (C-X-C motif) ligand 2 | 1.21 | 2.14 | 14.5 | 8E-35 | 9.7 | 8E-23 | Curated | Antibody array |
| CCL2   | Chemokine (C-C motif) ligand 2 | 2.25 | 3.31 | 12.9 | 5E-16 | 13.5 | 3E-18 | Curated | Antibody array |
| CXCL3  | Chemokine (C-X-C motif) ligand 3 | 0.09 | 0.15 | 10.1 | 1E-21 | 5.9 | 6E-13 | Curated | — |
| IL1B   | Interleukin 1, beta | 0.05 | 0.04 | 9.3 | 2E-19 | 21.5 | 9E-21 | Curated | Antibody array |
| THBS1  | Thrombospondin 1 | 0.4 | 0.75 | 8.6 | 6E-21 | 7.5 | 1E-12 | Highly likely | MS hSkMC |
| CHRNA1  | Cysteine-rich, angiogenic inducer, 61 | 9.29 | 12.53 | 8 | 9E-38 | 4.6 | 2E-30 | Curated | MS Murine |
| ADAMTS1 | ADAM metallopeptidase with thrombospondin type 1 motif, 1 | 5.87 | 6.66 | 7.2 | 1E-42 | 6.9 | 7E-35 | Curated | MS hSkMC |
| PLAUR  | Plasminogen activator, urokinase receptor | 0.32 | 0.46 | 7.1 | 2E-54 | 3.7 | 2E-26 | Curated | MS Murine |
| LIF    | Leukemia inhibitory factor | 0.06 | 0.09 | 6.5 | 4E-13 | 5 | 3E-08 | Curated | Antibody array |
| STC1   | Stanniocalcin 1 | 0.22 | 0.6 | 2E-28 | 8.6 | 2E-09 | 7.5 | 1E-12 | Highly likely | MS hSkMC |
| SERPINE1 | Serpin peptidase inhibitor, clade E, member 1 | 0.78 | 1.33 | 4.8 | 5E-26 | 4.3 | 1E-13 | Curated | Antibody array |
| F2R    | F2R like thrombin/tenasin receptor 3 | 0.18 | 0.29 | 1.7 | 4E-30 | 3.2 | 2E-09 | Curated | MS Murine |
| LDLR   | Low density lipoprotein receptor | 1.31 | 1.66 | 4.3 | 8E-83 | 2.5 | 6E-17 | Curated | MS Murine |
| INHBB  | Inhibin, beta B | 0.49 | 0.7 | 4.1 | 2E-46 | 4.7 | 9E-37 | Curated | MS Murine |
| ICAM1  | Intercellular adhesion molecule 1 | 2.1 | 2.56 | 3.9 | 2E-20 | 3.7 | 2E-14 | Highly likely | MS hSkMC |
| ANGPTL4 | Angiopoietin-like 4 | 0.52 | 0.55 | 3.7 | 1E-12 | 7.2 | 1E-24 | Curated | MS Murine |
| CHGB   | Chromogranin B | 2.58 | 3.38 | 3.6 | 2E-19 | 4.3 | 2E-20 | Curated | MS Murine |
| SRGN   | Serglycin | 7.81 | 11.94 | 2.2 | 2E-40 | 2 | 5E-18 | Curated | Antibody array |
| S100A8 | S100 calcium binding protein A8 | 1.01 | 1.24 | 2.3 | 6E-70 | 2.7 | 4E-47 | Highly likely | Curated |
| SERPINA1 | Serpin peptidase inhibitor, clade A, member 1 | 0.16 | 0.22 | 1.9 | 5E-03 | 2.3 | 8E-07 | Curated | Antibody array |
| ICAM1  | Intercellular adhesion molecule 1 | 2.1 | 2.56 | 3.9 | 2E-20 | 3.7 | 2E-14 | Highly likely | MS Murine |
| ANGPTL4 | Angiopoietin-like 4 | 0.52 | 0.55 | 3.7 | 1E-12 | 7.2 | 1E-24 | Curated | MS Murine |
| IL1B   | Interleukin 1, beta | 0.05 | 0.04 | 9.3 | 2E-19 | 21.5 | 9E-21 | Curated | Antibody array |
| IL1B   | Interleukin 1, beta | 0.05 | 0.04 | 9.3 | 2E-19 | 21.5 | 9E-21 | Curated | Antibody array |
| THBS1  | Thrombospondin 1 | 0.4 | 0.75 | 8.6 | 6E-21 | 7.5 | 1E-12 | Highly likely | MS hSkMC |

**Note:** The table highlights the transcripts up-regulated just after 45 min acute exercise of 70% of VO₂ max. The values in the table represent fold changes (FC) and q-values, indicating the significance of the changes. The detected categories include curated, antibody array, and highly likely.
Some transcripts encode well-known myokines, whereas others have never been studied in skeletal muscle. Approximately half of these myokines have previously been detected by mass spectrometry or antibody arrays in medium conditioned by cultured human or murine skeletal muscle cells [9–20, 34–36].

after 12 weeks training, although below the 1.5-fold cut-off. for instance, of the transcripts that increased > 1.5-fold after the first bicycle test (A2/A1), more than half were significantly increased (FC > 1, p-value < 0.05) after 12 weeks training (B1/A1, Supplementary Table 1).

