Exercise training impacts skeletal muscle gene expression related to the kynurenine pathway

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Author Contributions:

GP and SMP obtained the funding for the trial. SMP, GP, TS, DK and KEB designed and ran the trial and collected all the tissue samples. Assays were performed by DJA and JPN. DJA, JPN and JJH were responsible for conceptualization of the analysis, data analysis, and manuscript preparation.

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Abstract

Exercise positively impacts mood and symptoms of depression; however, the mechanisms underlying these effects are not fully understood. Recent evidence highlights a potential role for skeletal muscle-derived transcription factors to influence Trp metabolism, along the kynurenine pathway, which has important implications in depression. This has important consequences for older adults whose age-related muscle deterioration may influence this pathway and may increase their risk for depression. Although exercise training has been shown to improve skeletal muscle mass in older adults, whether this also translates into improvements in transcription factors and metabolites related to the kynurenine pathway has yet to be examined. The aim of the present study was to examine the influence of a 12-week exercise program on skeletal muscle gene expression of transcription factors, kynurenine aminotransferase (KAT) gene expression, and plasma concentrations of tryptophan metabolites (kynurenines) in healthy older men > 65 yrs. Exercise training significantly increased skeletal muscle gene expression of transcription factors (PGC1-α, PPARα, PPARȝ: 1.77, 1.99, 2.18-fold increases, respectively, $p < 0.01$) and KAT isoforms 1-4 (6.5, 2.1, 2.2, 2.6-fold increases, respectively, $p \leq 0.01$). Concentrations of plasma kynurenines were not altered. These results demonstrate that 12 weeks of exercise training significantly altered skeletal muscle gene expression of transcription factors and gene expression related to the kynurenine pathway, but not circulating kynurenine metabolites in older men. These findings warrant future research to determine whether distinct exercise modalities or varying intensities could induce a shift in the kynurenine pathway in depressed older adults.

Keywords: Skeletal muscle; PGC-1α; Kynurenine; Physical activity; Aging

Introduction

According to the World Health Organization depression is the single largest contributor to global disability (18). A disproportionately large number of those suffering are older adults, whose disability due to depression is augmented by age-related declines in their physical health and mobility. Traditional pharmaceuticals used in the treatment of depression (i.e., selective serotonin reuptake inhibitors) are often ineffective at reducing primary symptoms (14); these drugs are also associated with adverse side effects (8) and high rates of relapse (10). Exercise may be a beneficial alternative or adjunctive treatment strategy to the pharmaceutical treatment of depression. In addition to its positive effects on physical health and mobility in aging, exercise can reduce symptoms of depression (4, 6, 12). However, the mechanisms underlying these effects are not fully understood, and this information is critical towards determining the effectiveness of exercise as a treatment strategy for depression in older adults.

Preliminary research in younger adults suggests that skeletal muscle may play an important role in the mood-enhancing effects of exercise (1, 15). Specifically, exercise upregulates the expression of skeletal muscle-derived transcription factors which are responsible for promoting the expression of key enzymes which influence tryptophan (Trp) metabolism. Importantly, Trp is the precursor for serotonin (5-HT) synthesis and alterations in Trp metabolism may contribute to the low central 5-HT concentrations observed in Major Depressive Disorder (MDD) (2). Approximately 95% of Trp metabolism occurs via the kynurenine pathway (11). Trp is first degraded into the metabolite kynurenine (KYN). This metabolite is capable of crossing the blood brain barrier (BBB) and can therefore undergo further metabolism peripherally as well as within the brain. Kynurenine is metabolized along one of two distinct branches of the kynurenine pathway: a *neuroprotective* branch and a neurotoxic branch. The *neuroprotective* branch depends on the enzyme kynurenine aminotransferase (KAT) which

92 results in the production of the non-BBB transportable metabolite kynurenic Acid (KYNA). In
93 contrast, the neurotoxic branch depends on the enzyme kynurenine monooxygenase (KMO)
94 which shifts the pathway towards the production of potentially neurotoxic metabolites including
95 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN).

