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Characterization of Exercise-Associated Skeletal Muscle Secreted Factors Through Aging

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CHARACTERIZATION OF EXERCISE-ASSOCIATED SKELETAL MUSCLE SECRETED FACTORS THROUGH AGING

by

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MASTER OF SCIENCE

ABSTRACT

Exercise is a powerful behavioral intervention against Central Nervous System (CNS) aging[1–3] and plays an essential role in maintaining healthy neurocognitive function and immune metabolism in the aging brain[3]. We have developed a new transgenic mouse model that moderately over-expresses transcription factor master regulator of proteostasis, Transcription Factor E-B (TFEB), in skeletal muscle (cTFEB;HSACre mice). Despite living sedentary lifestyles, our model of enhanced skeletal muscle proteostasis shows significantly ameliorated proteotoxicity, increased BDNF levels and neurogenesis while improving neurocognitive function and decreasing neuroinflammation in the aging CNS[4,5]. These neuroprotective effects are markedly reminiscent of those observed in the aging CNS post-exercise[6,7], suggesting enhancing muscle proteostasis may be sufficient to replicate the local and systemic benefits of exercise. Indeed, skeletal muscle is a powerful endocrine organ, secreting bioactive molecules, cytokines, known as 'myokines', such as irisin/FNDC5 an cathepsin B that can modify CNS metabolism and function, which very likely contribute to the exercise-associated benefits on cognition[6,8]. Exercise activates the production and secretion of these myokines from skeletal muscle into circulation, directly implicating skeletal muscle metabolism in the CNS's response to exercise[9–11].

However, to date, the precise origin and function of these exercise-responsive, pro-neurogenic circulating factors remain largely unexplored. Therefore, **I hypothesize that changes in skeletal muscle-originating circulatory factors will be similar between our sedentary cTFEB;HSACre mice and wild-type voluntary running (exercised) mice.**

Keywords: Exercise, Aging, Central Nervous System, Circulatory Factors, Inflammation, Proteostasis, Muscle

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CHAPTER 1: INTRODUCTION

Aging

Aging is known as the progressive time-dependent functional loss that affects most organisms, as stated by the 'Hallmarks of Aging'[12]. Normal aging results in neurobiological alterations, including increased levels of oxidative stress and inflammation as well as decreased neurogenesis (the birth of new neurons) and synaptic plasticity[12,13]. Along with aging also comes the natural decline in muscle mass and quality[14]. Maintenance of muscle mass and health is important as it plays a central role in the degradation and upkeep of protein synthesis[14]. This progressive loss of muscle mass with aging shows negative effects on the health of aged individuals[14]. For example, muscle health is regulated by the upkeep of protein status, that being their synthesis or degradation controlled by an autophagosome-lysosomal mechanism, known as macro autophagy, hereafter referred to as autophagy[14] . When this system is not functioning correctly, we can see that there is an increase in the buildup of protein aggregates that cause downstream negative effects. Autophagy[14,15], the process of degrading and recycling all types of intracellular components, is an important process for the upkeep of proteins and organelles using the lysosome as the primary degrader[15]. The functional decline of muscle health with aging, showing an increase in aggregate harmful proteins within our body can be linked to autophagy pathways being interrupted through time.

Exercise

Many studies show exercise is a promotional method of healthy aging [1,2], particularly affecting the skeletal muscle [2]. Exercise induces expression and activates the function of Transcription Factor E-B[16], a master regulator of protein homeostasis (proteostasis) and cellular catabolism, in skeletal muscle, increasing autophagy and lysosomal biogenesis[4]. Exercise is also powerful behavioral intervention against central nervous system (CNS) aging and plays an essential role in maintaining healthy neurocognitive function and immune metabolism in the aging brain[1,3,6]. Physical activity is a positive regulator of hippocampal plasticity, stimulating adult neurogenesis, increasing levels of Brain-Derived Neurotrophic Factor (BDNF), and ultimately preserving neurocognitive function in the aging CNS[1–3,7,9]. Moreover, physical activity decreases the risk of developing neurodegenerative diseases, such as Alzheimer's Disease[1], Parkinson's Disease[9,17], and Huntington's Disease[18], and is associated with better prognosis, and positively affects cognitive function in neurodegenerative patients[2,7,8,17,18]. Exercise being known as any activity requiring physical effort which is used to sustain or improve physical and mental health, heavily affecting skeletal muscle. This way of working on the muscle is causing not only a local effect but also a systemic effect. This interaction is causing a release of a host of myokines that target receptors located throughout the body to perform a biological function, that being to decrease inflammation. Myokines, factors secreted from skeletal muscle function to provide a path to communicate with neighboring organs and initiate a biological response[19], have been identified with the improvement of cognition and many growth effects in the body[19].

There are two main types of exercise, one being endurance training, and the other being resistance training. Given the potent activation of autophagy and TFEB signaling in skeletal muscle during endurance exercise, this presents an interesting opportunity to investigate its role in the anti-inflammatory effects of exercise.

Autophagy

Autophagy is an important process of maintaining cellular balance through recycling degraded and harmful material, such as misfolded or aggregated proteins[20]. Autophagy is known to be a conserved intracellular process that captures and transports cargo, that being proteins and/or organelles, to lysosomes for degradation to occur[21,22]. For this process to initiate, there must be a stimulus of low cellular nutrient levels or low energy levels, which then will recruit Class III PI3K complex's to generate phosphatidylinositol 3-phosphate (PI3P) for the formation of the autophagosomal phagophore at the autophagic cargo site[21,22]. After the consumption of energy, the phagophore will engulf the cargo and deliver this to the lysosome for degradation[21]. With this, we know that autophagy features seem to deteriorate through aging, and along with this comes the decrease of functional autophagic genes needed to mediate lifespan extension[21]. Additionally, the decline of autophagy with age has demonstrated to be an intense risk for developing neurodegenerative diseases due to dysregulation in theupkeep of proteostasis and accrual of proteotoxicity over time[21]. A factor that is known to be heavily involved in the autophagy and lysosomal biogenesis pathway is known as TFEB or Transcription Factor E-B. Although little is known about the response of TFEB through aging, we created a transgenic mouse model that will over-express TFEB in the

skeletal muscle to observe the effects that it has on the health of the organism through its life.

TFEB overview

Transcription Factor E-B (TFEB), a master regulator not being acted upon by other gene regulatory elements[23], is an important factor that plays an essential role in the regulation of basic cellular processes, including lysosomal biogenesis which in turn affects autophagy[4]. This transcription factor is known to be in the microphthalmia family (MiT family), which has been shown to have important interactions with cellular process including affecting metabolic pathways and is affected through high stress situations or metabolically demanding states such as exercise[4]. The resting state of TFEB is located in the cytoplasm, where it remains phosphorylated and awaiting a stimulus to become active[4]. Once dephosphorylated by a stressed condition, TFEB then will translocate to the nucleus, where there it will bind and induct a transcriptional change[4]. Lysosomes are crucial components to maintaining cellular homeostasis by handling and recycling misshaped proteins and damaged cellular materials, they are known for this work[15]. Assisting the lysosome, an autophagosome, is sent to collect the damaged or harmful waste and return it to the lysosome where it can be broken down either for degradation or recycling the energy. With TFEB being heavily involved in autophagy processes and lysosomal biogenesis, this provides an opportunity to study the effects of TFEB over-expression on an aging model to discover what transcriptional changes are occurring through time.

Transgenic mouse model

Usage of the mouse model organism has been utilized since the early 1900s when it was first deemed advantageous to use due to its short generation time, comparatively large litters, ease of husbandry, and phenotypic variants[24]. The mice themselves are easy to alter genotypically, allowing the use of this model organism in many kinds of research, answering questions at different areas of interest. To do this, the development of a transgenic mouse model is needed. This is where the mouse has had its genome altered, allowing researchers to study gene functions and outcomes. To generate this TFEB over-expression, we generated mice with the β-actin promoter in combination with the CMV enhancer and with a floxed 3x-FLAG-eGFP STOP cassette placed ahead of a 3x-FLAG-human TFEB transgene, allowing for expression of TFEB in the presence of a Cre-recombinase **(Figure 1)**. To maintain TFEB expression strictly to skeletal muscle, we crossed fxSTOP-TFEB mice with Human Skeletal Actin (HSA)-Cre mice to achieve a widespread expression of Cre-recombinase in myogenic cells[25].

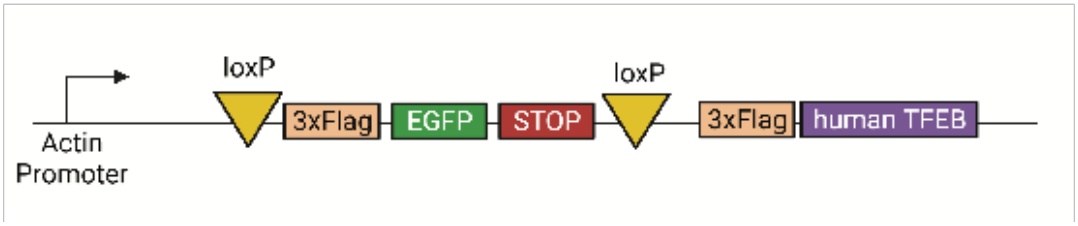


Figure 1: Diagram of floxSTOP-HumanTFEB Vector

Hypothesis

Exercise is known to initiate a cascade of protective and beneficial effects on the organism, especially on the function of the central nervous system through aging

timepoints. Skeletal muscle, being one of the primary respondents to exercise, manages these events by releasing a host of cytokines that are influenced by exercise. During physical activity, we see an increase in a lysosomal biogenesis regulator known as TFEB in skeletal muscle, which increases lysosomal activity thus enhancing autophagic pathways. By generating a transgenic mouse model that moderately over-expresses TFEB in the skeletal muscle, we are able to see similar like effects of exercise on a sedentary organism. However, to date, our understanding of the mechanisms underlying these geroprotective effects of exercise on the aging central nervous system remains poorly understood. **I hypothesize that changes in skeletal muscle-originating circulatory factors will be similar between our sedentary cTFEB;HSACre mice and wild-type voluntary running (exercised) mice.**

METHODS

Mouse collection/tissue processing

All mice are from Jackson Laboratory in the strain line C57BL6/BJ, also known as the B6 mice. The mice used for this study have been previously aged to twenty to twenty-four months of age, which represents a time point similar to a human individual around seventy-years-old. At this point in time, the mice have been able to reach maturity and present with any aging phenotypes that we are interested in. For the young cohort of mice, the mice are aged to four months before collection, which would represent a young adult ranging around twenty-four years of age. From both aforementioned cohorts, the mice are injected with 1.0ml of Avertin (anesthesia), before collections begin.

Figure 2 is an overview of how blood is collected and processed from the mice used in the study. The blood is collected via cardiac puncture, from the left ventricle. This provides the means to collect whole blood, then process the blood into serum and examine circulatory factors within the blood. Extracting serum from whole blood, I utilize a 4° centrifuge and spin the sample down, after allowing the whole blood to coagulate at room temperature for fifty minutes. Spinning down the sample at 1400 RPM allows the separation of layers to occur, presenting the clear serum layer at the top of the tube. Upon collecting the serum, I will proceed with examining the quality of the serum by observing the color. If the serum presents itself as a clear or yellow color, then the serum is deemed usable. If the serum is a bright red, then this is a sign that the red blood

cells have lysed within the serum, which can lead to a disruption in the reading of proteins in the sample, so the sample is deemed unusable.

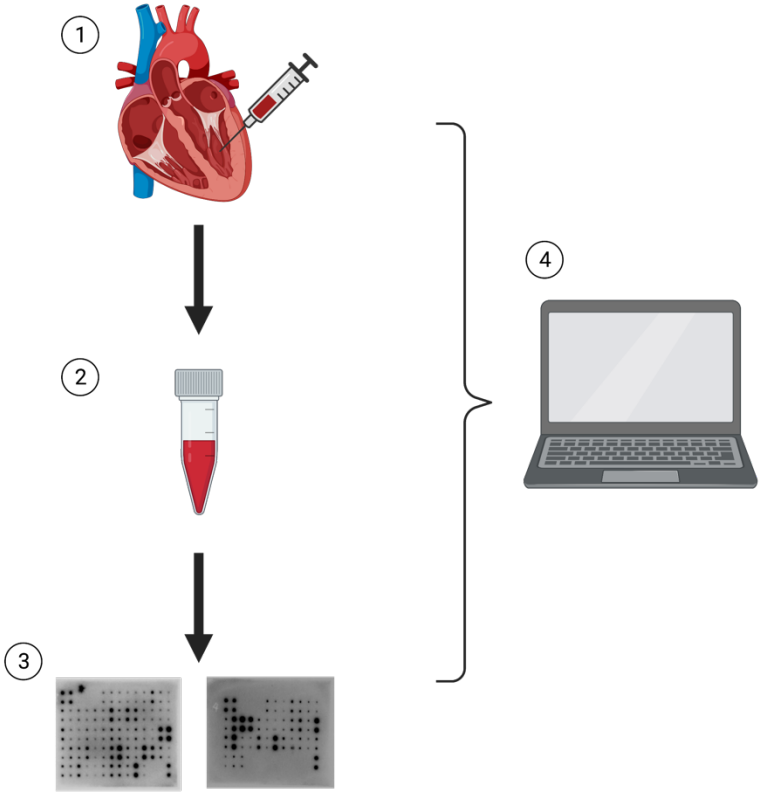


Figure 2: Overview of collection of whole blood from the left ventricle of hearts. Mice are knocked out using avertin, and during the collection process, **(1)** a 22g needle is used to collect whole blood from. **(2)** The blood is then allowed to coagulate at room temperature for 50 minutes. **(3)** Serum collected after coagulation is then placed directly onto cytokine array for downstream analysis **(4)**.

Figure 3, an overview of how the muscle is used for cytokine analysis. The next step is collecting the quadriceps muscle, which I will be using to extract protein. The extraction process begins by cutting a piece of tissue that ways 50 mg and placing that into RIPA buffer and a protease inhibitor and then lysing the tissue down into a liquid state. Following this, the sample must be spun down at 4C at 15000 rcf for a total of 2 minutes. This would allow the separation of layers and you can proceed with extracting

the protein lysate after this step. I will then look to see the quantity of the protein in the sample by performing a BCA protein assay analysis. This involves taking the entire tissue collected and extracting a piece that is ~50µg in weight and adding that to a total RIPA buffer solution, which is prepared with a protease inhibitor, after finding the sample has enough protein to use, I am able to introduce the sample directly onto the cytokine array itself for quantification of 96 specific cytokines known to deal heavily with inflammation and immune response changes.