The table below shows the fold change (FC), q-value, and evidence of detection in conditioned medium (CM) for selected transcripts.

| Symbol | Gene name | FPKM A1a | FPKM B2 | A2/A1 | B2/B1 | Metaz-SecKBc | Detected in CM |
|--------|-----------|----------|---------|-------|-------|--------------|----------------|
| ADM5  | Adrenomedullin (5 putative) | 0.35 | 0.28 | 1.3 | 4E-06 | 1.9 | 0.05 | Curated |
| STC2  | Stanniocalcin 2 | 0.31 | 0.17 | 1.8 | 8E-08 | 2 | 12 | Curated MS hSkMC |
| IFI50 | Interferon, gamma-inducible protein 30 | 2.02 | 2.43 | 1.7 | 4E-15 | 1.9 | 0.08 | Curated MS hSkMC |
| SERPINA3 | Serpin peptidase inhibitor, clade A, member 3 | 0.65 | 0.29 | 1.3 | 3E-04 | 2.7 | 0.05 | Curated |
| NFAM1 | NFAT activating protein with ITAM motif 1 | 0.17 | 0.22 | 1.7 | 1E-06 | 1.9 | 0.08 | Highly likely |
| SEMA7A | Semaphorin 7A, GPl membrane anchor | 0.27 | 0.14 | 1.7 | 1E-15 | 1.9 | 0.06 | Curated MS hSkMC |
| GABRE | Gamma-aminobutyric acid type A receptor epsilon subunit | 0.63 | 0.77 | 1.7 | 4E-17 | 1.8 | 0.21 | Highly likely |
| IL1R1 | Interleukin 1 receptor, type I | 2.49 | 2.86 | 1.7 | 7E-12 | 2.1 | 0.05 | Curated |
| FGFR2A | Fg fragment of IgG receptor Il | 0.38 | 0.61 | 1.2 | 8E-04 | 1.6 | 0.05 | Highly likely |
| QPCT | Glutamyl-piptide cyclotransferase | 0.37 | 0.68 | 1.6 | 1E-05 | 1.3 | 0.05 | Curated |
| TNF | Tumor necrosis factor | 0.37 | 0.60 | 1.6 | 1E-04 | 2 | 0.04 | Curated MS hSkMC |
| SERPINA4 | Solute carrier family 39 member 14 | 1.61 | 2.23 | 1.6 | 5E-15 | 1.7 | 0.02 | Highly likely |
| VWA1 | Vangl-Kiebrand factor A domain containing 1 | 1.52 | 2.80 | 1.2 | 1E-19 | 1.3 | 0.05 | Highly likely |
| GBP1 | Guanylate binding protein 1, interferon-inducible | 3.34 | 3.63 | 1.2 | 6E-13 | 1.5 | 0.05 | Highly likely |
| SMPDL3A | Sphingomyelin phosphodiesterase, acid-like 3A | 10.06 | 10.15 | 1.2 | 1E-17 | 1.7 | 0.09 | Highly likely |
| LILRB3 | Leukocyte immunoglobulin-like receptor B3 | 0.26 | 0.36 | 1.2 | 1E-06 | 1.5 | 0.05 | Highly likely |
| NPTX2 | Neuronal pentraxin II | 1.09 | 0.90 | 1.2 | 5E-10 | 1.7 | 0.05 | Highly likely |
| VEGFC | Vascular endothelial growth factor C | 0.77 | 0.80 | 1.2 | 1E-06 | 1.5 | 0.05 | Highly likely |
| TIMP1 | Timp metalloproteinase factor 1 | 0.63 | 0.97 | 1.2 | 1E-06 | 1.6 | 0.05 | Curated |
| CSF2RB | Colony stimulating factor 2 receptor beta common subunit | 0.34 | 0.53 | 1.2 | 7E-04 | 1.7 | 0.05 | Highly likely |
| FLT1 | Fms-related tyrosine kinase 1 | 3.59 | 4.40 | 1.2 | 1E-13 | 1.5 | 0.05 | Curated |
| PSFB | Platelet-derived growth factor beta polypeptide | 5.5 | 8.35 | 1.2 | 1E-15 | 1.3 | 0.05 | Highly likely |
| POSTN | Periostin, osteoblast specific factor | 0.45 | 0.75 | 1.2 | 1E-03 | 1.5 | 0.05 | Curated |
| LILRA6 | Leukocyte immunoglobulin-like receptor A6 | 0.21 | 0.26 | 2.1 | 1E-04 | 1.1 | 0.05 | Highly likely |
| LIPA | Lipase, adipose tissue | 0.06 | 0.11 | 1.2 | 1E-07 | 2.9 | 0.05 | Curated |
| VWA1 | Von Willebrand factor A domain containing 1 | 1.52 | 2.80 | 1.2 | 1E-19 | 1.3 | 0.05 | Highly likely |
| VWA1 | Vangl-Kiebrand factor A domain containing 1 | 1.52 | 2.80 | 1.2 | 1E-19 | 1.3 | 0.05 | Highly likely |
| S100A14 | S100 calcium binding protein A14 | 0.37 | 0.68 | 1.2 | 1E-05 | 1.3 | 0.05 | Highly likely |
| FZD8 | Frizzled-type receptor 8 | 0.36 | 0.68 | 1.2 | 1E-05 | 1.3 | 0.05 | Highly likely |
| GDF27 | Growth differentiation factor 27 | 0.36 | 0.68 | 1.2 | 1E-05 | 1.3 | 0.05 | Highly likely |
| SERPINA3 | Serpin peptidase inhibitor, clade A, member 3 | 0.57 | 0.75 | 1.2 | 1E-06 | 1.5 | 0.05 | Highly likely |