96 Both aerobic (9) and resistance (13) exercise training may bias metabolism of Trp
97 towards the neuroprotective branch by increasing KAT activity. Specifically, skeletal muscle-
98 derived transcriptional coactivators including PGC-1α (peroxisome proliferator-activated receptor
99 gamma coactivator 1-alpha), PPARα (peroxisome proliferator-activated receptor alpha) and PPARȝ
100 (peroxisome proliferator-activated receptor delta) promote KAT expression (1). Furthermore,
101 skeletal muscle PGC-1α overexpression is associated with a protective shift in Trp metabolism in
102 animal models (1). One prior study in younger adults demonstrated that aerobic exercise-training
103 can induce a protective shift in the kynurenine pathway related to increases in skeletal muscle
104 transcription factors (15). Although this has yet to be examined in older adults, previous research
105 has shown that, in rodents, aging is accompanied by a loss of exercise-induced expression of
106 skeletal muscle PGC-1α (5). If older humans demonstrate a similar loss it may negatively impact
107 the potential for exercise to influence the kynurenine pathway and ultimately impair the mood
108 enhancing properties of physical activity.

109 The present study examined whether, in a group of healthy, non-depressed older men, a
110 12-week combined (resistance + high intensity interval training) exercise training program would
111 enhance skeletal muscle gene expression of transcriptional coactivators and bias the kynurenine
112 pathway towards the neuroprotective branch. We hypothesized that the exercise training program
113 would increase skeletal muscle gene expression of PGC-1α, PPARα, and PPARȝ, which would

relate to an increase in KAT gene expression and a decrease in the ratio of QUIN/KYNA plasma metabolites indicating a shift in the kynurenine pathway towards the neuroprotective branch.

Materials and Methods

Study design and participants

This study is a secondary analysis on a subset of participants from a previously published randomized controlled trial (ClinicalTrials.gov NCT02281331). The original study was designed to evaluate the use of a multi-ingredient nutritional supplement combined with an exercise training program on lean body mass and strength in 49 non-depressed (17) healthy older men (≥65 yrs, Geriatric Depression Scale: 3.5±3.3 [mean±SD]). All participants had a body mass index within the normal to overweight range (between 18.5-30.0 kg/m^2), normal resting blood pressure or stage 1 hypertension (systolic blood pressure ≤140-159 mmHg; diastolic blood pressure ≤90-99 mmHg) and had not participated in any structured resistance or aerobic exercise training in the previous 6 months. A full description of the study design has been previously published (3). Briefly, participants either received an experimental protein-based nutritional supplement (n = 25) or a placebo (n = 24) twice per day for 6 weeks. All participants then completed a 12-week progressive exercise training program while continuing to take the supplement and/or placebo twice per day. Blood samples and muscle biopsies were collected at baseline (prior to beginning supplement or placebo), 7-weeks (pre-exercise training), and 20-weeks (post-exercise training) following an 8-12 hour overnight fast (no food or drink, except water, after midnight the previous night). Subjects were instructed to refrain from strenuous physical activity for 72 hours prior to collection of the blood and muscle samples. For this secondary analysis, blood samples and muscle biopsies were assessed at the original 7-week time

point (prior to the 12-week exercise intervention) and at the original 20-week time point (10-days following the cessation of the exercise intervention). Subjects were included for the current secondary analysis based on the availability of muscle tissue samples. As preliminary analysis revealed no differences in gene expression or plasma kynurenines between participants from the original supplement (n=11) and placebo (n=14) groups, subjects were collapsed across groups into a single subset of 25 participants.

Exercise Intervention

Between weeks 7-18 (inclusive) participants completed a supervised 12-week progressive exercise program at McMaster University. Each week participants performed two resistance training sessions (Mondays and Fridays) and a one high intensity interval training (HIIT; Wednesdays) session. Each resistance training session started with a 5 min warm up on a cycle ergometer followed by 3 sets of 4 resistance exercises. Monday sessions consisted of leg press, chest press, horizontal row, and leg extension. Friday sessions consisted of leg press, lateral pull down, shoulder press, and leg extension. The third set of each exercise was performed to volitional fatigue. Workloads were gradually increased from 65% 1 RM (10-12 repetitions) to 80% 1RM (6-8 repetitions) over the first 3 weeks of training. Loads were adjusted based on 1RM strength tests every 4 weeks or when subjects could complete ≥ 12 repetitions during the third set of each exercise. HIIT was performed on a cycle ergometer (ISO1000 Upright Bike; SCIFIT, Tulsa, OK) while wearing a heart rate (HR) monitor (H7 Heart Rate Sensor; Polar Electro Canada, Lachine, QC). Each HIIT session started with a 3 min warm-up at 25 W, followed by 10 X 60 sec intervals at a workload corresponding to ~90% maximal HR (HRmax) at a cadence of ≥ 90 rpm. Workload was adjusted as needed to maintain an average HR of ~90% HRmax over the

10 intervals. These high-intensity intervals were interleaved with 60s of recovery at 25W at a self-selected pace. Each HIIT session concluded with a 5 min cool-down at 25W.