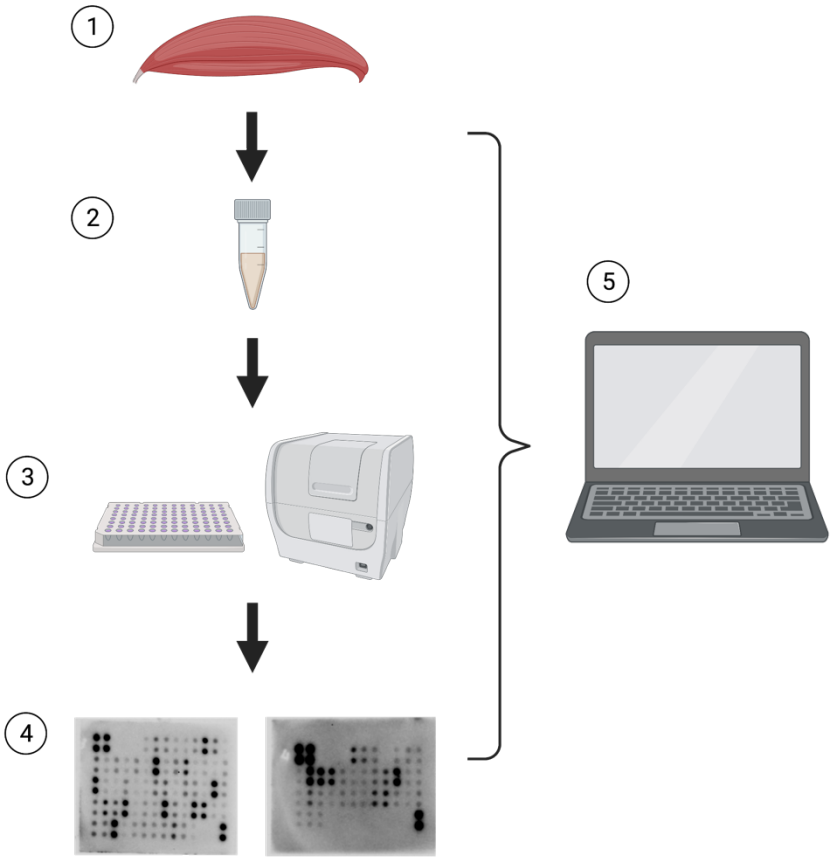


Figure 3: Overview of how the Quadricep muscle is used for analysis. **(1)** Quadricep muscle is collected after perfusion, and **(2)** turned into a muscle protein lysate. **(3)** Once the lysate has been formed, it is run through a BCA analysis to quantify how much protein is present. **(4)** After protein levels have been quantified, they are directly administered to the cytokine array and used for downstream analysis **(5)**.

Exercise protocol

Mice, young at three months of age and older at twenty months of age, were singly housed with access to a free-running wheel for 6 weeks. The wheel will count every time that it makes a complete rotation allowing us to keep track of how long they are running and how far they have run. The mice were separated by sex and age, having a total of three exercised young males and females to compare to sedentary control and sedentary cTFEB;HSACre mice. Following this, for the aged cohort, there is a total of four female mice that are being used, all of which are control mice. The tissue readily used in my study was protein muscle lysate from the young male and female, exercised cohort, which I used to compare protein expression changes to sedentary cTFEB;HSACre, and sedentary control mice. Further analysis of the aged cohort will begin when the tissues are collected from the running mice. This provides a good baseline for what we could expect to see when observing young expression changes to that of age with the introduction of exercise to the mice.

Cytokine array

Utilizing a cytokine array that is composed of 96 specific targets that are known to deal heavily with inflammation and immune system responses, I will be able to cultivate a list of targets to pursue as potential drug therapeutics that provide the protective and beneficial effects of exercise, without having the organism perform any physical activity. Use of this kit is beneficial to observe changes in many different kinds of proteins that all are known to deal with inflammation, immune, and growth responses within an organism. The cytokine array from ABCAM (ab193659) is able to utilize many biological liquid samples, two of which include serum and muscle protein lysate.

Introduction of the sample begins by diluting the total volume of the serum gathered down with the diluent buffer provided from the kit. Taking a total of 120µl of serum and mixing that with 1,080µl of 1X blocking buffer will give a total of 1.2ml of sample to introduce directly onto the membrane. This will sit at room temperature for an hour and a half rotating, this allows the proteins to distribute among the membrane. Then utilizing the washing buffer, we will wash the membranes three times with wash buffer 1, at a concentration of 1X, followed with two washes from wash buffer 2, at a 1X concentration. Following this, introduction of the primary of 1X Biotinylated Antibody Cocktail. After introduction of the primary directly onto the array, I will then wash and apply the secondary for a two-hour incubation at room temperature. Floating in the sample, while the rest of the material is washed away. Then place a total of 500µl of Detection Buffer, a 1:1 working dilution of Detection buffer D and C and allow it to incubate at room temperature for 2 minutes. **Figure 4** demonstrates what a cytokine array looks like after the sample has been placed onto the array, followed up by quantification of the dots on the array.

Image processing

Examining each dot on the cytokine array membrane requires the use of statistical software known as FIJI (Just ImageJ). Using this program, I am able to apply an add-on software that lays a grid out of what the array should look like and matches each dot to the grid that is made. Upon doing this, the reading will give me a value of how intense the pixels are in the given circle. All images of arrays will be imported into the imaging software called FIJI (Just ImageJ)[26] where I will quantify, using densitometry, how intense each dot on the array is presenting itself. This method is done by reading the pixel

strength on the image and providing a real value from the intensity. The individual data points are then normalized to the positive control located on each array and then also normalized to our 'baseline' control, which we determined as the sedentary male young mice. Visualization occurs using Prism GraphPad (version 9.4.0) and R (version 4.1.2). There I test for significant differences among expression values using a one-way ANOVA followed by independent post-hoc t-tests, looking for differences in the means of all groups.

Statistical analysis

Receiving the raw values, I am able to export the information into Microsoft Excel (version 16.62 2206100) to normalize the data to the positive controls by averaging the positive controls from the individual array together and then dividing the raw data values by the now averaged positive controls. Once all of the samples have been normalized to their array's positive control, I then generate a control strain line by averaging all of the normalized positive control values together. Using this now generated control strain line, I will divide the previously generated positive control normalized values by the newly generated control strain line to obtain the values that are now normalized to both the positive control and the control strain line. After that, I was able to import the data, after normalization has occurred, into GraphPad Prism (version 9.4.0) [27] and R (version 4.1.2)[28]. GraphPad Prism (version 9.4.0) is statistical software that is used commonly throughout the scientific community in regard to visualizing data and testing for significant differences in many ways. Particularly for my thesis, I utilized this software to test for significance among differentially expressed proteins in 3 different groups. Those groups, mentioned before, are control sedentary mice, cTFEB;HSACre

sedentary mice, and exercised control mice. All of which are at two different aged time-points, young being from three to four months of age and older adult mice being from twenty to twenty-four months of age. To categorize differentially expressed proteins between two groups, I performed an independent t-test, which is used to test for significant differences among the means of two groups that were not collected at the same time or day. Then when wanting to compare the same cytokine expression between the three groups together, I used an ANOVA analysis. An ANOVA test allows for the comparison of means between 3 or more groups, to test for any relationship between the sample groups. Upon doing so, I am able to determine which cytokines are being affected by the presence of TFEB over-expression in the skeletal muscle and can confidently suggest that by having TFEB over-expression in the skeletal muscle, the inflammatory and growth markers are being restored through aging time points compared to a control sedentary individual. Post-hoc analysis was performed using a two-stage set-up Benjamini, Krieger, and Yekutieli exam. This method examines the distribution of P values to find the fraction of the null hypothesis that is true. Then it uses this knowledge to determine when the P-value is low enough to be called a discovery.

RESULTS

Serum expression

Examining the 96 cytokine assay targets, measuring the expression of the proteins from sedentary control, and cTFEB;HSACre sedentary (experimental group) I was able to generate a list of a few of the differentially expressed proteins found to be significantly different between the two tested groups. When examining this data, the serum results experienced a heavy load of expression variation within individuals in a group, making it difficult to detect significance between the two groups (p-value < 0.05), visualization of quantitative protein changes are seen using a heatmap in **Figure 4**. However, some markers came back significant between groups. **Figure 5** represents two of the cytokines, IL-1 Alpha and DTK, that were found to be significantly different between aged males (20 to 24 months) when comparing control sedentary mice and cTFEB;HSACre sedentary mice. IL-1 Alpha is among one of the pro-inflammatory markers on the cytokine array panel that is known to stimulate the activity of genes involved in inflammation and immunity. This protein being lower in the cTFEB;HSACre sedentary mice could indicate reasoning as to why the mice are experiencing more beneficial central nervous system effects than that compared to the control mice[29]. DTK, also known as a tyrosine kinase receptor, plays an important role in mediating cell-to-cell communications, helping to control a wide range of biological functions including cell growth, motility, differentiation, and metabolism.

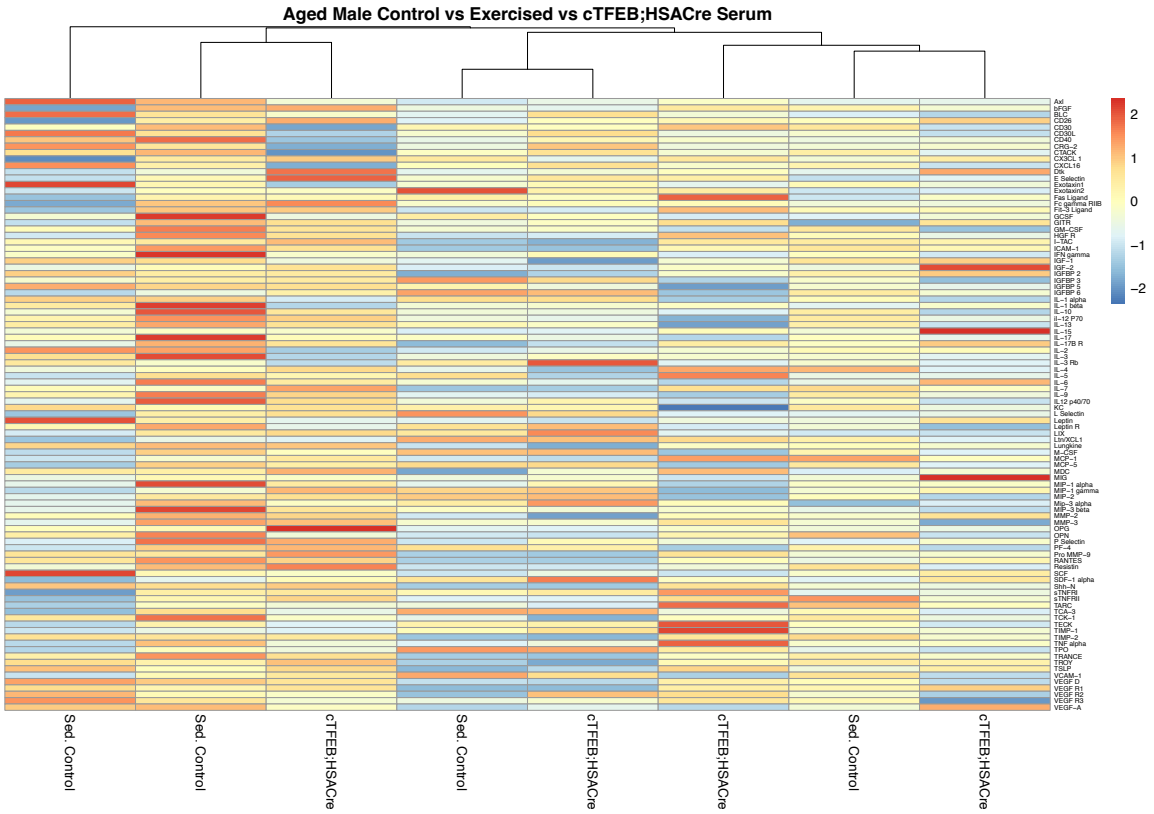


Figure 4: Quantitative protein expression changes in aged male serum, both sedentary control and sedentary cTFEB;HSACre mice, with an n = 4 per group. Heatmap generated with R package called pheatmap, version 1.0.12.

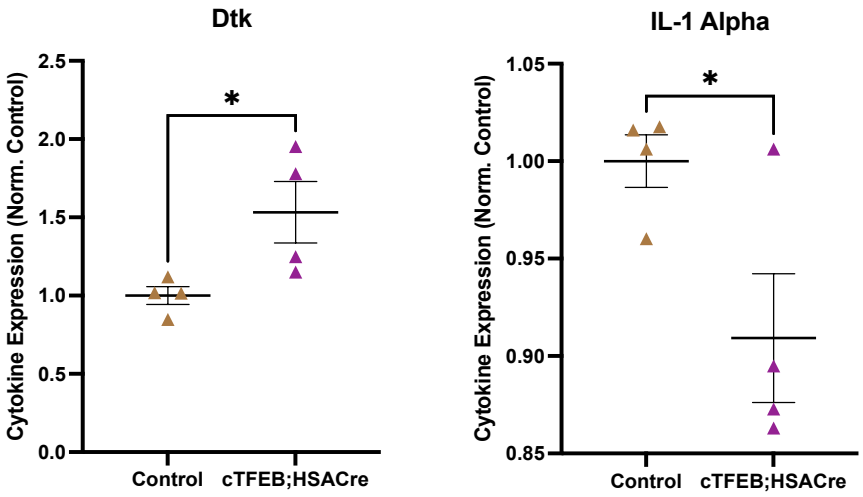


Figure 5: Overview of cytokine array for Aged Male Serum Analysis. From the analysis of 20–24-month-old male mice serum when normalized to control sedentary aged-matched males, two cytokines came to be significantly different from the groups when being compared. IL-1 Alpha, p-value < 0.05, and Dtk, p-value < 0.05. Control expression value is at 1, anything below 1 is decreased and anything above 1 is increased in cTFEB;HSACre mice.