a Fragments per kilobase of transcript per million mapped reads.
b Fold change.
c False discovery rate.
d Annotation in MetaSecKB.
e Expression level below EdgeR threshold for quantification.
f Detected in conditioned medium from murine skeletal muscle cells or explants with mass spectrometry or antibody arrays in medium conditioned by cultured human or murine skeletal muscle cells [9–20, 34–36].
g Detected in conditioned medium from human skeletal muscle cells with antibody array [9, 10, 18, 19].
h Detected in conditioned medium from murine skeletal muscle cells or explants with mass spectrometry analysis [13–16, 20, 34–36].
i Detected in conditioned medium from human skeletal muscle cells with mass spectrometry analysis [9–12].
### Table 2 – Transcripts up-regulated 2 h after 45 min acute exercise of 70% of VO2\textsubscript{max.}

| Symbol  | Gene name                          | FPKM A1\textsuperscript{a} | FPKM B1 | A2/A1 | B3/B1 | Metaz-SecKB\textsuperscript{b} | Detected in CM |
|---------|-----------------------------------|-----------------------------|---------|-------|-------|-------------------------------|----------------|
| CD163L1 | CD163 molecule-like 1             | 0.16                        | 0.29    | 1.6   | 5E-03 | 1.76                         | Curated        |
| STC1    | Stanniocalcin 1                   | 0.12                        | 0.22    | 1.67  | 6E-02 | 1.12                         | Curated        |
| SAA2    | Serum amyloid A2                  | 0.21                        | 0.26    | 1.67  | 1E-01 | 2.13                         | Curated        |
| TGFB3   | Transforming growth factor, beta 3| 2.91                        | 4.05    | 1.68  | 2E-14 | 1.59                         | Curated        |
| SRGN    | Serglycin                         | 7.81                        | 11.94   | 1.68  | 1E-12 | 1.26                         | Curated        |
| FKBP7   | FK506 binding protein 7           | 1.14                        | 1.24    | 1.69  | 1E-12 | 1.44                         | Curated        |
| LPL      | Lipoprotein lipase                | 20.1                       | 25.10   | 1.69  | 5E-12 | 1.74                         | Curated        |
| SEMA3G  | Semaphorin 3G                     | 4.88                        | 7.13    | 1.7   | 4E-11 | 1.34                         | Curated        |
| PDE7A   | Phosphodiesterase 7A              | 9.57                        | 9.09    | 2.29  | 4E-02 | 1.62                         | Curated        |
| INHBE   | Inhibin, beta E                   | 0.42                        | 0.66    | 1.57  | 8E-05 | 1.34                         | Curated        |
| FCGR2A  | Fc fragment of IgG receptor IIa   | 0.38                        | 0.61    | 1.54  | 4E-06 | 1.94                         | Curated        |
| SAA1    | Serum amyloid A1                  | 1.64                        | 4.05    | 2.58  | 8E-03 | 1.74                         | Curated        |

\textsuperscript{a} FPKM: Fragments per Kilobase of transcript per Million mapped reads. 
\textsuperscript{b} Metaz-SecKB: Meta-analysis of sequencing data generated by the Scalable Energy Cost and Energy Budget (SecKB) project.