Muscle biopsy

Following an overnight fast (~10 h), a percutaneous muscle biopsy was obtained from the *vastus lateralis* under local anesthetic (2% lidocaine) using a 5 mm Bergstrom needle adapted for manual suction. Subjects refrained from exercise for 72 hours before the collection of all muscle biopsy samples. Upon excision, a portion of the muscle sample was directly frozen in liquid nitrogen and stored at −80°C until mRNA analysis was performed.

RNA isolation and reverse transcription

RNA was isolated from 15–25 mg of muscle tissue using the Trizol/RNeasy method. All samples were homogenized with 1 mL of Trizol Reagent (Life Technologies, Burlington, ON, Canada), in Lysing Maxtrix D tubes (MP Biomedicals, Solon, OH, USA), with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals,Solon, OH, USA) for a duration of 40 sec at a setting of 6 m/sec. Following a five-minute room temperature incubation, homogenized samples were stored at -80°C for one month until further processing. After thawing on ice, 200 µl of chloroform (Sigma-Aldrich, Oakville, ON, Canada) was added to each sample, mixed vigorously for 15 seconds, incubated at RT for five minutes, and spun at 12000 g for 10 min at 4°C. The RNA (aqueous) phase was purified using the E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA) as per manufacturer's instructions. RNA concentration (ng/ml) and purity (260/280) was determined with the Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA). Samples were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in 20uL reaction volumes,

as per manufacturer's instructions, using an Eppendorf Mastercycler epGradient Thermal Cycler (Eppendorf, Mississauga, ON, Canada) to obtain cDNA for gene expression analysis.

Quantitative real time PCR

All QPCR reactions were run in duplicate in 25 µl volumes containing RT Sybr Green qPCR Master Mix (Qiagen Sciences, Valencia, CA, USA), prepared with the epMotion 5075 Eppendorf automated pipetting system (Eppendorf, Mississauga, ON, Canada), and carried out using an Eppendorf Realplex2 Master Cycler epgradient (Eppendorf, Mississauga, ON, Canada). Primers (listed in Table 1) were re-suspended in 1X TE buffer (10mM Tris–HCl and 0.11 mM EDTA) and stored at −20°C prior to use. Messenger RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and expressed as fold change from pre, as described previously (16). Briefly, Ct values were first normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH expression was not different between the baseline and the post-training timepoints. Ct values normalized to GAPDH were expressed as delta-delta Ct (ΔΔCt).

Venous blood sampling

Blood samples (~10 mL) were obtained from an antecubital vein immediately prior to the muscle biopsy procedure. Samples were collected into lithium heparin-coated tubes, mixed by inversion, and centrifuged at 1500 rpm for 10 min at 4°C. Aliquots of plasma were stored at −80°C until analysis.

Plasma analyses

Plasma kynurenine was measured using the Kynurenine ELISA (ImmuSmol, Pessac, France). Plasma kynurenic acid and quinolinic acid were measured using the KYNA ELISA and QUIN ELISA respectively (Cloud-Clone Corp TX, USA). All samples were measured undiluted

in triplicate and all standards were run in duplicate. Absorbance of each plate was measured at 450 nm, with a reference wavelength of 540 nm using a Multiskan GO UV/Vis microplate spectrophotometer (Thermo-Fisher Scientific, Waltham, MA).

Statistical analyses

The change in skeletal muscle gene and protein expression, and plasma kynurenines, were assessed using multivariate repeated measures ANOVA with a within-subjects factor of time (pre, post). Statistical significance was set at $p \leq 0.05$. Univariate post-hoc analyses were then performed to determine which variables changed over time. Change scores are presented as means ± SD. The association between the changes in skeletal muscle gene and protein expression, and plasma kynurenines, were analyzed using Pearson Correlations. Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, version 23.0; IBM Corp., Armonk, NY).

