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Supplementary Materials for

Therapeutic exercise attenuates neutrophilic lung injury and skeletal muscle wasting

D. Clark Files,* Chun Liu, Andrea Pereyra, Zhong-Min Wang, Neil R. Aggarwal, Franco R. D'Alessio, Brian T. Garibaldi, Jason R. Mock, Benjamin D. Singer, Xin Feng, Raghunatha R. Yammani, Tan Zhang, Amy L. Lee, Sydney Philpott, Stephanie Lussier, Lina Purcell, Jeff Chou, Michael Seeds, Landon S. King, Peter E. Morris, Osvaldo Delbono

*Corresponding author. E-mail: dfiles@wakehealth.edu

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Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/7/278/278ra32/DC1)

Supplementary Materials

Supplementary Materials and Methods:

Randomized study of early mobility in humans with acute respiratory failure

Enrollment, Randomization, and Study Initiation Time Window

The study was approved by the Wake Forest Human Subjects Committee and the Institutional Review Board. All subjects or their legally authorized representatives gave consent before randomization. Subjects were randomized within 48 hours of the institution of mechanical ventilation. The study was conducted from July 2007 to July 2009.

Randomization

Study subjects were randomized to receive early mobility therapy or usual care, using blocked randomization to ensure equal accrual to each treatment. Randomization was done through the data management system website, which resided on a secured server internal to the medical center. Severity of illness scores for the first 50 patients enrolled (cohort 1) were tabulated as Apache II scores and the second 50 patients (cohort 2) were tabulated as Apache III scores (Table S3).

Inclusion/Exclusion Criteria

The inclusion criteria included age greater than 18 and the need for mechanical ventilation by endotracheal tube. Baseline characterization of the need for mechanical ventilation was separated into 3 groups: coma - need for mechanical ventilation is primarily for airway protection; acute respiratory failure - lung failure without known existing heart or lung disease; acute on chronic-lung failure with known underlying heart or lung disease. The exclusion criteria were hospitalization > 72 hours, inability to walk without assistance before the acute ICU illness

(use of a cane or walker were allowed), cognitive impairment before the acute ICU illness (non-verbal), a pre-admission immunocompromised state (prednisone >20 mg/d for 2 weeks before hospitalization, CD4 count <50), acute stroke, body mass index (BMI) >45, neuromuscular disease that could impair weaning (myasthenia gravis, Amyotrophic Lateral Sclerosis (ALS), Guillain-Barre Syndrome), new hip fracture, unstable cervical spine, or pathological fracture, mechanical ventilation >48 hours, CPR or DNR on admission, hospitalization within 30 days before admission without return to prior level of physical function, cancer therapy within last 6 months, or re-admission to the ICU within current hospitalization.

PROM therapy was administered to all upper and lower extremity joints by one of the Mobility Team nursing assistants. Five repetitions of PROM were provided for each joint. For the upper extremities, PROM included finger flexion and extension; wrist flexion, extension, ulnar and radial deviation; elbow flexion, extension, supination, and pronation; shoulder flexion, abduction, internal and external rotation. Lower extremity PROM included toe flexion and extension; ankle dorsiflexion, plantar flexion, inversion, and eversion; knee flexion and extension; hip flexion, abduction, adduction, internal and external rotation.

Therapy sessions were function-based and targeted toward identified functional deficits. This intervention was based on our previously published protocol *(1)*. The session components included bed mobility, transfer training, and balance training. As the subject progressed, the physical therapy increasingly focused on functional activities such as transfer to the edge of the bed; safe transfers to and from the bed, chair, or commode; seated balance activities; pre-gait standing activities (forward and lateral weight shifting, marching in place), and ambulation.

Mouse activity measurements

A line was drawn in the middle of the cage, and the number of times each mouse body fully crossed the midline was counted as one unit of travel activity. Rearing activity was monitored and recorded as one unit of rearing activity when each animal placed both forelimbs on the side of the mouse cage. Each mouse was observed in a cage for 5 minutes at the same time of day (2-3 pm) to capture these events.