**Note:** The table lists the up-regulated transcripts with their respective fold changes and q-values, highlighting their significance in the context of acute exercise. The table includes a variety of genes involved in metabolic and regulatory processes, emphasizing the transient response to exercise at 2 hours post-exercise.
Cultured muscle cells (Figure 3B). The relative expression levels of the all mRNAs except wnt family member 9A (MTRNR2L4), Fmnl 3 (FAM20C), thrombospondin 4, (THBS4), complement component 8 gamma polypeptide (C8G), humanin-like 4 (MTRNR2L4), wnt family member 9A (WNT9A), Fms related tyrosine kinase 1 (FLT1), and lipocalin 10 (LCN10). All mRNAs except WNT9A, FLT1, and LCN10 were expressed in cultured muscle cells (Figure 3B). The relative expression levels of the investigated mRNAs showed differences between in vitro differentiated myotubes and skeletal muscle biopsies. MTRNR2L4 and THBS4 were highly expressed in biopsies, whereas in cultured myotubes the expression was low. In myotubes STC2 was highly expressed, which was not the case in skeletal muscle biopsies.

To investigate mRNA expression during myogenic differentiation, myoblasts were differentiated to multinucleated myotubes for 7 days (Figure 3C–F). During myogenesis, THBS4 (4-fold), FAM20C (2.3-fold), CHSY1 (1.5-fold), TNFRSF25 (3.5-fold), and KALAD1 (2-fold) all increased significantly, whereas STC2 was the only gene that was down-regulated (−3.3-fold; Figure 3C). MTRNR2L4, SMPDL3, CSF1, C8G, and IL4R were not significantly changed (data not shown).

To gain further insight into the exercise-related regulation of gene expression, cultured myotubes were subjected to EPS for 24 h to induce myotube contraction (Figure 3G). EPS increased the expression of PPARGCA1A and IL6, 1.3- and 3-fold, respectively, as previously shown [27]. However, only CSF1 was significantly up-regulated by 24 h EPS (1.3-fold, p < 0.05), whereas FAM20C expression was reduced −1.25-fold (p < 0.05).

3.6. CSF1 is secreted from human skeletal muscle cells

Because CSF1 expression was enhanced in skeletal muscle after acute exercise (Figure 4A) and in cultured myotubes after 24 h EPS (Figure 3G), we further focused on CSF1. The expression of CSF1 was also slightly (9%) increased in muscle after 12 weeks. Interestingly, the expression of CSF1 receptor (CSF1R) in skeletal muscle increased after 12 weeks exercise training (Figure 4B). We also measured CSF1 concentration in plasma samples from participants, before (n = 26) and after (n = 22) the 12 weeks intervention. Plasma concentration of CSF1 was significantly increased after exercise, as compared to baseline (Figure 4B).

Table 2 (continued)