Muscle lysate analysis

Analysis of the factors that are being altered in the skeletal muscle follows a similar protocol to that of the serum. For this, collection of the quadriceps muscle is required from all samples and is turned into a protein lysate. After normalization to 50µg of protein, I then placed the sample directly onto the array for better visualization of the proteins in the sample. Muscle lysates were isolated from sedentary control mice and sedentary cTFEB;HSACre mice at different time points (young and aged) mentioned before and including both males and females. When examining aged males' protein expression **(Figure 6)**, using a heatmap generated with the pheatmap R package (version 1.0.12) with an n = 4 per group, in the sedentary control group and the sedentary cTFEB;HSACre group, there were multiple cytokines that came up to be differentially expressed. Demonstrating that with the influence of TFEB over-expression in the skeletal muscle, there is a change on the expression of immune and inflammatory markers being regulated.

Upon examination of the cytokine array output, I was able to find significantly differentially expressed proteins between the two groups **Figure 7**. Comparison between the control sedentary mice and the cTFEB;HSACre sedentary mice was used and confirmed that with the over-expression of TFEB in the skeletal muscle, there was a change in expression of pro-inflammatory markers, such as IL-15[30], Shh-N, OPG, IGF-2, and PF4.

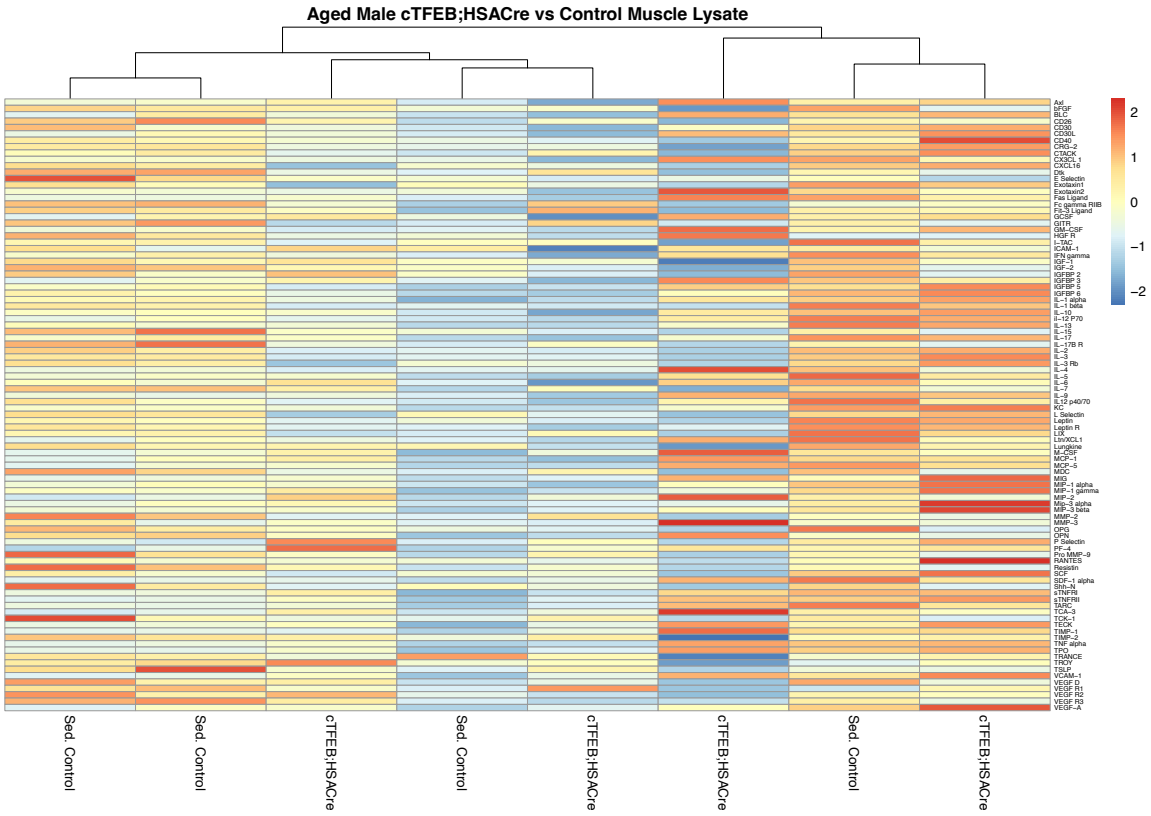


Figure 6: Quantitative protein expression changes, in aged males both sedentary control and sedentary cTFEB;HSACre, found using the pheatmap function in R, version 1.0.12. Proteins that are higher in the sample come back as red, whereas if the protein is lower in the sample the color will be blue.

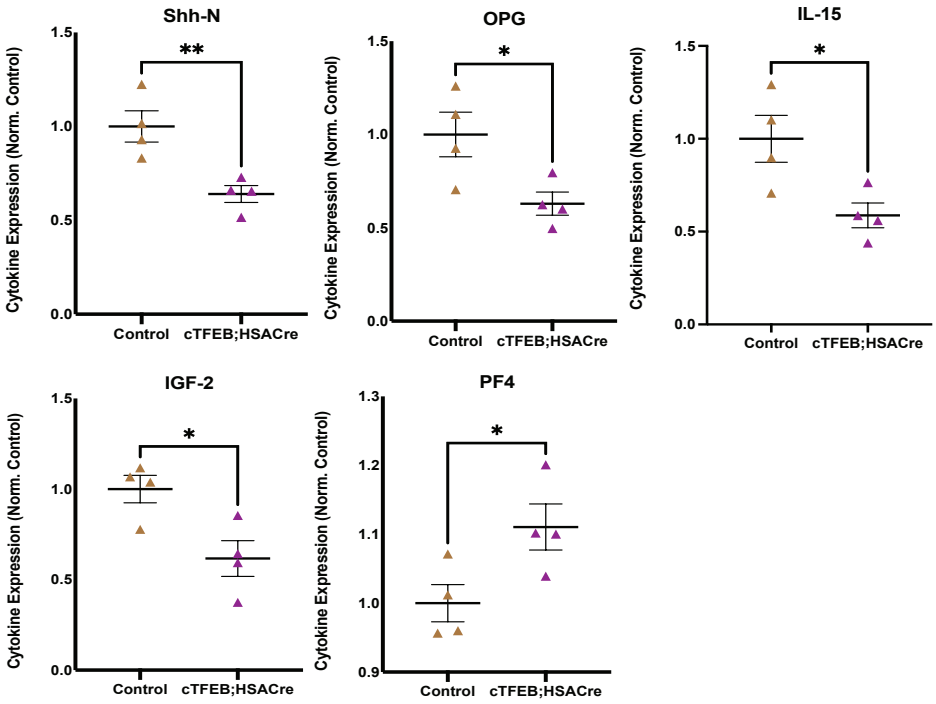


Figure 7: Aged Males, 20 to 24 months-of-age, comparison from control sedentary to cTFEB;HSACre sedentary groups, n = 4 per group, p-value < 0.05. Tissue used is quadricep protein lysate. All data normalized to sed. control mice with an expression of 1. Anything ranging below 1 is decreased and above is increased.

I next performed an analysis of aged female lysate starting with a heatmap to visualize any quantitative changes in protein expression **(Figure 8)**, between the sedentary controls and sedentary cTFEB;HSACre mice with four samples per group. There is a total of seven cytokines that came out to be significantly different between the two groups **(Figure 9)**. This is demonstrating that with the influence of TFEB over-expression in the skeletal muscle, we are able to see a change happening with what immune and inflammation markers are being expressed. Cytokines that came out to be

significant in the aged female protein lysates are strongly related to immune system responses, and responsible for inflammation responses and prevention. Surprisingly we see differentially expressed proteins with TFEB over-expression between the muscles of aged males and females.

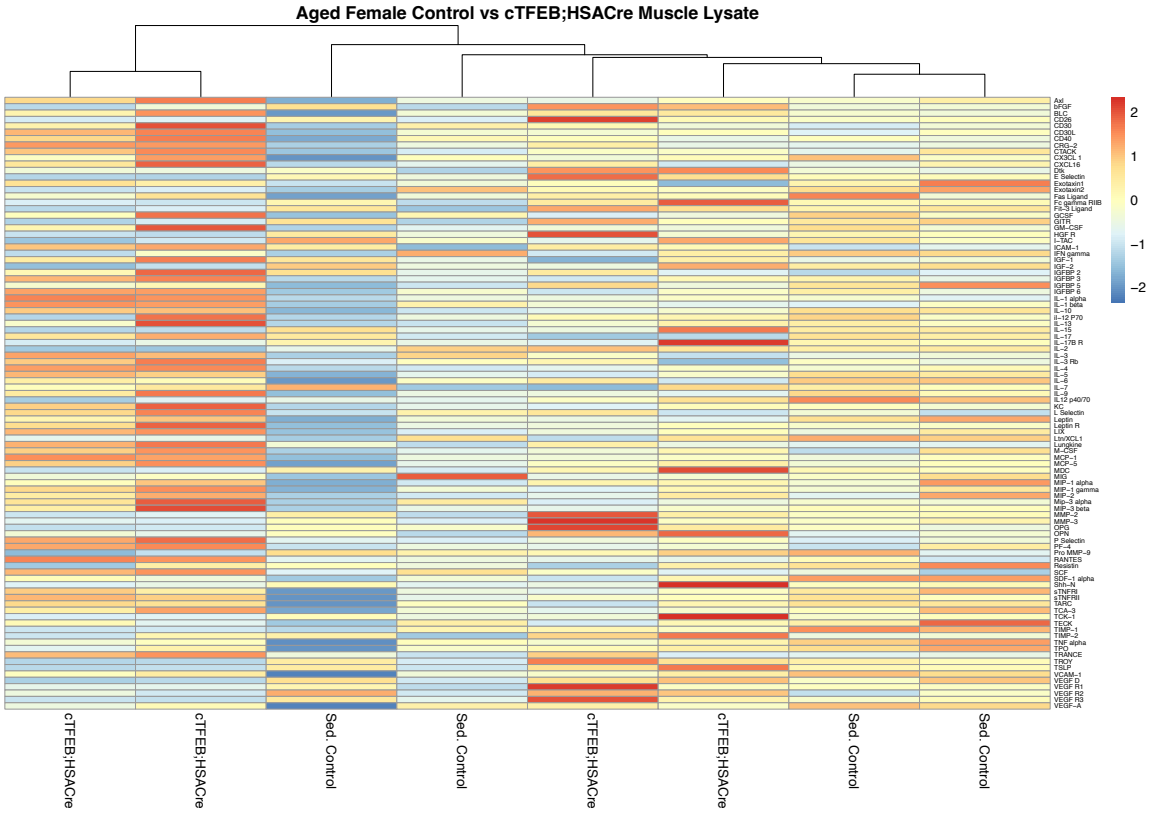


Figure 8: Figure 5: Quantitative protein expression changes, in aged females both sedentary control and sedentary cTFEB;HSACre, found using the pheatmap function in R, version 1.0.12. Proteins that are higher in the sample come back as red, whereas if the protein

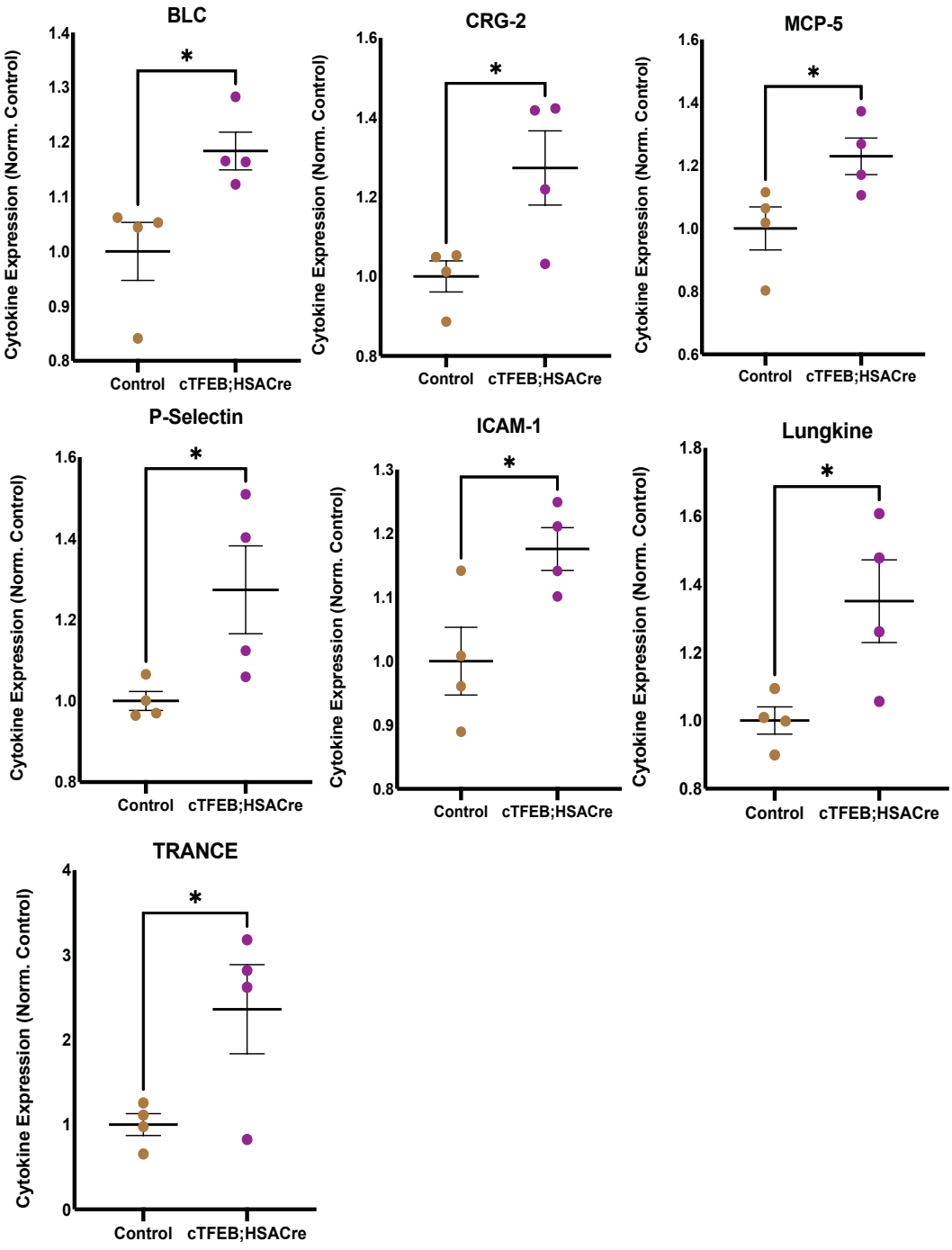


Figure 9: Aged Female Muscle Protein Lysate Cytokine Expression Analysis. All data is normalized to sed. control mice with an expression value of 1. With an n = 4, and performing an independent t-test for each individual cytokine, six came up as significantly different between the two groups, control and cTFEB;HSACre. P-value < 0.05.