Lung, bronchoalveolar lavage (BAL), and blood collection and analysis

At specified time points after Sham or ALI conditions, mice were anesthetized and blood was collected from the inferior vena cava in EDTA-containing tubes. Post mortem, bronchoalveolar lavage (BAL) fluid was collected for analysis of total cell and differential counts. Cell-free BAL supernatants or plasma were analyzed by ELISA for G-CSF protein (R&D Systems). Lungs were prepared for histological sectioning with 1% agarose inflation followed by formalin fixed paraffin embedding (FFPE), as previously reported *(10)*.

Muscle ex-vivo force measurements

After euthanasia by cervical dislocation, both soleus muscles were dissected under stereoscopic observation and the surrounding connective tissue removed. The muscle was mounted between a 407A force transducer (Aurora Scientific; compliance: 0.01 micron/mN, resonant frequency: 4.0 kHz) and an adjustable holder to control muscle position and length. It was continually perfused with buffered recording solution (mM: NaCl 121, KCl 5, CaCl2 1.8, MgCl2 0.5, NaH2PO4 0.4, NaHCO3 24.0, glucose 5.5, *d*-tubocurarine chloride hydrate 0.015) bubbled with a mixture of 5% CO2 and 95% O2 in a contiguous chamber to maintain pH 7.4. The muscle was allowed to balance for 15 minutes before direct stimulation by an electrical field generated between two

parallel platinum electrodes connected to a stimulator. The muscle length was adjusted until a single stimulus pulse or a train of pulses elicited maximum force during twitch or tetanus (optimal length [Lo]) under isometric conditions. After attaining Lo, the muscle was allowed to rest for 8 min, and subsequently the force–frequency relationship was recorded at 1, 5, 10, 20, 50, 100, and 150 Hz using 400-ms trains of pulses. The interval between trains of pulses varied according to the magnitude of the stimulus (0.5 - 8 min). Whole soleus muscle fatigability was studied by applying a sequence of pulses at 150 Hz. The pulse duration was 400 ms, the interval was 1 ms, and the total duration of the stimulation protocol was 5 minutes. The Fatigue Index (FI) was calculated as the ratio between the tension developed by the muscle at each minute and the maximum force developed at the initial time point (0 minutes). All the experiments were performed at 22°C, and the muscle responses were recorded and analyzed with ASI600A Digital Controller software (Aurora Scientific Inc.).

Muscle characterization and morphometric analysis

Skeletal muscles and diaphragms were removed from the animals and snap frozen in liquid nitrogen for RNA or protein extraction. For fiber size measurements, the soleus was removed and affixed at resting length and then frozen in isopentane equilibrated in dry ice and mounted for cryosectioning in OCT (Tissue-Tek). The harvested diaphragm was “rolled” onto a plastic rod, the rod was removed and the tissue frozen. 12 μm cryosections of the specified muscles were obtained. For fiber typing, type I or II fibers were identified by the ATPase method at pH 9.4 to quantify fiber size of type I versus type II fibers as previously described *(10)*, and sections were co-incubated with an anti-laminin antibody (#MAB1914P, Millipore). 100 to 250

individual muscle fibers were counted, and the cross-sectional area was quantified using Image J software (NIH).

Lung morphometric analysis and digital pathology

The left lung of each mouse, formalin-fixed and paraffin-embedded, was sectioned and stained with hematoxylin and eosin. Slides were scanned at 20x on a Nanozoomer-XR® (Hamamatsu); image files were converted to the Aperio.svs format and were analyzed using the image management software and analysis algorithms available within the Aperio Spectrum framework (Leica Biosystems). The Genie machine learning module was trained to identify regions categorized as lung injury, bronchial epithelium, alveolar space, and blank (no tissue) areas. The extent of lung injury was recorded as the percentage of total tissue area occupied by lung injury. The Nuclear Algorithm module, which identifies individual nuclei, was run within the damaged areas to quantify the density of cellular infiltrate, mainly inflammatory cells, as a cell count per unit area. An index combining extent and intensity of injury was calculated by multiplying the percentage of injured lung times the cell density in the injured areas *(50)*.