Results

Multivariate repeated measures ANOVA showed a significant multivariate effect for the eleven latent variables $F(11,6) = 5.03$, $p = .03$, *Cohens d* = 6.07. Univariate level post-hoc tests were then conducted to identify the specific dependent variables that changed over time.

Participant Characteristics – Physiological & Performance Parameters

A complete description of participant physiological and performance parameters has been previously published (3). In brief, participants in the supplement and control groups had baseline body mass indices (BMI) of 28.9 and 28.1 respectively. BMI was not significantly altered in either group following the intervention. Aerobic fitness was assessed by a VO_2peak test on a cycle ergometer. Significant improvements from baseline to post intervention were observed for both the supplement and control groups by respective changes in VO_2peak of 23.8 ± 0.8 – 26.2 ±

1.2 ml/kg/min and 24.4 ± 0.9 – 26.4 ± 1.4 ml/kg/min and peak power 154±5 - 164±7 W and 158 ± 7 – 178 ± 10 W. Isotonic muscle strength was assessed by 1 repetition maximums (1RMs) for the following exercises: leg press, chest press, lateral pull-down, horizontal row, shoulder press, and leg extension. Significant improvements, from baseline to post-intervention, were demonstrated in both the supplement and control groups by changes in the sum of all 1RMs which included 23% and 21% increases respectively.

Skeletal muscle gene and transcription factor expression

Figure 1A illustrates significant exercise training-induced increases in transcriptional coactivators PGC-1α (1.77-fold increase; $p < .01$; Cohens d=1.71), PPARα (1.99-fold increase; $p < .001$, *Cohens d* = 3.22), and PPARδ (2.18-fold increase; $p < .001$; *Cohens d* = 3.59). Significant increases were also shown for all 4 KAT isoforms including KAT1 (6.5-fold increase; $p = .01$; *Cohens d* = 1.39), KAT2 (2.1-fold increase; $p < .01$; *Cohens d* = 1.88), KAT3 (2.2-fold increase; $p < .01$; *Cohens d* = 1.81), and KAT4 (2.6-fold increase; $p < .01$; *Cohens d* = 1.75) (Figure 1B). Importantly, increases in each transcription factor (PGC-α, PPARα, PPARδ) were significantly correlated with increases in each KAT isoform (KAT 1-4) (see Table 2).

Plasma kynurenines

The change in plasma kynurenines following intervention showed moderate effect sizes, however, no changes reached statistical significance (Figure 1C). Kynurenine was reduced from 651.3 ng/ml to 603.3 ng/ml (7.4% decrease) ($p = .09$; *Cohens d* = .90), KYNA was increased from 154.0 ng/ml to 159.7 ng/ml (3.7% increase) ($p = .59$; *Cohens d* = .27) and QUIN was reduced from 163.7 ng/ml to 157.3 ng/ml (3.9% decrease) ($p = .31$; *Cohens d* = .53). The QUIN/KYNA ratio was reduced by 5.7% (p = .47; Cohens d = .38). Changes in skeletal muscle

gene and transcription factor expression was not correlated with the change in any of the plasma kynurenines.

Discussion

The present study is the first to evaluate the influence of a 12-week exercise training program on skeletal muscle transcription factors and aspects of the kynurenine pathway in older men. The exercise intervention resulted in significant increases in the expression of the skeletal muscle transcription factors PGC-1α, PPARα, and PPARȝ, which is in accordance with previous work in young adults. Critically, these changes correlated positively with increased gene expression of all KAT isoforms; however, the plasma concentrations of kynurenines were not significantly altered in response to the exercise training program. Despite the lack of change in plasma kynurenines, the significant increase in skeletal muscle gene expression is encouraging given that previous work in animal models showed a loss in exercise-induced increases with aging (5). These changes would be expected to help facilitate a shift towards the neuroprotective branch of the kynurenine pathway. Although this was not achieved in the current study, we will herein discuss potential limitations in the study design related to the timing of sample collection that may have obscured a true physiological effect.