After completion of the analysis of the aged cohort, I was able to generate a list of potential factors to pursue as protective therapeutics from inflammation and immune system responses during aging. I then analyzed the younger cohort of mice, aging from three to six months of age, including both males and females from sedentary control mice, sedentary cTFEB;HSACre mice, and an additional exercised control mouse cohort. This data was gathered from the quadriceps muscle protein lysate to compare the effects of TFEB over-expression in the skeletal muscle of sedentary mice to an exercise intervention in control mice. Starting with the young male mice from the aforementioned groups, I was able to see a visual change in cytokine expression from being a control sedentary mouse to having TFEB over-expressed in the muscle or mice being exposed to exercise interventions.

Our results confirm that exercise is a powerful regulator of inflammatory and immune responses. With exercise interventions, there is a fluctuation of protein levels of known inflammatory markers in the samples used, including that of the interleukin family and also growth factors like the TNF families. I am able to see outcomes that TFEB is potentially leading to a similar state, providing similar gene expression levels in a sedentary cTFEB;HSACre mouse to that of an exercised control mouse. **Figure 10** shows the overall expression of proteins in the form of a heatmap after normalized to control sedentary mice of young male mice. Following this, in **Figure 11,** there are individual cytokines that come up significantly between the groups control sedentary, n = 4, and control exercised, n = 3, with a p-value ranging below 0.05.

There are a total of 33 cytokines that came up significantly different when performing an independent t-test on every single cytokine across the groups. All of which

promote signaling factors for communication and prevention of inflammatory states in the organism. **Figure 12** highlights significant factors that are being affected through the comparison of the sedentary control group, to that of the sedentary cTFEB;HSACre group, n = 4, and exercised control groups.

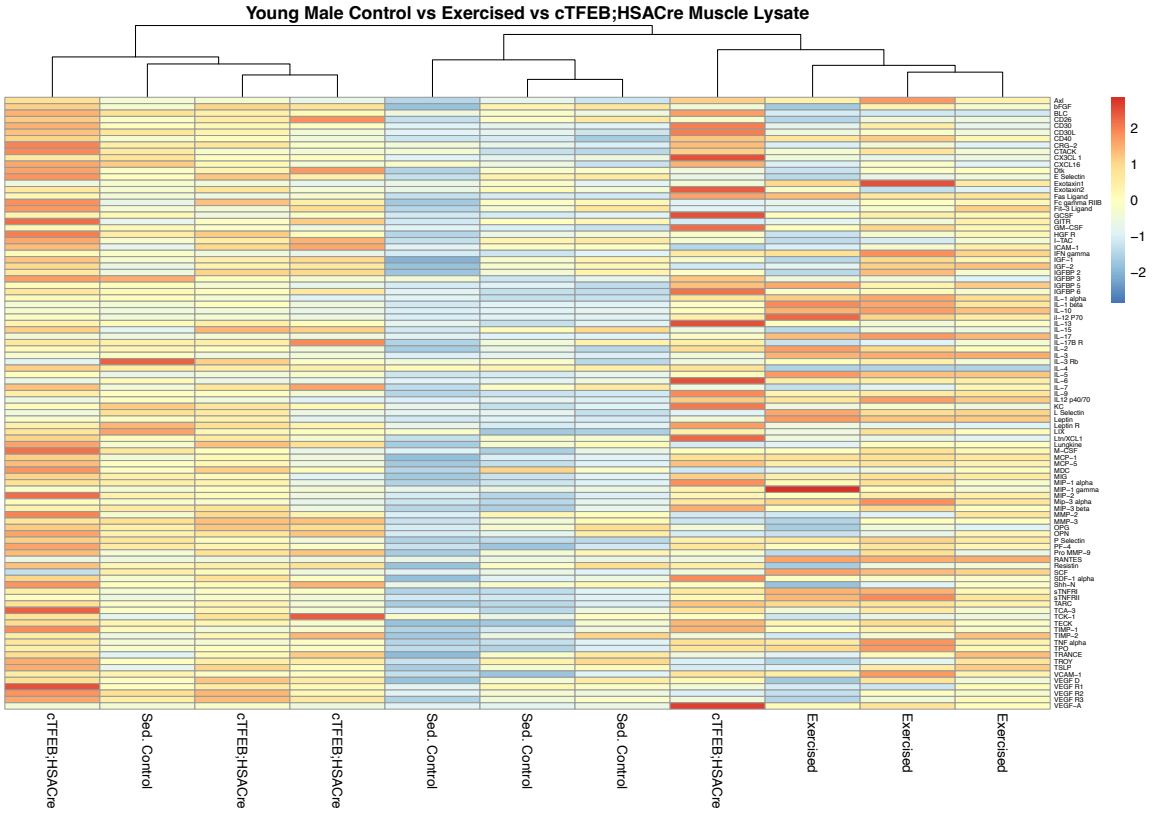
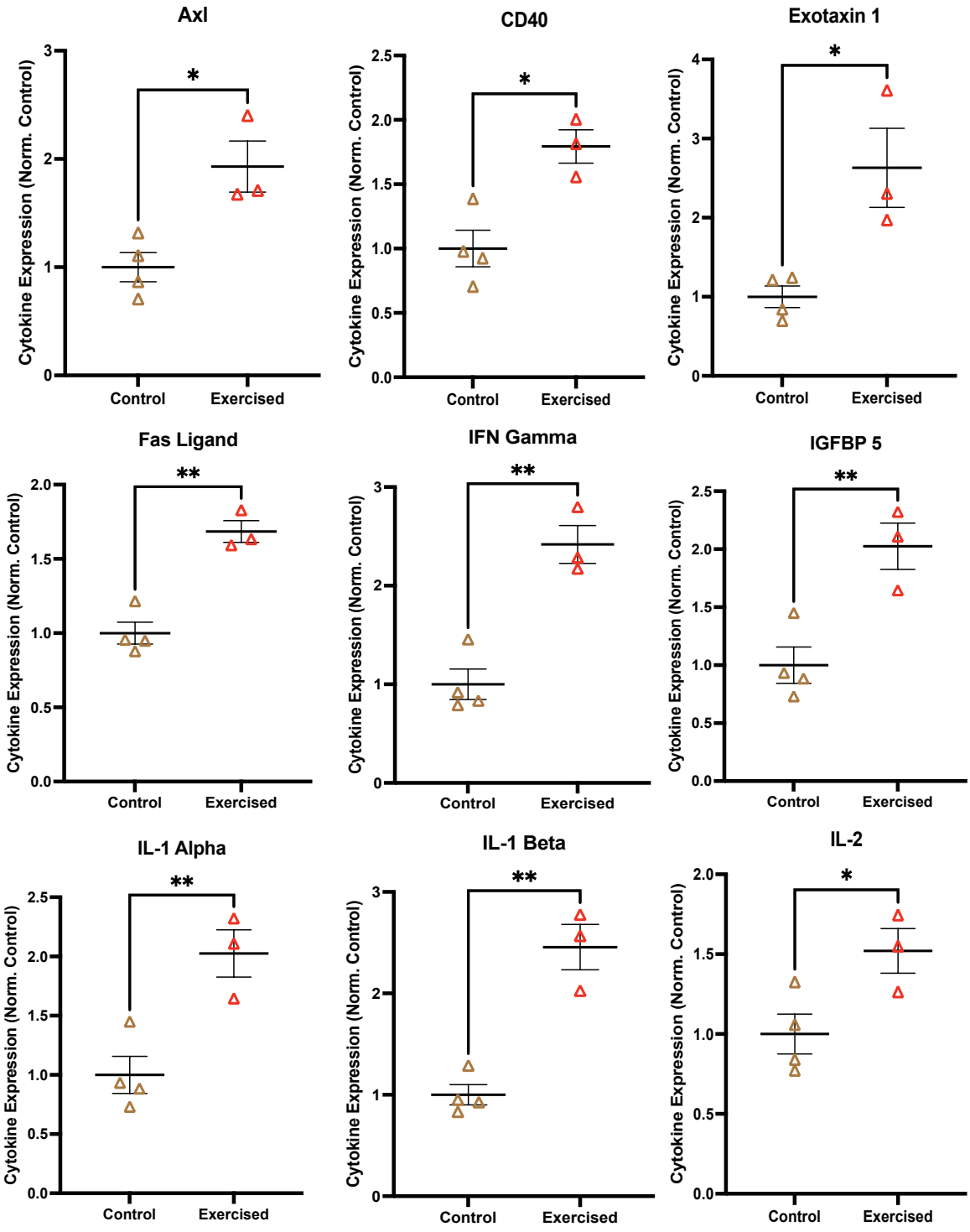


Figure 10: Overview of cytokine expression changes between sedentary cTFEB;HSACre mice and Exercised control mice young male mice. All data is normalized to sedentary control young males. Heatmap generated through the use of pheatmap package in R (1.0.12).


Axl
CD40
Exotaxin 1
Fas Ligand
IFN Gamma
IGFBP 5
IL-1 Alpha
IL-1 Beta
IL-2
Cytokine Expression (Norm. Control)
Control
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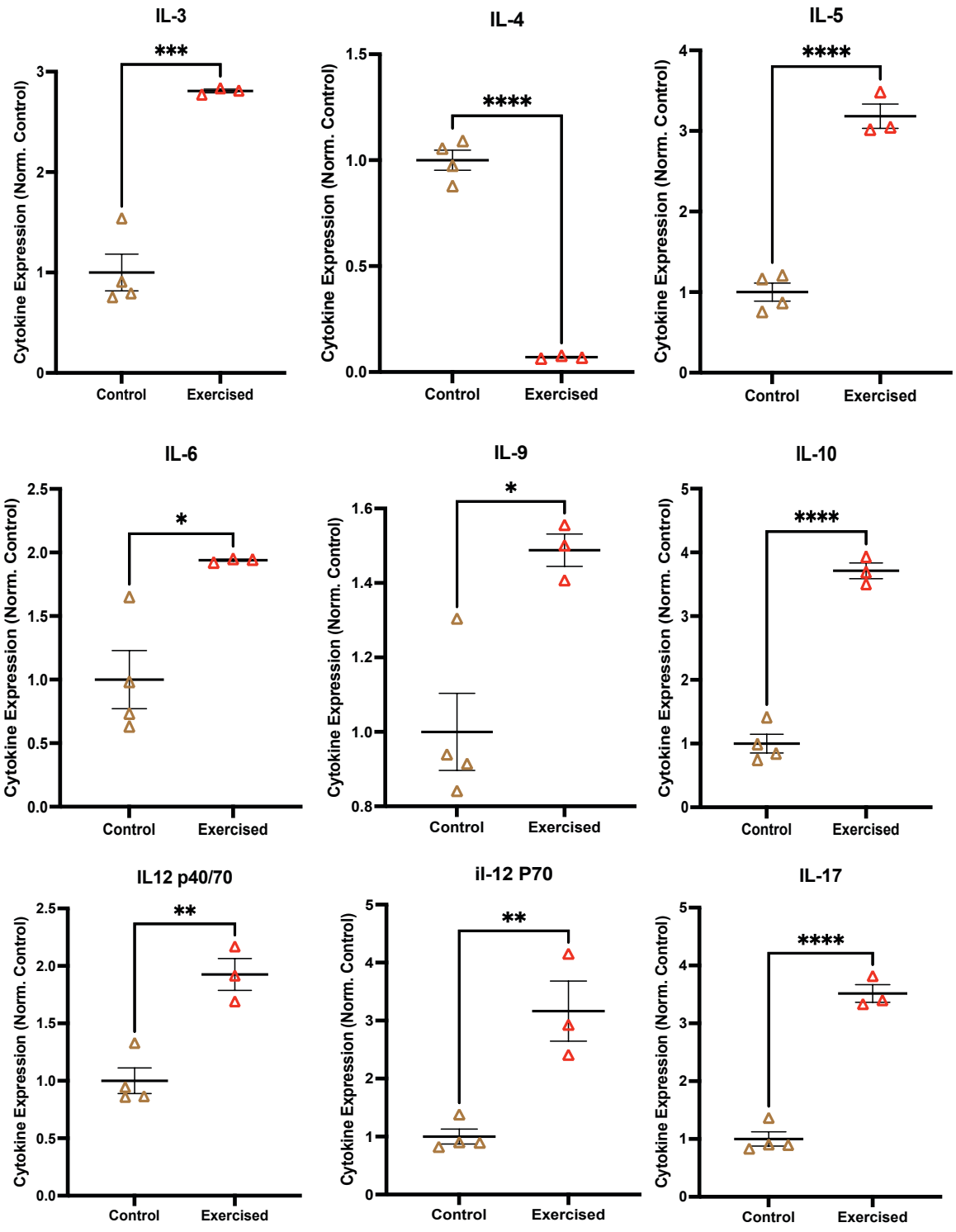
IL-3
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Cytokine Expression (Norm. Control)
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Exercised
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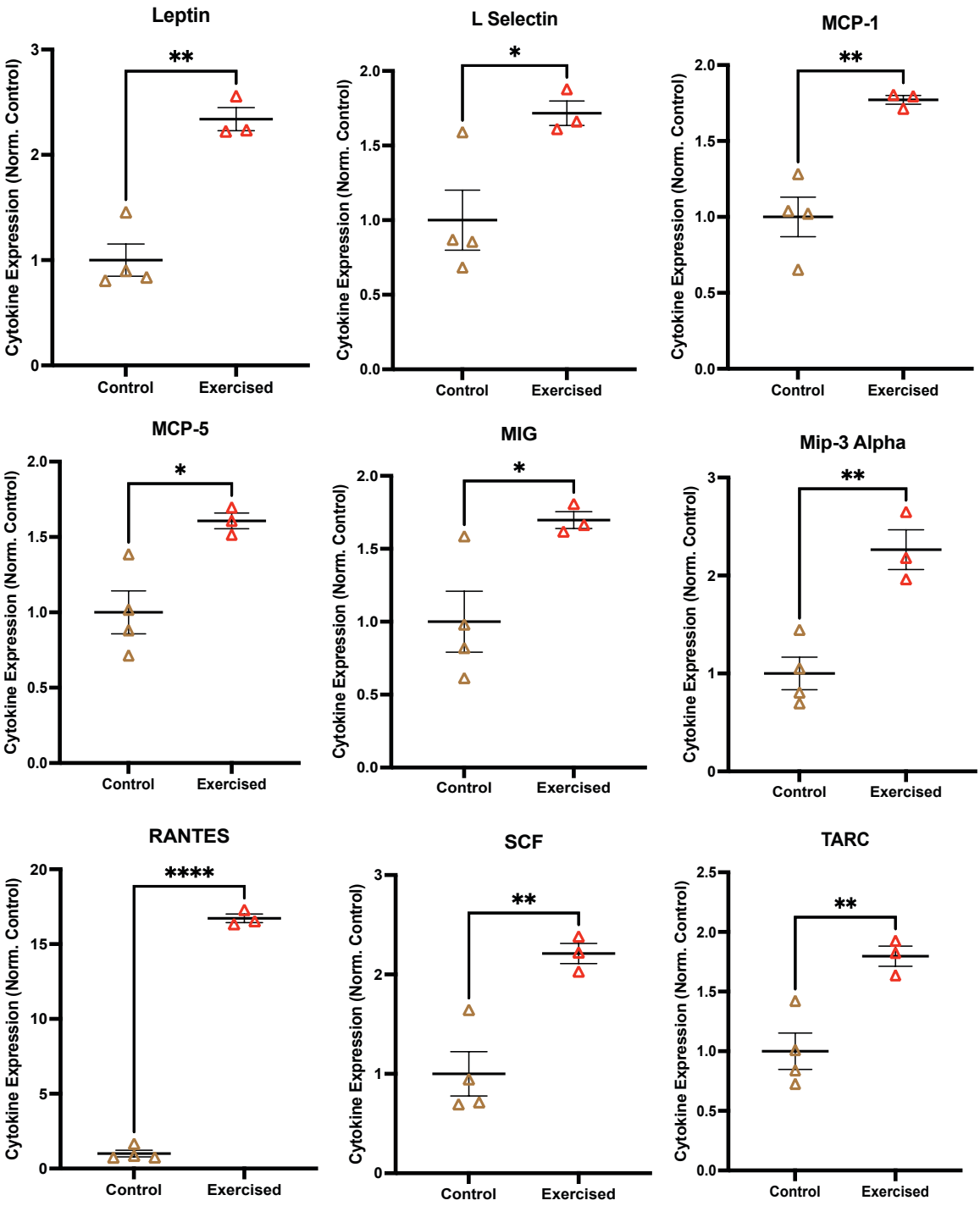
Cytokine Expression (Norm. Control)
Control
Exercised
IL12 p40/70
**
Cytokine Expression (Norm. Control)
Control
Exercised
il-12 P70
**
Cytokine Expression (Norm. Control)
Control
Exercised
IL-17
****
Cytokine Expression (Norm. Control)
Control
Exercised