| Symbol          | Gene name                          | FPKM A1 | FPKM B1 | A3/A1 | B3/B1 | Metaz-SecKB | Detected in CM |
|-----------------|------------------------------------|---------|---------|-------|-------|-------------|----------------|
| TNFRSF12A       | Tumor necrosis factor receptor superfamily member 12A | 6.53    | 10.35   | 1.58  | 6E-03 | 1.05        | 8E-01          | Highly likely  |
| CCL2            | Chemokine (C-C motif) ligand 2      | 2.25    | 3.31    | 1.58  | 4E-02 | 1.35        | 1E-01          | Curated        |
| GLA             | Galactosidase alpha                | 2.94    | 3.10    | 1.57  | 3E-06 | 1.37        | 6E-05          | Highly likely  |
| MTRNR2L4        | MT-RNR2-like 6                     | 19.29   | 22.12   | 1.56  | 8E-08 | 1.27        | 5E-03          | Curated        |
| IFI30           | Interferon, gamma-inducible protein 30 | 2.02    | 2.43    | 1.56  | 9E-04 | 1.4E-04     | 4E-04          | Curated  MS isMC |
| PLA2G15         | Phospholipase A2, group XV        | 8.45    | 9.28    | 1.55  | 2E-12 | 1.39        | 4E-09          | Curated  MS Murine |
| LOXL2           | Lysyl oxidase-like 2               | 1.1     | 2.64    | 1.55  | 2E-07 | 1.4         | 3E-06          | Curated  MS isMC |
| METRN1L         | Meteorein, glial cell differentiation regulator-like | 1.37   | 1.88    | 1.54  | 5E-03 | 1.33        | 2E-02          | Curated  MS Murine |
| GFT1            | Glutamine-fructose-6-phosphate transaminase 2 | 1.04   | 1.15    | 1.54  | 3E-03 | 1.31        | 2E-02          | Highly likely  |
| ST3GAL1         | ST3 beta-galactoside alpha-2,3-sialyltransferase 1 | 14.1   | 14.03   | 1.53  | 1E-14 | 1.41        | 3E-11          | Curated  MS Murine |
| ADPOQ           | Adiponectin, C1Q and collagen domain containing | 0.29    | 0.28    | 1.52  | 1E-01 | 1.19        | 5E-01          | Curated        |
| NRG2            | Neuregulin 2                       | 0.41    | 0.40    | 1.51  | 5E-03 | 1.47        | 6E-06          | Curated        |
| CEACAM1         | Carcinoembryonic antigen-related cell adhesion molecule 1 | 0.62  | 0.69    | 1.51  | 3E-04 | 1.62        | 2E-08          | Curated  a |
| MTRNR2L4        | MT-RNR2-like 4                     | 68.77   | 74.18   | 1.5   | 3E-09 | 1.36        | 5E-15          | Curated        |
| CD300LG         | CD300 molecule like family member g | 4.55    | 7.60    | 1.5   | 2E-09 | 1.15        | 3E-02          | Highly likely  |
| NPTX2           | Neuronal pentraxin II              | 1.09    | 0.90    | 1.5   | 1E-03 | 1.57        | 1E-04          | Curated        |
| TLR9            | Toll like receptor 9               | 0.62    | 0.40    | 1.3   | 3E-02 | 1.71        | 3E-06          | Highly likely  |
| FCGR3A          | Fc fragment of IgG, low affinity IIIa, receptor (CD16a) | 0.59   | 0.62    | 1.29  | 1E-01 | 1.52        | 2E-04          | Curated        |
| PLAUR           | Plasminogen activator, uronikase receptor | 0.3   | 0.46    | 1.4   | 3E-02 | 1.51        | 2E-03          | Curated  MS Murine |
| CCL2F5          | Cardiotrophin-like cytokine factor 1 | 0.22    | 0.32    | 1    | 1    | 1.98        | 9E-11          | Curated        |
| FX1             | Four jointed box 1                 | 0.18    | 0.35    | 1    | 1    | 1.69        | 3E-05          | Curated        |
| HLA-G           | Major histocompatibility complex, class I, G | 0.21   | 0.28    | 0.57  | 2E-01 | 1.53        | 1E-01          | Highly likely  |
| LILRB3          | Leukocyte immunoglobulin like receptor B3   | 0.3    | 0.36    | 1    | 1    | 1.51        | 2E-04          | Curated        |

a Fragments per kilobase of transcript per million mapped reads.

b Fold change.
c False discovery rate.
d Annotation in MetaSecKB.
e Annotated as secreted in Swissprot, but not in MetaSecKB.
f Expression level below EdgeR threshold for quantification.
g Detected in conditioned medium from murine skeletal muscle cells with antibody array [9,10,18,19].
h Detected in conditioned medium from human skeletal muscle cells with antibody array [13–16,20,34–36].
i Detected in conditioned medium from human skeletal muscle cells with mass spectrometry analysis [8–12].
### Table 3 — Transcripts up-regulated after 12 weeks exercise intervention.