Isolation of skeletal muscle nuclear and cytoplasmic subfractions

Nuclear and cytoplasmic fractions of mouse skeletal muscle lysates were modified according to previously published protocols*(51, 52)*. 50 mg of frozen gastrocnemius muscle was ground in liquid nitrogen and homogenized on ice in 2 ml lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol) in addition to protease and phosphatase inhibitor cocktails. The samples were filtered through a 40 μm cell strainer, centrifuged at 5000 rpm for 5 min at 4°C, and the supernatants collected as nuclear-free

cytosolic fractions. The remaining pellet was washed 3 times in ice-cold lysis buffer and resuspended in a high-salt buffer containing 360 µl of lysis buffer plus 49.8 µl of 5M NaCl with protease and phosphatase inhibitors. The nuclear prep was rotated for 2 h at 4°C to release the nuclear fraction and centrifuged at 13000 rpm for 15 min at 4°C. The supernatants were collected and stored as cytosolic-free nuclear fractions.

Western blotting

For Western blotting, muscles were homogenized using a bead blender (BBX24B Bullet Blue Blender, Next Advance) with lysis buffer containing 60 mM Tris pH 6.8, 1% SDS, and 12% glycerol, then cleared by centrifugation. Protein concentration was determined by Bio-Rad DC assay (Bio-Rad). Proteins were boiled and separated by SDS-PAGE and the gel then electrophorectically transferred to a PVDF membrane (Millipore). Specific proteins were detected with the following antibodies: Trim63 (MuRF1) (AF5366 R&D Systems), GAPDH (AM4300 Ambion), p65 (4764S Cell Signaling Technology), phospho-p65 (3039S Cell Signaling Technology). Horseradish peroxidase–conjugated secondary antibodies were then incubated with the blot and the antibody complexes detected by chemiluminescence (ECL; Amersham, GE Healthcare). Western blot densitometry was carried out using Image J software.

Real Time QPCR

Total RNA was isolated from muscles using the Trizol reagent per the manufacturer's suggestions (Invitrogen). The RNA was reverse transcribed into cDNA and amplified with the appropriate primers using a one-step kit (Lo-Rox Bio-78005 Bioline) and a Thermo cycler (7500 Fast real time PCR system, Applied Biosystems). All mRNA expression was normalized to

GAPDH. TaqMan probe-based primers (Applied Biosystems) were used in all reactions. Accession numbers are listed after each gene: GAPDH, Mm99999915_g1; Trim63 (MuRF1), Mm01185221_m1.

Inflammatory biomarker array, individual ELISAS, and multiplexed ELISAS

50 µl of mouse plasma was used for a chip-based fluorescent cytokine array using a semi-quantitative approach with 144 murine cytokines (RayBiotech Inc. AAM-CYT-G2000). The glass chip was analyzed in a blinded fashion using a laser detector at RayBiotech, and the results were provided to our lab. G-CSF was quantified in EDTA plasma prepared samples using a mouse quantikine ELISA kit (R&D Biosystems, MCS00). IL-17A, IL-17F, and IL-23 were multiplexed on a multiplex chip-based platform (RayBiotech Inc., QAM-TH17-1). The experiment was performed in our lab and sent to Raybiotech for blinded analysis. The results were provided to our lab.

Supplementary Tables and Figures:

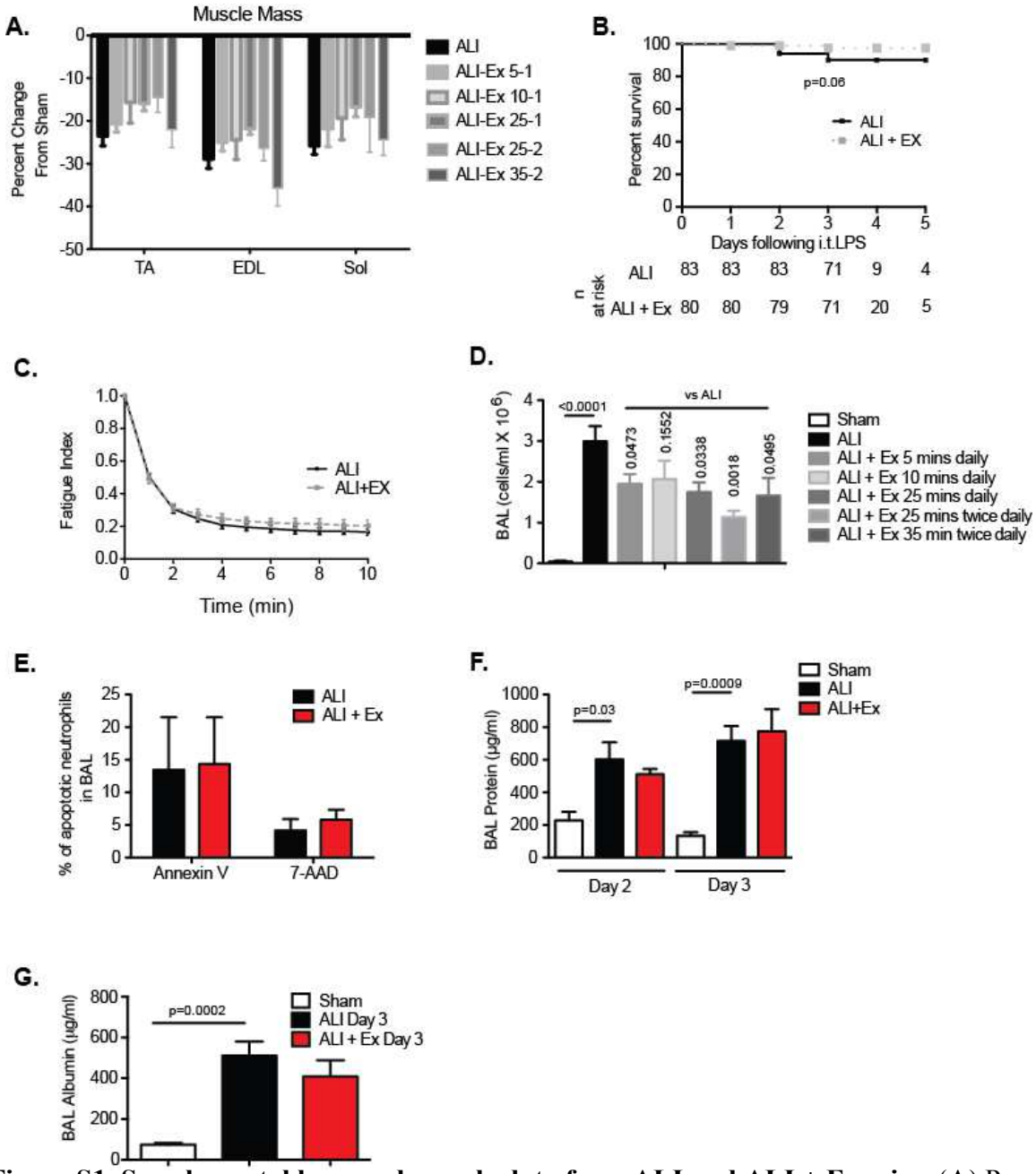

Figure S1. **Supplemental lung and muscle data from ALI and ALI + Ex mice.** (A) Percent change in wet muscle mass of the tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (Sol) in ALI and ALI + Ex mice at varying durations of exercise compared to Sham mice at day 3. (B) Survival curves for ALI and ALI + Ex experiments. (C) Fatigue index of the soleus muscle over a five minute stimulation protocol. (D) BAL cell counts of Sham, ALI, and ALI + Ex mice at varying durations of exercise. (E) Annexin V and 7-AAD labeling of BAL neutrophils of ALI and ALI + Ex mice by flow cytometry. (F) BAL total protein and (G) albumin in Sham, ALI, and ALI + Ex mice. n=5/group