Leptin
**
Cytokine Expression (Norm. Control)
3
2
1
0
Control
Exercised
L Selectin
*
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
Exercised
MCP-1
**
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
Exercised
MCP-5
*
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
Exercised
MIG
*
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
Exercised
Mip-3 Alpha
**
Cytokine Expression (Norm. Control)
3
2
1
0
Control
Exercised
RANTES
****
Cytokine Expression (Norm. Control)
20
15
10
5
0
Control
Exercised
SCF
**
Cytokine Expression (Norm. Control)
3
2
1
0
Control
Exercised
TARC
**
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Exercised

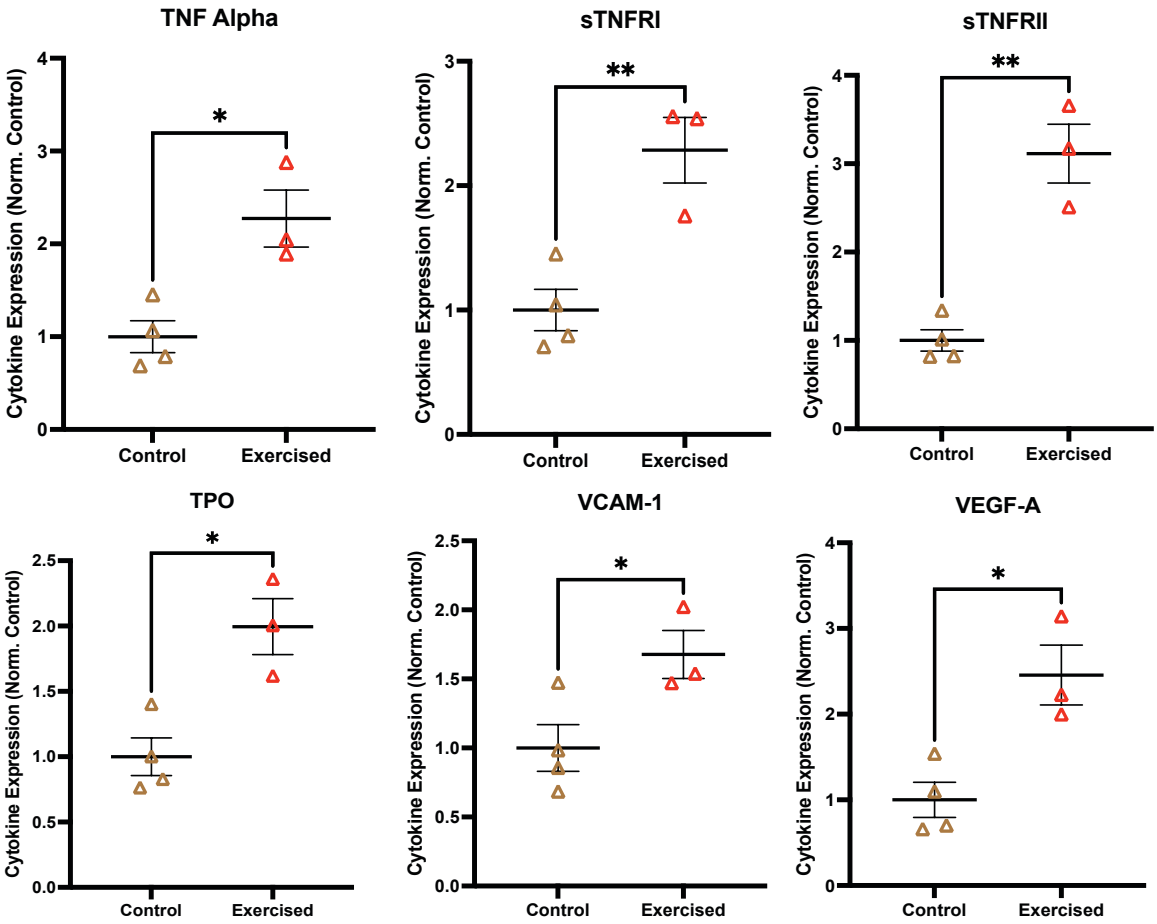
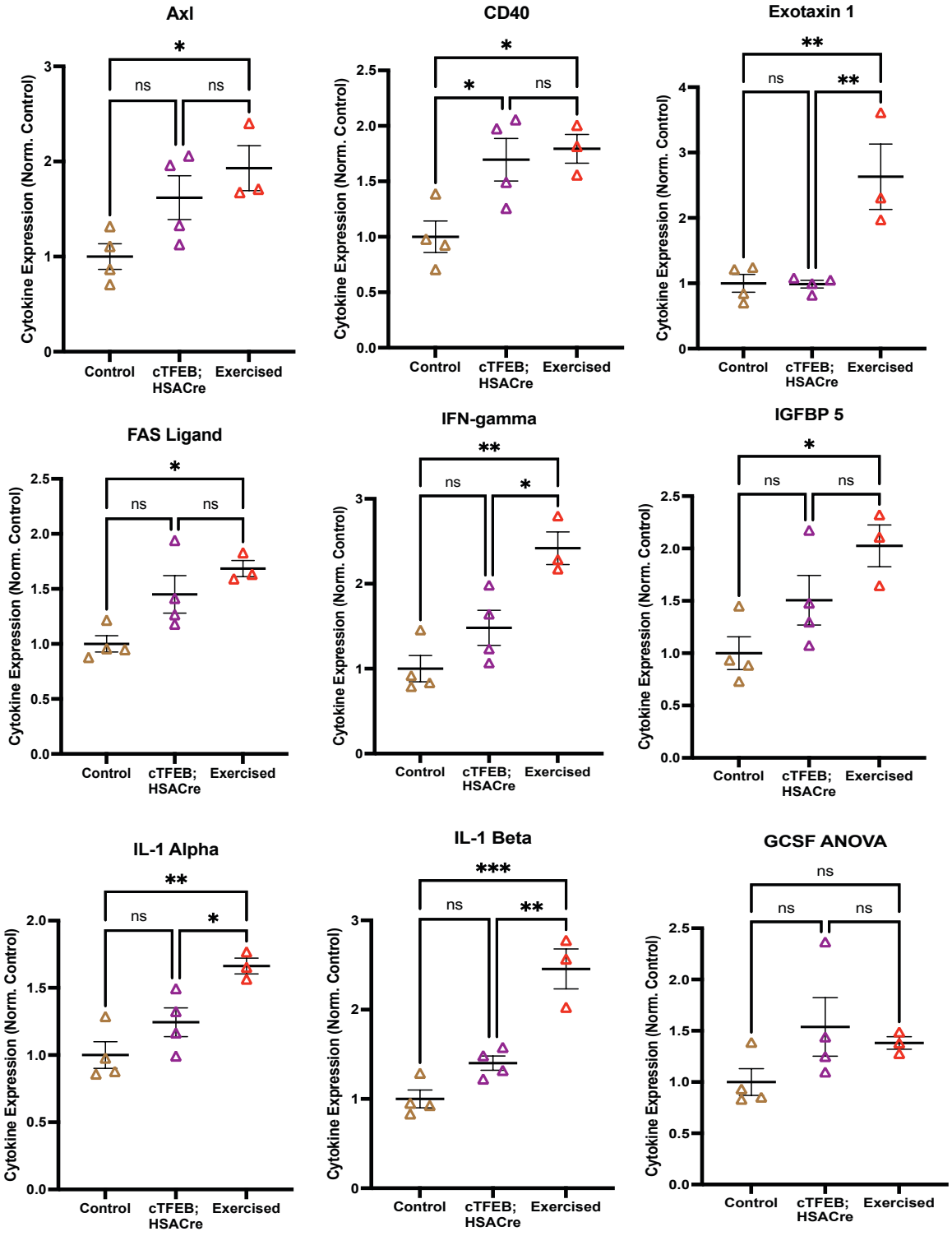
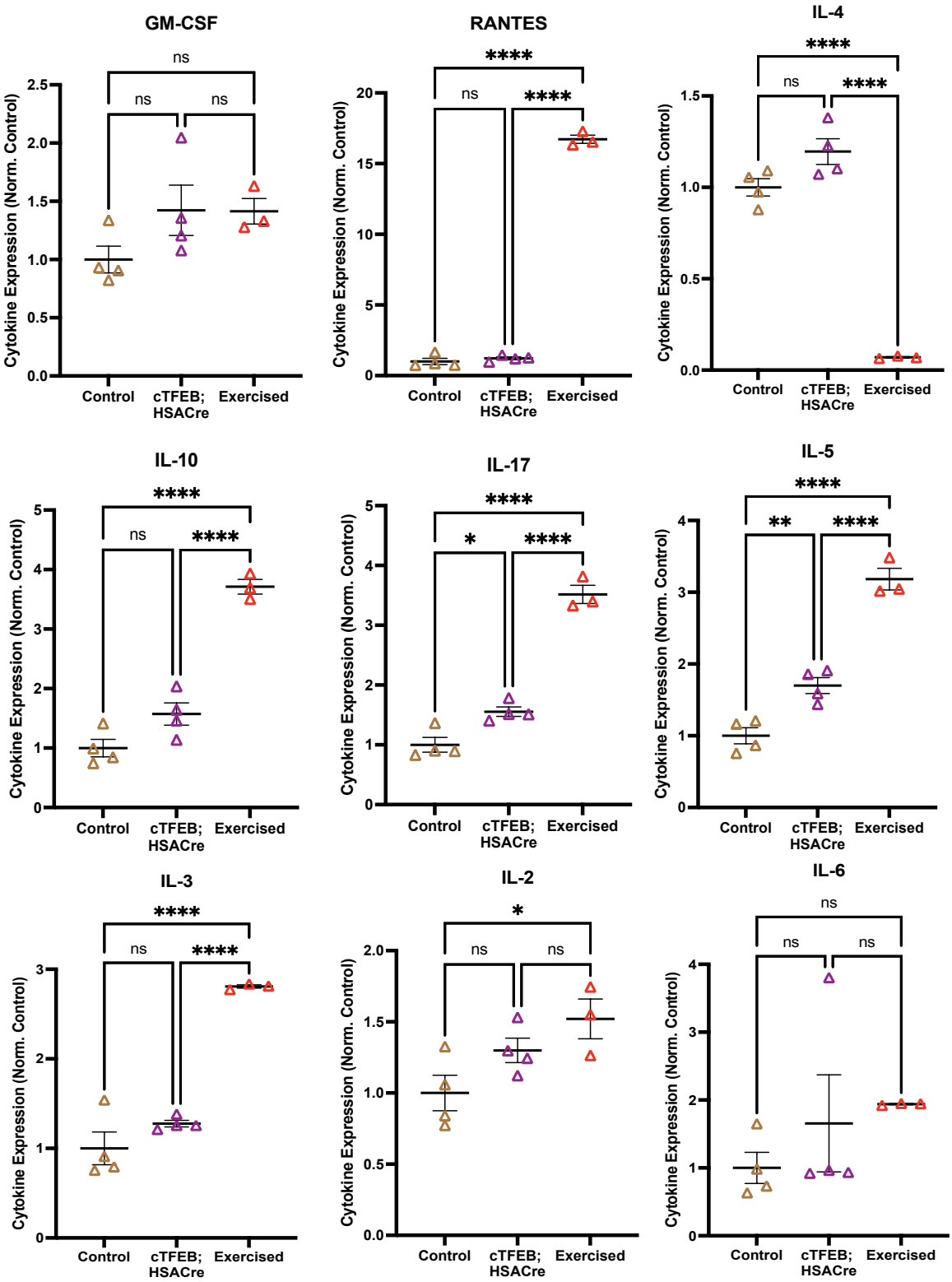
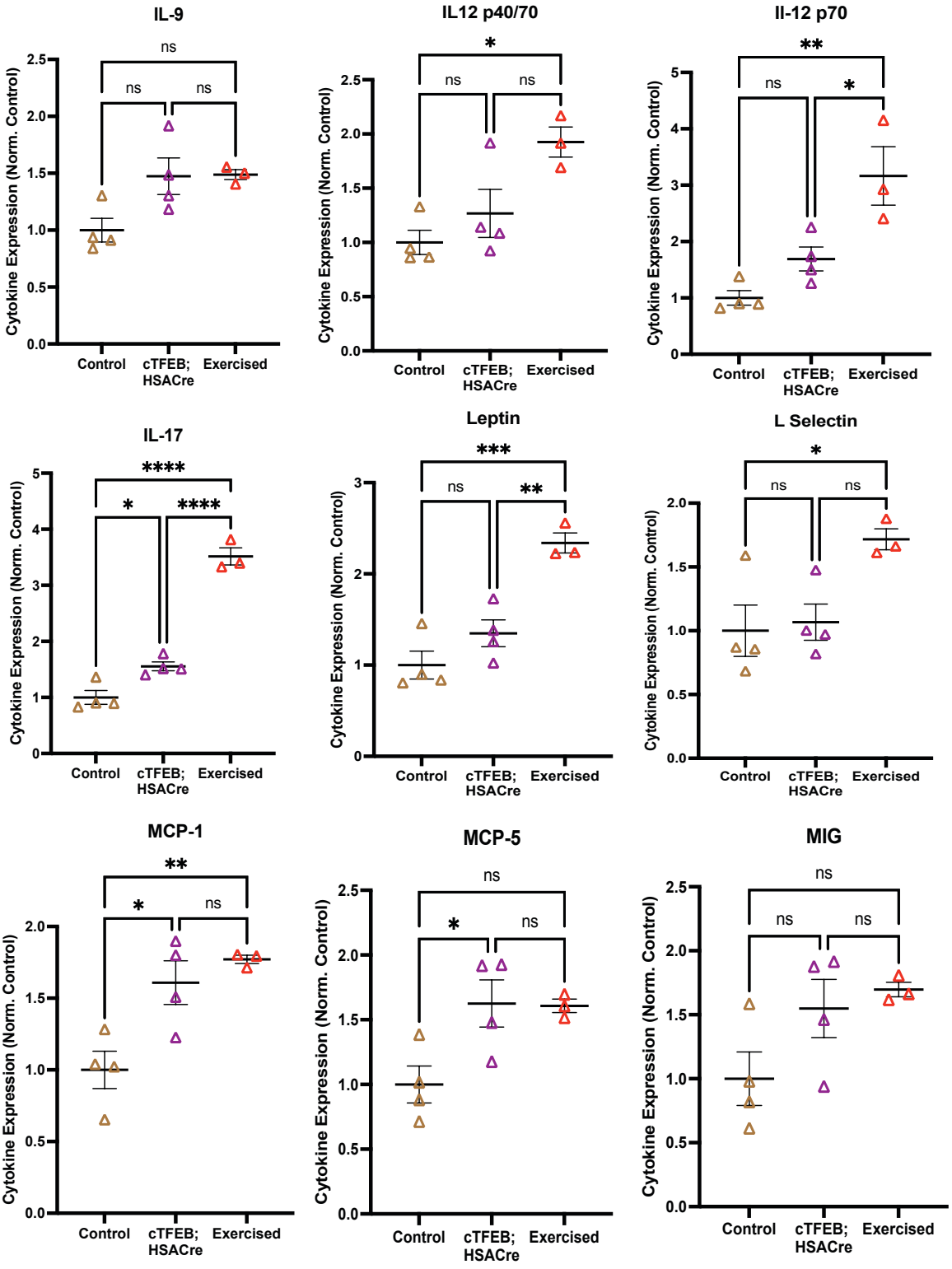