| Symbol | Gene name | FPKM A1 | B1/A1 | MetaSecK | Detected in CM |
|--------|-----------|---------|-------|-----------|----------------|
| SFRP5  | Secreted frizzled-related protein 5 | 0.31 | 4.84 | 1E-01 | Curated |
| MIR05A | Matrix-remodelling associated 5 | 0.48 | 2.75 | 3E-14 | Curated |
| THY1   | Thy-1 cell surface antigen | 1.7 | 2.44 | 3E-14 | Highly likely |
| CPXM1  | Carboxypeptidase Y (M14 family), member 1 | 0.2 | 2.43 | 2E-04 | Curated |
| COL1A1 | Collagen, type I, alpha 1 | 4.92 | 2.4 | 8E-18 | Curated |
| COL3A1 | Collagen, type III, alpha 1 | 16.73 | 2.4 | 4E-17 | Curated |
| COL4A1 | Collagen, type IV, alpha 1 | 14.09 | 2.36 | 2E-26 | Curated |
| THBS4  | Thrombospondin 4 | 21.74 | 2.21 | 1E-19 | Curated |
| SFRP2  | Secreted frizzled-related protein 2 | 0.33 | 2.18 | 1E-07 | Curated |
| LOXL2  | Lysyl oxidase-like 2 | 1.1 | 2.18 | 2E-24 | Curated |
| COL4A2 | Collagen, type IV, alpha 2 | 13.41 | 2.17 | 4E-25 | Curated |
| BGN    |biglycan | 0.36 | 2.1 | 2E-15 | Curated |
| OGN    | Osteoglycin | 0.62 | 2.09 | 6E-07 | Curated |
| CCL21  | Chemokine (C-C motif) ligand 21 | 0.35 | 2.07 | 6E-02 | Antibody array |
| COL6A6 | Collagen, type VI, alpha 6 | 0.1 | 2.04 | 3E-07 | Curated |
| AM0C1  | Adhesion molecule, interacts with CXADR antigen 1 | 0.3 | 2.0 | 5E-09 | Highly likely |
| IG2F   | Insulin-like growth factor 2 | 0.7 | 1.98 | 3E-21 | Curated |
| LOX    | Lysyl oxidase | 0.3 | 1.96 | 4E-23 | Curated |
| TIMEM1 | Transmembrane protein 119 | 0.4 | 1.96 | 3E-13 | Highly likely |
| CDH24  | Cadherin 24, type 2 | 0.27 | 1.97 | 3E-08 | Highly likely |
| PXDN   | Peroxidoxid | 3 | 1.94 | 5E-29 | Curated |
| VPR1   | Vasointestinal intestinal peptide receptor 1 | 6.1 | 1.9 | 3E-10 | Highly likely |
| WISP1  | WNT1 inducible signaling pathway protein 1 | 0.14 | 1.86 | 8E-10 | Curated |
| ADAMTS7| ADAM metalloprotease with thrombospondin type 1 motif, 7 | 0.52 | 1.85 | 9E-19 | Curated |
| ASPN   | Asporin | 2.45 | 1.83 | 1E-15 | Curated |
| NRP2   | Neuregulin 2 | 0.53 | 1.83 | 3E-19 | Curated |
| ST5SIA2| ST5 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 | 0.09 | 1.82 | 5E-03 | Highly likely |
| GPCT   | Glutaminyl-cysteine cyclotransferase | 0.57 | 1.81 | 3E-07 | Curated |
| IL10RA | Interleukin 10 receptor, alpha | 0.7 | 1.86 | 5E-12 | Highly likely |
| COL14A1| Collagen, type XVII, alpha 1 | 0.62 | 1.78 | 7E-10 | Curated |
| OMD    | Osteomodulin | 0.31 | 1.78 | 8E-06 | Curated |
| SFRP4  | Secreted frizzled-related protein 4 | 2.51 | 1.77 | 5E-05 | Curated |
| F2R    | Coagulation factor II receptor | 1.5 | 1.77 | 2E-26 | Highly likely |
| PTN    | Pleiotrophin | 0.62 | 1.77 | 1E-07 | Curated |
| COL1A2 | Collagen, type I, alpha 2 | 16.05 | 1.76 | 1E-10 | Curated |
| SPARC  | Secreted protein, acidic, cysteine-rich | 71.53 | 1.76 | 5E-24 | Curated |
| ANGPTL7| Angiopoietin-like 7 | 0.34 | 1.74 | 4E-01 | Curated |
| LAM1B  | Laminin, beta 1 | 8.8 | 1.74 | 3E-16 | Curated |
| CTHRC1 | Collagen triple helix repeat containing 1 | 1.34 | 1.73 | 2E-10 | Curated |
| PAMR1  | Peptidase domain containing associated with muscle regeneration 1 | 0.86 | 1.73 | 5E-05 | Curated |
| LAM4   | Laminin, alpha 4 | 2.95 | 1.72 | 1E-32 | Curated |
| MEST   | Mesoderm specific transcript | 1.24 | 1.71 | 2E-07 | Highly likely |
| NID2   | Nidogen 2 (osteonidogen) | 2.27 | 1.71 | 3E-22 | Curated |
| WAA1   | von Willebrand factor A domain containing 1 | 1.52 | 1.71 | 1E-15 | Curated |
| LAMC3  | Laminin, gamma 3 | 0.09 | 1.7 | 1E-03 | Curated |
| GPRIR2G | G protein-coupled receptor 162 | 0.44 | 1.69 | 8E-10 | Highly likely |
| ADCCAP1R1 | Adenylate cyclase activating polypeptide 1 receptor type I | 0.3 | 1.68 | 1E-11 | Highly likely |
| KCP    | Klinin/chordin-like protein | 0.2 | 1.67 | 1E-07 | Curated |
| ITH43  | Inter-alpha-trypsin inhibitor heavy chain 3 | 0.46 | 1.66 | 9E-05 | Curated |
| HEPH   | Heparin | 0.26 | 1.66 | 2E-08 | Highly likely |
| IGFBP3 | Insulin-like growth factor binding protein 3 | 3.48 | 1.66 | 5E-16 | Curated |
| VMD1   | Vimentin membrane outer layer 1 homolog | 1.54 | 1.65 | 3E-01 | Curated |
| CD163L1| CD163 molecule-like 1 | 0.16 | 1.65 | 1E-05 | Curated |
| CD300LG | CD300 molecule-like family member g | 4.55 | 1.64 | 1E-23 | Highly likely |
| FGR2R2 | Fc fragment of IgG, low affinity llb, receptor | 0.44 | 1.63 | 3E-05 | Highly likely |
| AGNN   | Agrin | 1.88 | 1.63 | 9E-19 | Curated |
| ADAMTS15| ADAM metalloprotease with thrombospondin type 1 motif, 15 | 0.78 | 1.63 | 3E-20 | Curated |
| KAZALD1| Kazal-type serine peptidase inhibitor domain 1 | 2.08 | 1.62 | 7E-14 | Curated |
| APLN   | Apelin | 1.61 | 1.62 | 9E-07 | Curated |
| EMLN3  | Elastin microfiber interactor 3 | 0.35 | 1.61 | 1E-03 | Curated |
| COL5A2 | Collagen, type V, alpha 2 | 2.94 | 1.59 | 2E-11 | Curated |
| ECMN3  | Extracellular matrix protein 2 | 1.86 | 1.58 | 4E-15 | Curated |
| THBS1  | Thrombospondin 1 | 0.4 | 1.58 | 2E-05 | Highly likely |
| FGFR3  | Fibroblast growth factor receptor 3 | 0.13 | 1.58 | 2E-04 | Curated |
| ADAMTS8| ADAM metalloprotease with thrombospondin type 1 motif, 8 | 0.26 | 1.58 | 1E-03 | Curated |
| SERPIN1| Serpin peptidase inhibitor, clade E, member 1 | 0.78 | 1.57 | 7E-06 | Antibody array |
| ADAMTS3| ADAMTS-like 3 | 0.84 | 1.57 | 2E-13 | Curated |
| NID1   | Nidogen 1 | 4.32 | 1.57 | 4E-16 | Antibody array |
CSF1 was significantly increased immediately after exercise, and was reduced to below baseline after 2 h recovery (Figure 4C). Acute exercise may influence plasma volume and protein concentration [37], however total protein concentration was measured and did not change significantly during acute exercise in our study. Interestingly, the concentration of CSF1 also increased after 12 weeks exercise training. Interestingly, these data are in concordance with our study [50].