As hypothesized, we observed an exercise training-induced increase in skeletal muscle transcriptional coactivators. This was related to an increase in the expression of KAT isoforms, upon which the *neuroprotective* branch depends. However, for these changes to be regarded as reflective of a true neuroprotective shift, we would also have needed to observe a decrease in plasma concentrations of QUIN and an increase in plasma concentrations of KYNA. Although the QUIN/KYNA ratio was reduced by a medium effect size following exercise training, the changes in circulating KYNA and QUIN were not statistically significant. This lack of

significant alterations in metabolites may be due to several factors, including the timing of sample collection, exercise training parameters, and the use of non-depressed participants. Blood samples were collected following muscle biopsies 10-days following the cessation of the exercise intervention. As plasma kynurenic acid has been shown to be rapidly excreted by the kidneys following exercise, the most dramatic elevations in plasma concentrations would likely have been apparent near the end or immediately following an exercise bout (7). It may be possible that the changes in plasma kynurenines were purely transient in nature and therefore not detected in the current analysis. Future research should evaluate the temporal dynamics of the kynurenine pathway during and following an acute bout in this population. Further, although significant increases in KAT *gene* expression were demonstrated, limited tissue sample availability precluded the examination of KAT *protein* expression, and it is therefore not possible to confirm whether corresponding increases in KAT protein were achieved.

Although both aerobic (9) and resistance (13) exercise has been shown to bias Trp metabolism towards the neuroprotective branch, research is limited regarding the most effective modality, volume, and intensity (particularly in the older adult population). It may be possible that the multi-modal exercise intervention employed in the current study, which consisted of both resistance training and high intensity interval training, was not sufficient to induce substantial enough changes to produce lasting effects. Alternatively, the training volume of twice per week and/or the intensity within those training sessions may not have been sufficient.

Although these results provide evidence for the ability of exercise to enhance skeletal muscle gene expression related to the kynurenine pathway in older adults, our sample was relatively small and consisted of only men who were not depressed. Older adults with depression may have an even greater skeletal muscle transcriptional deficit and thus be more apt to

experience a dramatic shift in the kynurenine pathway with exercise. Further, as plasma kynurenines were assessed 10 days following the final exercise bout, it is not possible to comment on potential transient changes in the acute phase following exercise. Larger scale exercise trials, which assess both transient and resting state biochemical changes are needed to examine how changes in these fundamental biochemical processes impact depressive symptoms.

Conclusion

In conclusion, older men who engaged in a new 12-week combined exercise training program had significant increases in skeletal muscle transcriptional coactivators and gene expression related to the kynurenine pathway. Plasma concentrations of kynurenines were, however, not significantly altered. Despite this, the significant exercise training-induced increase in the expression of skeletal muscle transcription factors and KAT in older adults is encouraging given the potential implications related to kynurenine pathway regulation. Future studies are warranted to explore the impact of various exercise modalities and intensities on transient changes of such factors in depressed older adults.

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List of Abbreviations

5-HT: Serotonin

BBB: Blood brain barrier

HIIT: High intensity interval training

KAT: Kynurenine aminotransferase

KMO: Kynurenine monooxygenase

KYN: Kynurenine

KYNA: Kynurenic acid

PGC1-α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

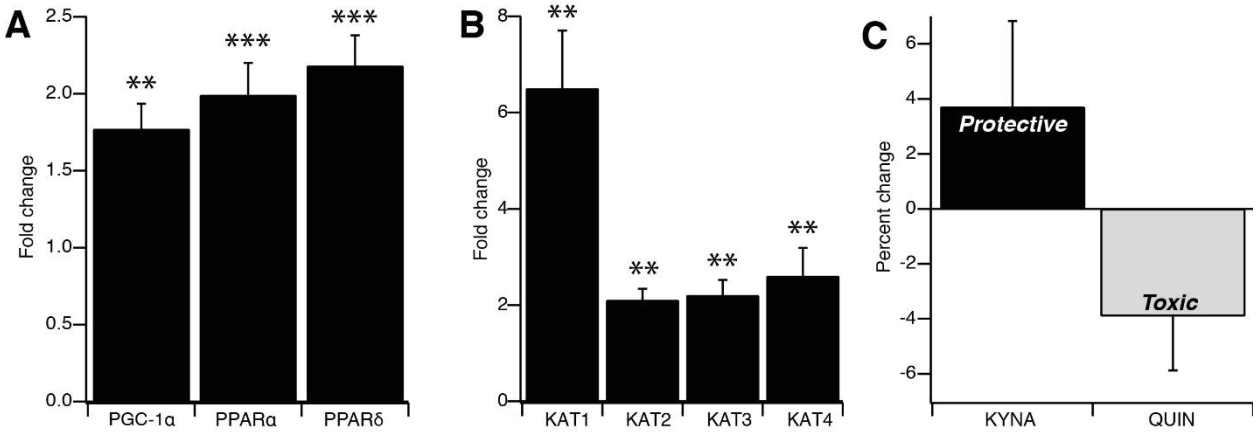
PPARδ: peroxisome proliferator-activated receptor delta