Figure 11: Cytokines that are significantly different between young male control sedentary mice (n = 4) and exercised control mice (n = 3). Data is normalized to the control sedentary mice, and we can see that with exercise, there is an interaction on pro-inflammatory markers causing them to be expressed differently than that of the control mice. Independent t-test was performed with a p-value < 0.05.

Upon performing the analysis of all three groups together, control sedentary, control exercised, and cTFEB;HSACre sedentary mice, there were factors that came up significant (p-value < 0.05) through the use of a one-way ANOVA followed by a post-correction exam that compared the mean of one group to every other group, determining what is changing between the groups **Figure 13**. We can see that there are cytokines that are being affected by the presence of TFEB in the skeletal muscle, some matching that of an exercise-like status. Cytokine functions include communication, differentiation, longevity, and inflammation responses.


Axl
CD40
Exotaxin 1
FAS Ligand
IFN-gamma
IGFBP 5
IL-1 Alpha
IL-1 Beta
GCSF ANOVA
Cytokine Expression (Norm. Control)
Control
cTFEB; HSACre
Exercised
ns
*
**
***


GM-CSF
Cytokine Expression (Norm. Control)
ns
ns
ns
Control
cTFEB; HSACre
Exercised
RANTES
****
ns
****
IL-4
****
ns
****
IL-10
****
ns
****
IL-17
****
*
****
IL-5
****
**
****
IL-3
****
ns
****
IL-2
*
ns
ns
IL-6
ns
ns
ns


IL-9
ns
ns
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
IL12 p40/70
*
ns
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
Il-12 p70
**
ns
*
Cytokine Expression (Norm. Control)
5
4
3
2
1
0
Control
cTFEB; HSACre
Exercised
IL-17
****
*
****
Cytokine Expression (Norm. Control)
5
4
3
2
1
0
Control
cTFEB; HSACre
Exercised
Leptin
***
ns
**
Cytokine Expression (Norm. Control)
3
2
1
0
Control
cTFEB; HSACre
Exercised
L Selectin
*
ns
ns
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
MCP-1
**
*
ns
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
MCP-5
ns
*
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
MIG
ns
ns
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised

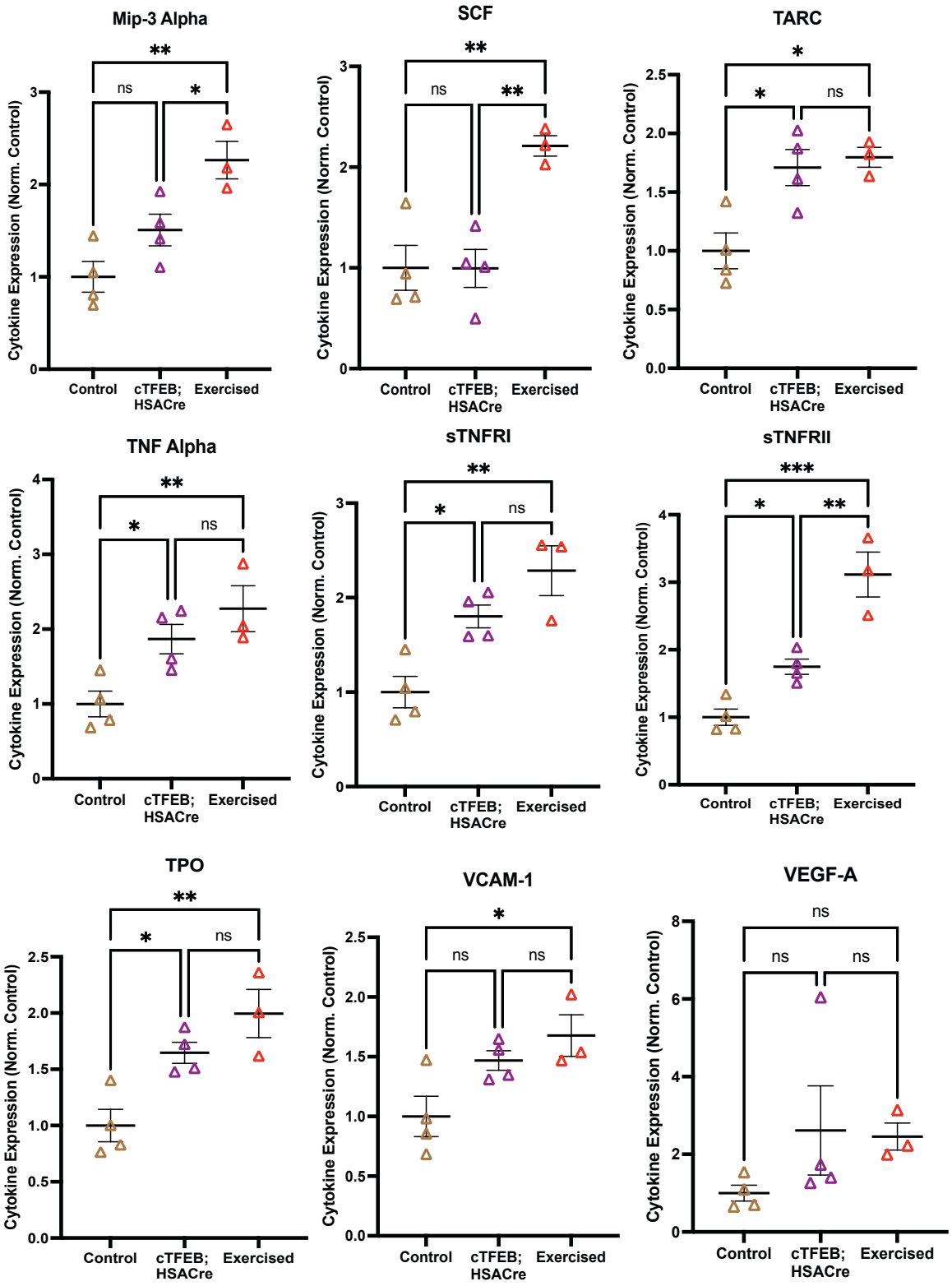


Figure 12: Overview of cytokines changing between groups of young male mice. Groups included are control sedentary mice, cTFEB;HSACre sedentary mice, and exercised control mice. One-way ANOVA analysis was used for each cytokine with an alpha level

of 0.05 with a post-hoc follow up exam. Following this test, t-test were used to test for differences among the means of every other group, p-value <0.05.

Analyzing the young female groups, including control sedentary mice (n = 4), cTFEB;HSACre sedentary mice (n = 4), and lastly the exercised control mice (n = 3), there was more variation apparent between samples within the groups. When comparing the groups together, there are cytokines that are differentially expressed between control sedentary mice, exercised control mice, and cTFEB;HSACre mice. We can see using a heatmap to visualize changes in protein expression when normalized to control sedentary female mice in **Figure 13.** Interestingly enough, we can see that different proteins are being expressed at different levels when comparing the male to the female data.