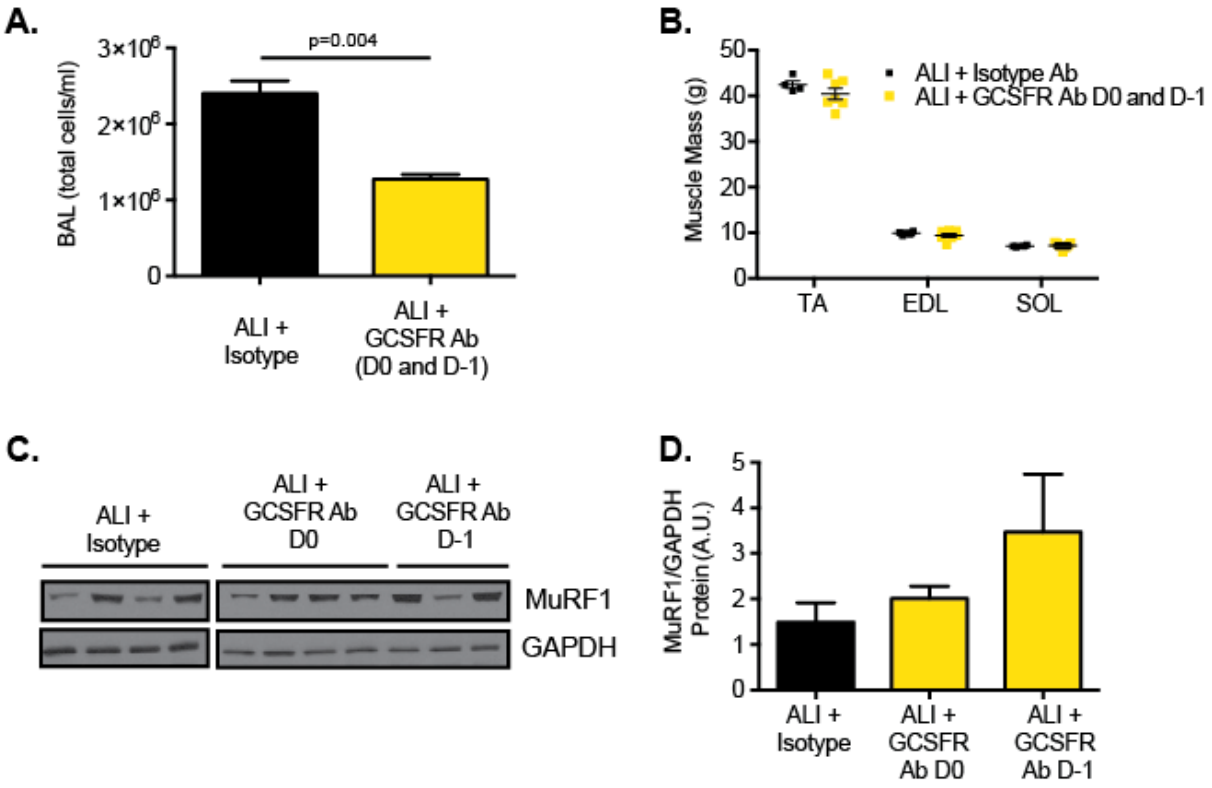

Figure S2. Prophylactic blockade of the G-CSFR in ALI mice. (A) BAL cell counts from ALI + isotype and ALI + GCSFR Ab starting at day 0 or day -1 (experimental time points performed separately but data combined) and harvested at day 3. (B) Wet weight of the tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (SOL) of ALI + isotype and ALI + GCSFR Ab starting at day -1 and day 0 (experimental time points performed separately but data combined) and harvested at day 3. (C) Western blots of MuRF1 normalized to loading control GAPDH in ALI + isotype, ALI + GCSFR Ab day 0, and ALI + GCSFR Ab day -1. (D) Western blot quantification by densitometry.

5 mins daily			Speed (m/min)				
	Phase	Time (min)	Day 0	Day 1	Day 1.5	Day 2	Day 2.5
	1	5	0	5	0	5	0

10 mins daily			Speed (m/min)				
	Phase	Time (min)	Day 0	Day 1	Day 1.5	Day 2	Day 2.5
	1	5	0	5	0	5	0
	2	5	0	7	0	7	0

25 mins daily			Speed (m/min)				
	Phase	Time (min)	Day 0	Day 1	Day 1.5	Day 2	Day 2.5
	1	5	0	5	0	5	0
	2	5	0	7	0	7	0
	3	5	0	9	0	7	0
	4	5	0	7	0	7	0
	5	5	0	5	0	5	0

25 mins BID			Speed (m/min)				
	Phase	Time (min)	Day 0	Day 1	Day 1.5	Day 2	Day 2.5
	1	5	0	5	5	5	5
	2	5	0	7	7	7	7
	3	5	0	9	9	9	9
	4	5	0	7	7	7	7
	5	5	0	5	5	5	5

35 mins BID			Speed (m/min)				
	Phase	Time (min)	Day 0	Day 1	Day 1.5	Day 2	Day 2.5
	1	5	0	5	5	5	5
	2	5	0	7	7	7	7
	3	5	0	9	9	7	7
	4	5	0	7	7	7	7
	5	5	0	5	5	5	5
	6	5	0	5	5	5	5
	7	5	0	5	5	5	5

Table S1. Exercise protocols in lung-injured mice. BID=twice daily

Table S2. **Inflammatory biomarker array for ALI + Ex versus ALI mice**.