In addition to 24 h EPS, short-term EPS enhanced CSF1 expression significantly (p < 0.05). This suggests that CSF1 is an exercise-responsive myokine secreted from human skeletal muscle cells.

4. DISCUSSION

In the present study, we used mRNA sequencing as an untargeted screening to identify exercise-responsive myokines. We searched for secretory transcripts that were either up- or down-regulated, although we chose to focus on myokines that were increased by exercise. In total, we detected almost 250 genes encoding putative myokines that were up-regulated after acute and/or long-term training. About half of these proteins have not been detected by previous proteomics studies on skeletal muscle cell cultures. To our knowledge, there are no published studies that have used mRNA sequencing to search for new myokines, which is a useful approach to substantially expand our knowledge of the skeletal muscle secretome. Most transcripts followed different patterns of expression; some were “fast” or “slow” responders to exercise, whereas others responded to long-term exercise, or both. Moreover, several transcripts responded differently to acute exercise after long-term exercise.

Several other studies have focused on the transcriptional response in skeletal muscle to physical activity. Catoire et al. used microarray to measure gene expression after 1 h one-legged cycling or 12 weeks of combined exercise training [17]. The authors identified 52 putative myokines that were significantly (p < 0.01) up-regulated in the exercising leg after acute exercise and 66 that were induced after 12 weeks training. Interestingly, these data are in concordance with our RNA-seq data; of the 52 acute transcripts identified by Catoire et al., 50 were also increased in our dataset (A2/A1, p < 0.05). Furthermore, of the 66 putative myokines induced after 12 weeks, 62 were also significantly up-regulated in our participants (B1/A1).
After acute exercise, several cytokines, chemokines, growth factors and ECM remodeling enzymes were up-regulated. Several of these are known contraction-regulated myokines, including IL6, IL8, LIF, CCL2, CX3CL1, SERPINE1, ANGPTL4, and VEGF [17,38]. Because many myokines and cytokines are difficult to detect by the use of mass spectrometry, antibody arrays have been used as a tool for myokine discovery. Raschke et al. used a cytokine antibody array to detect proteins released from cultured human muscle cells in response to electrical stimulation [19]. In total, they identified 45 proteins that were induced by EPS, and many of them were cytokines, chemokines, or growth factors. About half were up-regulated after acute or long-term exercise in our participants (p < 0.05, B1/A1).