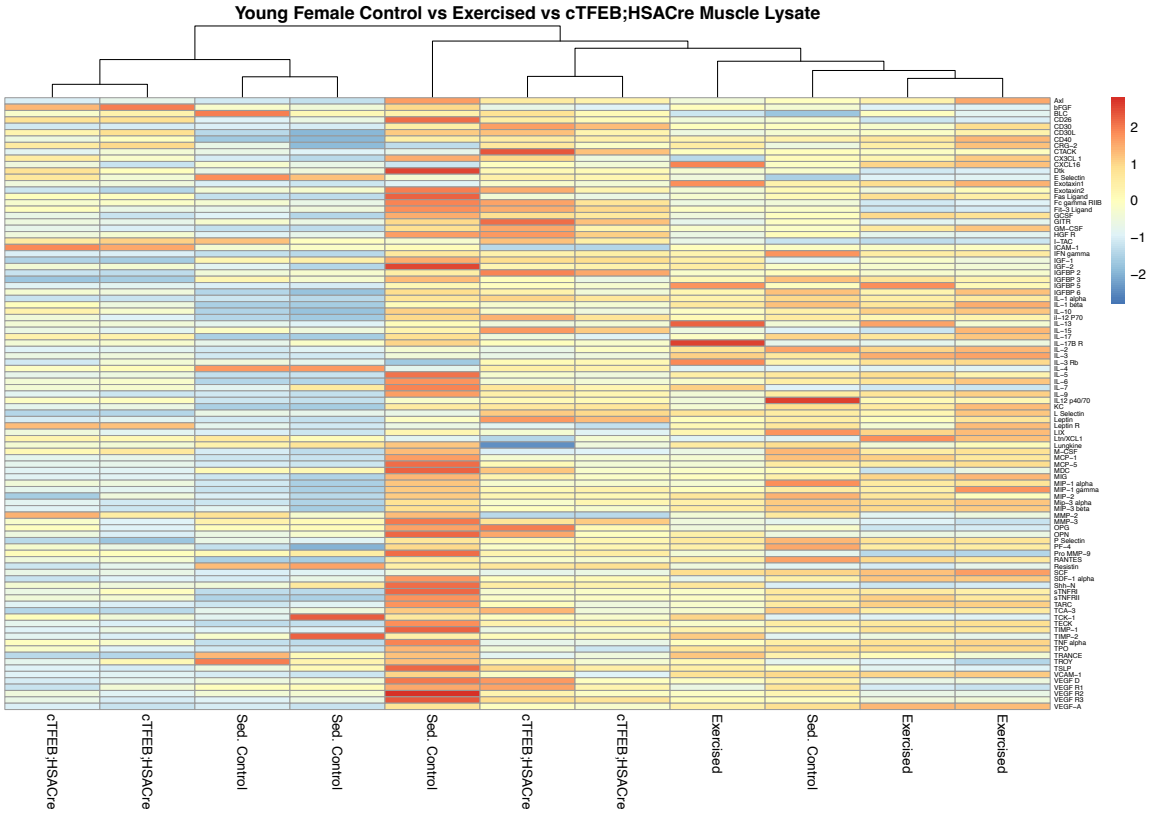
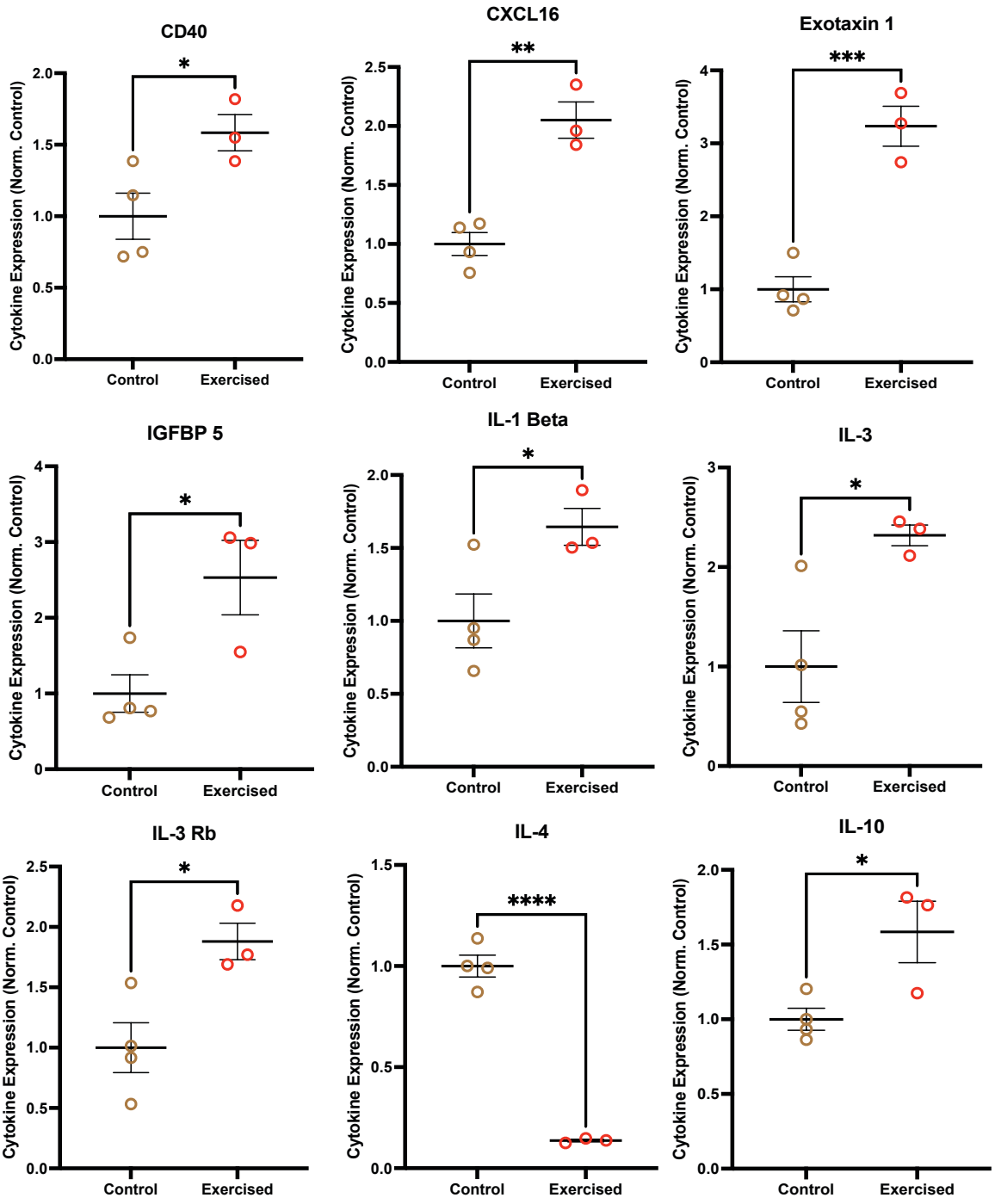


Figure 13: Overview of protein expression changes after normalization to the control sedentary group, value at 1, of all 96 cytokines from young female mice. Generated through R package pheatmap (1.0.12).

When looking further into which cytokines are significantly expressed differently, I was able to generate a list of proteins by comparing sedentary control mice to exercised control mice in **Figure 14**. From looking at this data we are able to see that there are differentially expressed proteins that all deal with growth, survivability, and inflammatory responses within the organism. Many cytokines appeared to be changing with the influence of exercise like interventions in the skeletal muscle, many of which I included in **Figure 16**, showing that with this influence of TFEB overexpression in the skeletal muscle, we are seeing a change in protein expression of many known inflammatory and growth markers such as many of the interleukin cytokines and tyrosine receptor cytokines. All of which are known to provide inflammation responses by lowering the level or causing inflammation and providing support and growth to cells.


CD40
*
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
Exercised
CXCL16
**
2.5
2.0
1.5
1.0
0.5
0.0
Exotaxin 1
***
4
3
2
1
0
IGFBP 5
*
IL-1 Beta
*
IL-3
*
IL-3 Rb
*
IL-4
****
IL-10
*

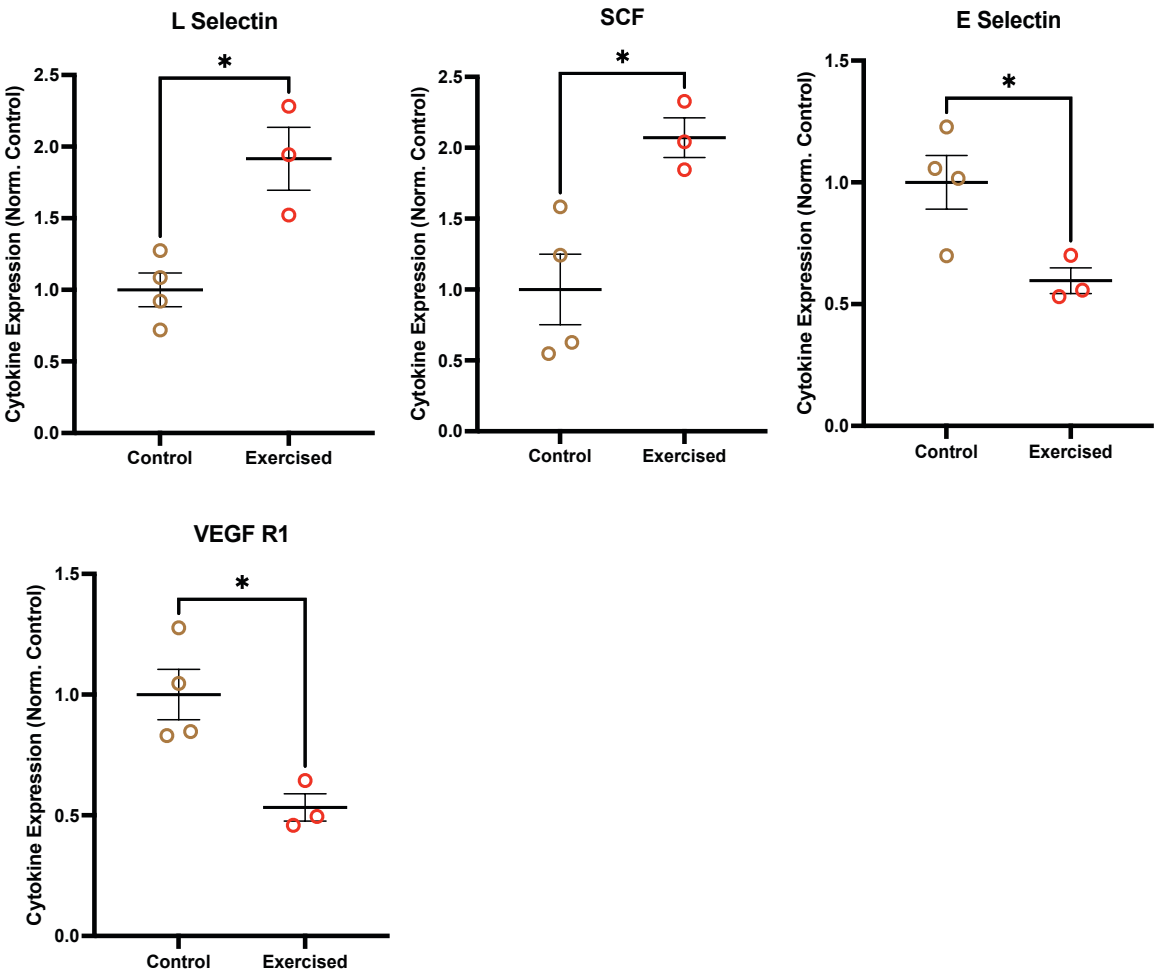
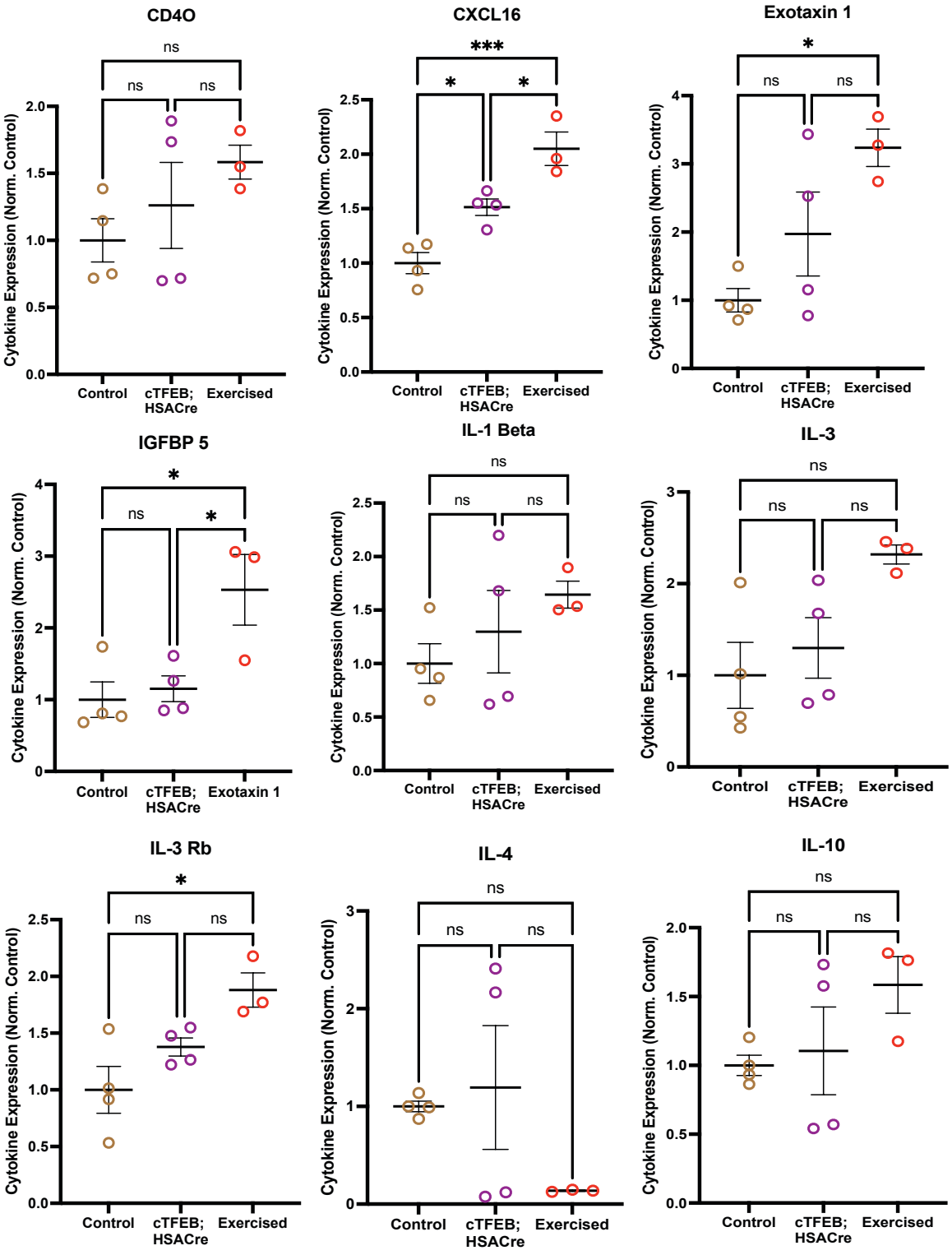


Figure 14: Cytokines that are significantly different when comparing young female data between the control sedentary group, n =4, and a exercised control group, n =3. P-value < 0.05, performed an independent t-test on each cytokine to determine significance.

With the addition of the cTFEB;HSACre group, we lose the significant values between the three groups, but we can see outcomes that with the over-expression of TFEB in the skeletal muscle we are able to increase the chances of protein expression in sedentary mice matching that of protein expression from exercised mice **Figure 15**.