PPARα: Peroxisome proliferator-activated receptor alpha

QUIN: Quinolinic Acid

Trp: Tryptophan

Figure 1: Exercise training-induced changes in biomarkers



Fold changes post vs. pre exercise training in (A) the expression of skeletal muscle transcription coactivators PGC-1α, PPARα, and PPARȝ; and (B) skeletal muscle gene expression of KAT isoforms 1-4. Panel C depicts the percent change in the plasma metabolites KYNA and QUIN post vs. pre exercise training. PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARα, peroxisome proliferator-activated receptor alpha; PPARȝ, peroxisome proliferator-activated receptor delta; KAT, kynurenine amino transferase; KYNA, kynurenic acid; QUIN, quinolinic acid.
Repeated Measures ANOVA; n=25. Error bars represent SEM.
**p≤0.01
**p≤0.001

Table 1: Primer sequences for quantitative real-time PCR analysis

Gene Name	Sense primer sequence (5′-3′)	Antisense primer sequence (5′-3′)
PGC1-α	CAGCCTCTTTGCCCAGATCTT	TCACTGCACCACTTGAGTCCAC
PPAR-α	CATCACGGACACGCTTTCAC	CCACAGGATAAGTCACCGAGG
PPAR-δ	ACTGAGTTCGCCAAGAGCATC	ACGCCATACTTGAGAAGGGTAA
KAT1	CCAGTGGATGGTCTACGACG	CTCCCGTTCAAAGCTCTCG
KAT2	AATTACGCACGGTTCATCACG	TCCTCTGCTCAATATGTCAGTCA
KAT3	ATCCTTGTGACAGTAGGAGCA	GGGCTCATAGCAGTCATAGAAAG
KAT4	AAGAGGGACACCAATAGCAAAAA	GCAGAACGTAAGGCTTTCCAT
GAPDH	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PGC-1α, Peroxisome proliferator activated receptor gamma coactivator 1-α; PPAR-α, Peroxisome proliferator-activated receptor α; PPAR-δ, Peroxisome proliferator-activated receptor α; KAT1, Kynurenine aminotransferase 1; KAT2, Kynurenine aminotransferase 2; KAT3, Kynurenine aminotransferase 3; KAT4, Kynurenine aminotransferase 4.

Table 2: Skeletal Muscle Gene and Transcription Factor Expression Correlation Matrix

	Δ*PGC-1α*	Δ*PPAR α*	Δ*PPAR$_{\delta}$*	Δ*KAT1*	Δ*KAT2*	Δ*KAT3*
Δ*PPAR α*	.53*	--				
Δ*PPAR$_{\delta}$*	.53*	.80**	--			
Δ*KAT1*	.41	.71**	.53*	--		
Δ*KAT2*	.55*	.60**	.54**	.88**	--	
Δ*KAT3*	.46*	.52*	.53*	.62**	.70**	--
Δ*KAT4*	.49*	.73**	.53*	.75**	.71**	.57**

PGC-1α, Peroxisome proliferator activated receptor gamma coactivator 1-α; PPAR-α, Peroxisome proliferator-activated receptor α; PPAR-δ, Peroxisome proliferator-activated receptor α; KAT1, Kynurenine aminotransferase 1; KAT2, Kynurenine aminotransferase 2; KAT3, Kynurenine aminotransferase 3; KAT4, Kynurenine aminotransferase 4.
Pearson Correlations; Data presented as r-values; n=25
*$p \leq 0.05$
**$p \leq 0.01$