After long-term training, a large proportion of the up-regulated transcripts were related to ECM. Induction of ECM related genes after exercise training has been reported by several others [17,39–41]. We have discussed the data related to ECM in more detail elsewhere [27]. We used primary human skeletal muscle cells as a model system for further investigation of 17 novel myokine candidates in vitro. Cultured human myotubes share many morphological and biochemical characteristics with skeletal muscle fibers in vivo [42,43]. They are multinucleated and have the ability to contract upon electrical stimulation. The relative expression levels of the 17 selected candidates were different in cultured cells as compared to muscle biopsies; some of the most highly expressed transcripts in biopsies were expressed at low levels in vitro and vice versa. IL6, STC2, and CSF1 were all higher expressed in cultured myotubes than in muscle tissue (relative to the expression of RPLPO). Moreover, three transcripts were not expressed in cultured myotubes. Gene expression patterns in cultured myotubes

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**Figure 3:** A–B) mRNA expression of selected genes in skeletal muscle biopsies (in A1, n = 26) or cultured human myotubes. mRNA expression in biopsies was determined with RNA-seq, and in myotubes by RT-PCR from 5–6 experiments using different donors. C–F) Primary human myoblasts were differentiated to multinucleated myotubes for 7 days. mRNA expression values are shown as fold change from the expression in myoblasts (day 0) and represent means ± SEM from 3–4 experiments using different donors. *p < 0.05 vs. D0, students t-test. G) Primary human skeletal muscle cells were differentiated for 5 days and subjected to electrical pulse stimulation (EPS; 1 Hz, 2 ms, 11.5 V) for 24 h. Data are shown as fold vs. control and represent means ± SEM from 5–7 experiments. *p < 0.05, students t-test. Gene expression values were normalized to RPLP0, bars depict means ± SEM.
a marked increase in number of blood monocytes. CSF1 may in tissues, and injecting mice with recombinant CSF1 induces resorbing osteoclasts [47]. CSF1 is a central regulator of macrophage progenitors into monocytes, macrophages, dendritic cells and bone-resorbing osteoclasts [47]. CSF1 is a cytokine and an important hematopoietic growth factor [46], inducing differentiation of myeloid progenitors into monocytes, macrophages, dendritic cells and bone-resorbing osteoclasts [47]. CSF1 is a central regulator of macrophage numbers in tissues, and injecting mice with recombinant CSF1 induces a marked increase in number of blood monocytes. CSF1 may influence macrophage survival, proliferation, differentiation, and function, and CSF1 has been linked to diseases like arthritis, cancer, nephritis, pulmonary fibrosis, atherosclerosis, and vascular injury [48–50].

Based on our data, we hypothesize that CSF1 mediates cross-talk between skeletal muscle cells and immune cells and could be involved in exercise-induced immune responses. It is also possible that CSF1 may have other functions during exercise. Several of the previously described myokines are cytokines with immune-regulatory functions. For instance, IL6 was originally identified as a proinflammatory cytokine secreted from T-cells and macrophages, whereas exercise-induced IL6 is not associated with muscle damage and inflammation, but has been linked to metabolic regulation [5,6].

Alternative functions of CSF1 during exercise may be related to muscle adaptation. Incubation of skeletal muscle cells or monocytes with CSF1 promotes increased VEGF production and angiogenesis [51,52]. VEGF promotes angiogenesis by stimulating proliferation of endothelial cells [53]. Our data demonstrate increased expression of both VEGFA and CSF1 after exercise, which might be important for skeletal muscle vascularization.

Several lines of evidence suggest that CSFs may reduce serum lipids and cholesterol levels. Human CSF1 was injected to 7 boys with chronic neutropenia. After 7 days treatment, absolute neutrophil numbers increased in 4 patients, but serum cholesterol levels were reduced in all of them [54]. Shimano et al. injected rabbits with recombinant CSF1 for 7 days, lowering plasma cholesterol levels by 33% [55]. Moreover, CSF1 may modulate lipoprotein metabolism by promoting macrophages to produce lipoprotein lipase (LPL) [56]. Thus, enhanced plasma concentration of CSF1 after acute exercise may influence lipid metabolism and lower cholesterol levels.

In summary, we identified numerous transcripts that were regulated in skeletal muscle after acute and/or long-term exercise. These
transcripts encode potential myokines, which may play key roles in local and systemic adaptations to exercise. Furthermore, we identified CSF1 as a novel myokine, which was increased after acute and long-term exercise, and secreted from cultured human myotubes in response to EPS.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.01.007.

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