CD4O
ns
ns
ns
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
CXCL16
***
*
*
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
Exotaxin 1
*
ns
ns
Cytokine Expression (Norm. Control)
4
3
2
1
0
Control
cTFEB; HSACre
Exercised
IGFBP 5
*
ns
*
Cytokine Expression (Norm. Control)
4
3
2
1
0
Control
cTFEB; HSACre
Exotaxin 1
IL-1 Beta
ns
ns
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
IL-3
ns
ns
ns
Cytokine Expression (Norm. Control)
3
2
1
0
Control
cTFEB; HSACre
Exercised
IL-3 Rb
*
ns
ns
Cytokine Expression (Norm. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised
IL-4
ns
ns
ns
Cytokine Expression (Norm. Control)
3
2
1
0
Control
cTFEB; HSACre
Exercised
IL-10
ns
ns
ns
Cytokine Expression (Norm. Control)
2.0
1.5
1.0
0.5
0.0
Control
cTFEB; HSACre
Exercised

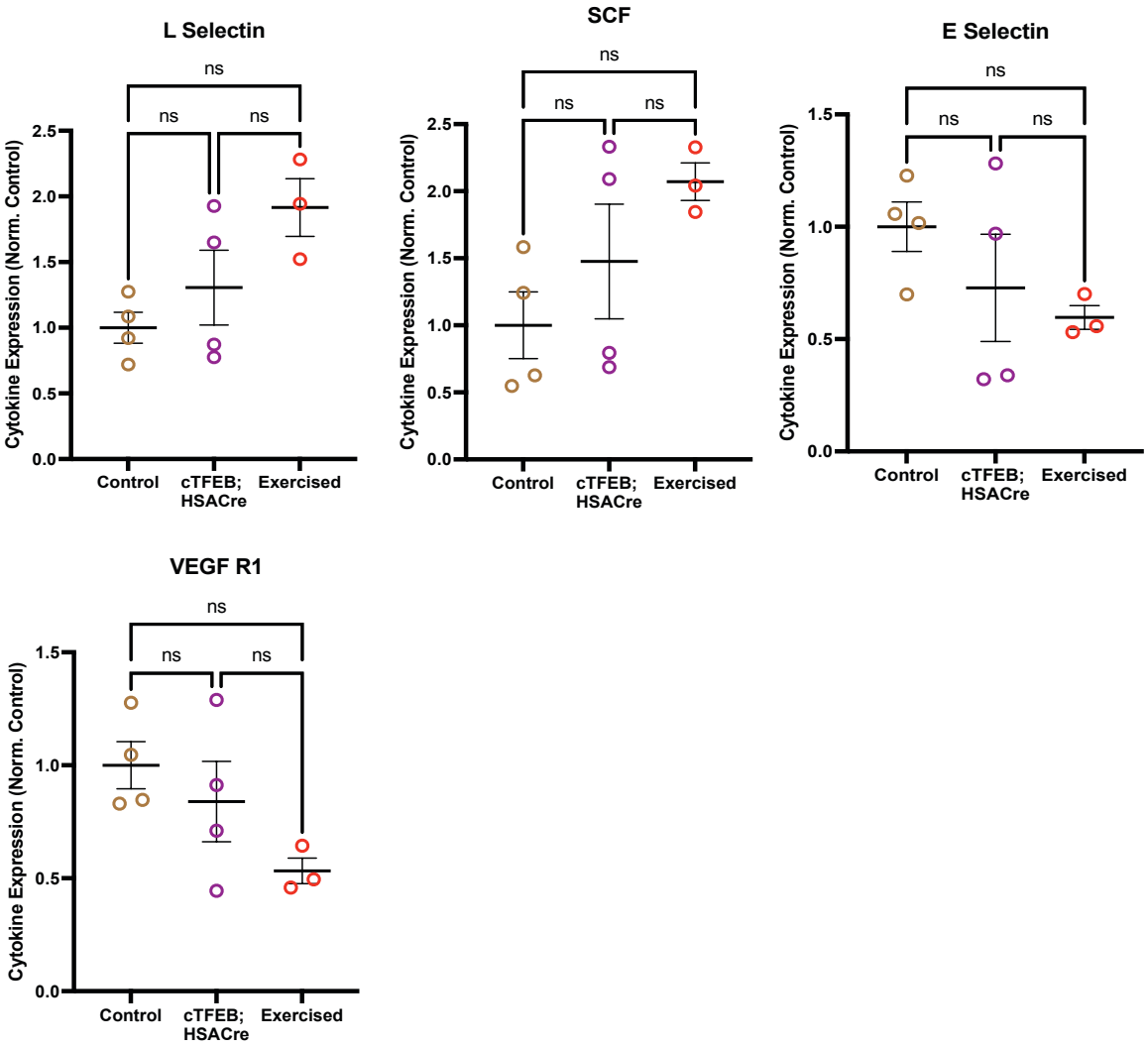


Figure 15: Young Female mice, quadricep protein lysate collected from each sample. Exercised control, n =3, Sedentary control, n = 4, and cTFEB;HSACre sedentary mice, n = 4. One-Way ANOVA was performed between the three groups, followed by performing individual t-test for each cytokine between the groups.

DISCUSSION

Exercise is a powerful behavioral intervention against Central Nervous System aging and plays an essential role in maintaining healthy neurocognitive function and immune metabolism in the aging brain[1–3,6,7,29]. By developing a new transgenic mouse model that moderately over-expresses a transcription factor master regulator of proteostasis, Transcription Factor E-B (TFEB), in skeletal muscle, we are able to see that with this over-expression of TFEB in the skeletal muscle there is a return to a healthier status of the body matching that similar to exercise states. With the continuation of studying the effects of TFEB on the skeletal muscle, we will be able to develop a drug therapeutic that is able to administer the protective and beneficial effects of exercise onto the body of an organism that has little to no access to exercise like means, for example, an elderly or ill individual. This would provide the benefits of exercise, enhanced proteostasis, and decreased inflammation, in a sedentary organism. I have generated a list of targets to pursue as potential therapeutics for central nervous system aging. This is beneficial as it can serve to further discover what circulatory factors are responsible for the rejuvenation of the central nervous system, allowing for targeting groups as potential therapeutics for individuals who are not able to acquire the benefits of exercise. **Figure 16** is a list compiled of some of the cytokines, by using STRING version 11.5, that function has been seen to interact with each other influencing growth and immune response through aging. We can take this list, continuing to develop more understanding

ideas of what the other cytokines do, and potentially pursue to acquire more information on what the effect is on the living organism through aging time points.

Future directions of this project: Continuation on analysis of aged timepoints, that being young and old, to further investigate protein expression changes in the serum of control, cTFEB;HSACre, and exercised mice for both male and female groups. Performing a gene ontology pathway analysis is another direction that this project could take. Understanding how the pathways interconnect and their function could help to understand how cytokines play a pivotal role in providing protective benefits. Another direction to continuing this project could include running a similar analysis in different muscle tissues, for example, the gastrocnemius muscle is known to be highly involved in exercise and could provide strong results to compare to the findings of this study to.

Taking further comparisons, analysis of brain sample lysate using the cytokine array to determine the differentially expressed proteins in the brain compared to circulation and muscle. This would provide strong evidence to suggest that these targets that are being affected by the over-expression of TFEB in the skeletal muscle, are interacting with the central nervous system and altering the way that it is maintaining health and is protected through aging in the mice. Comparison of RNA levels in the brain of known inflammatory markers to that of the results from the muscle lysate and serum analysis using the cytokine array kit could provide insight as to what is elevated in circulation and how it is changing in the brain.

With this, taking the list of cytokines that have been seen as statistically significant, the lab could introduce the cytokines directly onto neuronal cell cultures to observe the effects that it has on the development of a neuronal live cell. Continuing this

idea, by taking cytokines that are shown to be significantly impacted by the presence of TFEB, you could test the effects of the cytokine directly on the body by injecting the factor, stereotaxically, into the brain of mice and then in another group, inject the factor via tail vein, and then compare the results of having the factor injected directly into the brain and having the factor injected via tail vein. This would provide evidence to whether the effect of this cytokine in circulation is going to be able to provide similar results to that of being injected directly into the brain.

Il1a	Interleukin-1 alpha; Produced by activated macrophages, IL-1 stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. IL-1 proteins are involved in the inflammatory response, being identified as endogenous pyrogens, and are reported to stimulate the release of prostaglandin and collagenase from synovial cells
Il1b	Interleukin-1 beta; Potent proinflammatory cytokine. Initially discovered as the major endogenous pyrogen, induces prostaglandin synthesis, neutrophil influx and activation, T-cell activation and cytokine production, B-cell activation and antibody production, and fibroblast proliferation and collagen production; Belongs to the IL-1 family
Il2	Interleukin-2; Produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. Can stimulate B-cells, monocytes, lymphokine- activated killer cells, natural killer cells, and glioma cells
Il3	Interleukin-3; Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoiesis by controlling the production, differentiation, and function of 2 related white cell populations of the blood, the granulocytes and the monocytes-macrophages
Il4	Interleukin-4; Participates in at least several B-cell activation processes as well as of other cell types. It is a costimulator of DNA-synthesis. It induces the expression of class II MHC molecules on resting B-cells. It enhances both secretion and cell surface expression of IgE and IgG1. It also regulates the expression of the low affinity Fc receptor for IgE (CD23) on both lymphocytes and monocytes. Positively regulates IL31RA expression in macrophages
Il5	Interleukin-5; Factor that induces terminal differentiation of late-developing B-cells to immunoglobulin secreting cells

Il6	Interleukin-6; Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig- secreting cells Involved in lymphocyte and monocyte differentiation. Acts on B-cells, T-cells, hepatocytes, hematopoietic progenitor cells and cells of the CNS. Required for the generation of T(H)17 cells. Also acts as a myokine. It is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and to improve insulin resistance. It induces myeloma and plas [...]
Il9	Interleukin-9; Supports IL-2 independent and IL-4 independent growth of helper T-cells
Il10	Interleukin-10; Inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells
Il13	Interleukin-13; Cytokine. Inhibits inflammatory cytokine production. Synergizes with IL2 in regulating interferon-gamma synthesis. May be critical in regulating inflammatory and immune responses (By similarity). Positively regulates IL31RA expression in macrophages
Il17a	Interleukin-17A; Ligand for IL17RA. The heterodimer formed by IL17A and IL17F is a ligand for the heterodimeric complex formed by IL17RA and IL17RC (By similarity). Involved in inducing stromal cells to produce proinflammatory and hematopoietic cytokines (By similarity)
RANTES	Chemokine (c-c motif) ligand 5; C-C motif chemokine 5; Chemoattractant for blood monocytes, memory T-helper cells and eosinophils. Causes the release of histamine from basophils and activates eosinophils. May activate several chemokine receptors including CCR1, CCR3, CCR4 and CCR5. May also be an agonist of the G protein-coupled receptor GPR75. Together with GPR75, may play a role in neuron survival through activation of a downstream signaling pathway involving the PI3, Akt and MAP kinases. By activating GPR75 may also play a role in insulin secretion by islet cells
TNF alpha	Tumor necrosis factor superfamily, member 2; Tumor necrosis factor; Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, Under certain conditions it can stimulate cell proliferation and induce cell differentiation
VCAM-1	Vascular cell adhesion molecule 1; Important in cell-cell recognition. Appears to function in leukocyte-endothelial cell adhesion. Interacts with integrin alpha- 4/beta-1 (ITGA4/ITGB1) on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/ITGA4/ITGB1 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation

VEGF-A	Vascular endothelial growth factor A; Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Binds to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, heparan sulfate and heparin. May play a role in increasing vascular permeability during lactation, when increased transport of molecules from the blood is required for efficient milk protein synthesis (By similarity).
TRANCE	Tumor necrosis factor ligand superfamily member 11; Cytokine that binds to TNFRSF11B/OPG and to TNFRSF11A/RANK. Osteoclast differentiation and activation factor. Augments the ability of dendritic cells to stimulate naive T-cell proliferation. May be an important regulator of interactions between T-cells and dendritic cells and may play a role in the regulation of the T-cell-dependent immune response. May also play an important role in enhanced bone-resorption in humoral hypercalcemia of malignancy (By similarity)
IL-15	Interleukin-15; Cytokine that stimulates the proliferation of T- lymphocytes. Stimulation by IL-15 requires interaction of IL-15 with components of IL-2R, including IL-2R beta and probably IL-2R gamma but not IL-2R alpha
IGFBP 2	Pregnancy-associated plasma protein a; Pappalysin-1; Metalloproteinase which specifically cleaves IGFBP-4 and IGFBP-5, resulting in release of bound IGF. Cleavage of IGFBP-4 is dramatically enhanced by the presence of IGF, whereas cleavage of IGFBP-5 is slightly inhibited by the presence of IGF. Isoform 2 cleaves IGFBP-4 very slowly compared to PAPP-A, but its ability to cleave IGFBP-5 is unaffected
TNF alpha	Tumor necrosis factor superfamily, member 2; Tumor necrosis factor; Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, under certain conditions it can stimulate cell proliferation and induce cell differentiation
CD40	Tumor necrosis factor receptor superfamily member 5; Receptor for TNFSF5/CD40LG. Transduces TRAF6- and MAP3K8-mediated signals that activate ERK in macrophages and B cells, leading to induction of immunoglobulin secretion
CXCL16	C-X-C motif chemokine 16; Induces a strong chemotactic response. Induces calcium mobilization. Binds to CXCR6/Bonzo. Also acts as a scavenger receptor on macrophages, which specifically binds to OxLDL (oxidized low density lipoprotein), suggesting that it may be involved in pathophysiology such as atherogenesis; Belongs to the intercrine alpha (chemokine CxC) family

Dtk	Tyrosine-protein kinase receptor TYRO3; Receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm by binding to several ligands including TULP1 or GAS6. Regulates many physiological processes including cell survival, migration and differentiation. Ligand binding at the cell surface induces dimerization and autophosphorylation of TYRO3 on its intracellular domain that provides docking sites for downstream signaling molecules. Following activation by ligand, interacts with PIK3R1 and thereby enhances PI3-kinase activity
E Selectin	Selectin, endothelial cell; E-selectin; Cell-surface glycoprotein having a role in immunoadhesion. Mediates in the adhesion of blood neutrophils in cytokine-activated endothelium through interaction with PSGL1/SELPLG. May have a role in capillary morphogenesis
P Selectin	Selectin, platelet (p-selectin) ligand; P-selectin glycoprotein ligand 1; A SLe(x)-type proteoglycan, which through high affinity, calcium-dependent interactions with E- and P-selectins, mediates rapid rolling of leukocytes over vascular surfaces during the initial steps in inflammation. Critical for the initial leukocyte capture
MCP-5	C-C motif chemokine 12; Chemotactic factor that attracts eosinophils, monocytes, and lymphocytes but not neutrophils. Potent monocyte active chemokine that signals through CCR2. Involved in allergic inflammation and the host response to pathogens and may play a pivotal role during early stages of allergic lung inflammation; Belongs to the intercrine beta (chemokine CC) family

Figure 16: 25 cytokine's function that are shown to interact with each other using the software STRING, version 11.5, that shows protein pathway interactions. Further analysis on existing cytokines is required for future directions

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