Protein	Fold Change	p value
G-CSF	-2.558456243	0.024501041
GITR Ligand	-2.534858295	0.086364309
Lungkine (CXCL15)	-2.376051304	0.015742118
KC	-1.991386816	0.079436228
Pro-MMP-9	-1.941470059	0.438356025
Osteoporotegerin	-1.902215471	0.032799429
MIG	-1.797350324	0.298068913
Growth arrest specific 6	-1.694098075	0.044861939
IGF-I	-1.684530895	0.08897452
HGF R	-1.625343827	0.38073752
Dtk	-1.573214983	0.621466126
TSLP	-1.572659853	0.154526292
IL-17F	-1.55867563	0.054905174
CXCL10	-1.549098019	0.058637003
TRANCE	-1.545202	0.153053957
IL-1ra/IL-1F3	-1.530896216	0.057777533
Fcg RIIB	-1.428251731	0.165449058
HAI-1	-1.424437703	0.250682281
LIX	-1.40077628	0.108800576
IL-7	-1.394450916	0.431510145
HGF	-1.36814835	0.06972608
SCF	-1.359352519	0.280361418
VEGF R3	-1.354754942	0.02424935
CD27	-1.349140063	0.432030482
VEGF R2	-1.322095651	0.23447868
L-Selectin	-1.301076811	0.384294705
RAGE	-1.278934764	0.088441632
Cardiotrophin-1	-1.233986443	0.420590429
IL-17B	-1.233882174	0.017970281
BLC	-1.219176844	0.638843843
IL3 Rb	-1.213170925	0.370954691
Leptin	-1.213085362	0.542134194
Shh-N	-1.198467144	0.189774308
Pentraxin 3	-1.192935353	0.242693835
IL2	-1.182902041	0.550341035
DKK-1	-1.180763743	0.057022985
RANTES	-1.164269165	0.156113551
IL-2 R alpha	-1.15960903	0.136777745
CTACK	-1.145767333	0.269785283
IL-17B R	-1.144466476	0.725230663
sTNF RII	-1.138753603	0.202328875
M-CSF	-1.133723451	0.51192746
IL6	-1.12635902	0.396633186
IL4	-1.125409534	0.494373481
bFGF	-1.123603637	0.55481708
I-TAC	-1.120882051	0.533172888
TARC	-1.11218259	0.584183094
MCP-5	-1.107903411	0.462057325
IL-21	-1.104244756	0.280777655
Fractalkine	-1.100801694	0.278791926
TIMP-2	-1.096094664	0.822998866
VEGF D	-1.094590423	0.880569272
Neprilysin	-1.094437163	0.494838109
CD40	-1.090503032	0.657359748
MFG-E8	-1.089996328	0.783737653
MIP-1-gamma	-1.08748124	0.524560416
IL-28	-1.086631192	0.511393173
Eotaxin	-1.07744981	0.532351947
IL-11	-1.075981978	0.60074895
Eotaxin-2	-1.071908759	0.41249558
MIP-1-alpha	-1.071341913	0.810469174

Osteopontin	-1.067253733	0.532058531
IL-15	-1.064259241	0.605299542
MAdCAM-1	-1.061046143	0.854895237
VEGF R1	-1.058723076	0.58972622
DPPIV/CD26	-1.054315533	0.780335196
GM-CSF	-1.050019792	0.79017468
IL-17E	-1.045597716	0.840414421
Chordin	-1.041998369	0.845984518
IL3	-1.041483848	0.569905525
TREM-1	-1.033433205	0.665117561
Amphiregulin	-1.026927682	0.89256971
IGF-II	-1.02674563	0.878804758
IL-1 R4/ST2L	-1.025530854	0.945719208
TECK	-1.007410755	0.961028545
IL17	-1.006129563	0.879309596
PF4	1.000302376	0.998035561
Lymphotactin	1.000894784	0.987782449
VCAM-1	1.003300299	0.965770628
MIP-3-beta	1.005157885	0.961663573
TNF-alpha	1.013874069	0.912557116
E-Cadherin	1.013975253	0.307004056
Troy	1.015314725	0.92842148
Epiregulin	1.017588421	0.881183215
TIMP-1	1.020031533	0.869010565
IL9	1.023368042	0.981673387
CD30L	1.029660202	0.748476749
TACI	1.030987006	0.952322705
CD27 Ligand	1.031968552	0.887789795
MIP-3-alpha	1.03868728	0.923861804
IL10	1.044596185	0.763536665
TWEAK	1.044720749	0.803576641
IL12-p40/p70	1.048160871	0.505270524
SDF-1 alpha	1.052305419	0.678335922
ALK-1	1.054256225	0.824767336
IL12-p70	1.058250522	0.73541427
Flt-3 lLigand	1.058330401	0.673886175
4-1BB	1.059763589	0.383892365
MDC	1.065681081	0.85839591
Endoglin	1.078153722	0.587659044
MCP-1	1.080259595	0.34534634
IFN-gamma	1.081975111	0.301092458
E-Selectin	1.097166233	0.620562099
ICAM-1	1.108153744	0.697823349
TPO	1.115719416	0.161323616
6Ckine	1.119899744	0.781247878
IL5	1.129825595	0.162002007
ACE /CD143	1.133777548	0.699255821
Thymus CK-1	1.136328349	0.849765244
IGF-BP-5	1.158919593	0.128731671
CD36	1.178109311	0.50905588
VEGF	1.181546458	0.697417611
CD40 Ligand	1.182999646	0.318090578
EGF	1.18356428	0.340880557
Axl	1.188620818	0.551606362
IGFBP-2	1.194271136	0.559547649
FAS ligand	1.202475037	0.159687595
Granzyme B	1.203253987	0.535379454
IL1-beta	1.210264368	0.3459454
TWEAK R	1.227519313	0.538466115
Epigen	1.253537478	0.433129587
CTLA-4	1.292830896	0.337191376
MIP-2	1.294041899	0.01827715
IL-6 R	1.334898672	0.204467974
P-Selectin	1.337176482	0.081735741
GITR	1.353301173	0.276485971
CD30/TNFRSF8	1.353869245	0.312778462

Decorin	1.390500279	0.16908542
IL1-alpha	1.392331805	0.129998505
MMP-2	1.409504298	0.276204514
Resistin	1.478012311	0.118299539
IGF-BP-3	1.601139613	0.296731048
IL13	1.638313465	0.322839404
sTNF RI	1.647980085	0.349233581
Leptin R	1.652159608	0.368039322
TCA-3	1.69121269	0.502259658
Growth arrest specific 1	1.775002222	0.134937036
MMP-3	1.953046312	0.02824541
JAM-A	2.094362789	0.210507324
Galectin-1	2.107736394	0.008920341
Prolactin	2.136515163	0.109364024
IL-20	2.234110194	0.260463278
IGF-BP-6	2.344060163	0.103478889

	Exercise (n=50)	No Exercise (n=50)	*p-value*
Age (yrs), mean ±SD	53.6±18.6	51.9±18.6	0.638
Body mass index (mean ±SD)	29.4±8.0	27.9±8.0	0.351
Gender			0.155
Male, n (%)	26(52)	33(66)	
Race			1.000
Caucasian, n (%)	43(86)	43(86)	
African American, n (%)	7(14)	6(12)	
Hispanic, n (%)	0(0)	1(2)	
Admission Source			0.277
Direct admit, n (%)	0(0)	3(6)	
Emergency Department, n (%)	29(58)	29(58)	
Outside Hospital, n (%)	21(42)	18(36)	
Insurance, n (%)			0.606
Medicare, n (%)	26(52)	19(38)	
Medicaid, n (%)	11(22)	14(28)	
Private Insurance, n (%)	10(20)	12(24)	
Uninsured, n (%)	3(6)	5(10)	
Apache II (mean ±SD), cohort 1 (n=50)	22.5±10.1	21.0±7.4	0.646
Apache III (mean ±SD), cohort 2 (n=50)	57.8±23.01	63.2±17.3	0.360
Charlson Comorbidity Index, mean ±SD	4.7±3.2	4.7±2.9	0.921
Vasopressors at entry or during study, n (%)	14(28)	16(32)	0.663
Diagnosis			0.223
Coma, n (%)	9(18)	10(20)	
Acute respiratory failure, n (%)	31(62)	36(72)	
Acute on chronic, n (%)	10(20)	4(8)	

Table S3. Patient characteristics at enrollment of randomized pilot study of early mobility in patients with ARF.