Exercise and the Kynurenine pathway: Current state of knowledge and results from a randomized cross-over study comparing acute effects of endurance and resistance training

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Abstract

Introduction: *The essential amino acid tryptophan (TRP) is primarily degraded through the kynurenine (KYN) pathway, which is dysregulated in several chronic diseases. KYN pathway metabolites have immune- and neuro-modulatory properties and are involved in the de novo synthesis of nicotinamide adenine dinucleotide (NAD^+). Currently, little evidence exists demonstrating that physical exercise may influence this pathway. However, differences between acute and chronic stimuli as well as the influence of exercise modalities remain to be investigated. Here, we provide an overview of existing studies and present results of a randomized cross-over trial on acute effects of a single-bout of resistance and endurance exercise.*

Methods: *24 healthy male adults conducted both an acute endurance exercise (EE) and resistance exercise (RE) session. Blood samples were collected before, immediately after and one hour after cessation of each exercise session. Outcomes comprised serum levels of TRP, KYN, kynurenic acid (KA), quinolinic acid (QA) and calculated ratios. Gene expression of the enzymes indoleamine 2,3 dioxygenase (IDO) 1 and kynurenine aminotransferase (KAT) 4 was measured in peripheral blood mononuclear cells (PBMCs). Moreover, serum concentrations of the potential KYN pathway mediators interleukin (IL)-6 and cortisol were determined. Finally, we investigated baseline correlations between immune cell subsets, potential mediators and initial KYN pathway activation outcomes.*

Results: *The KYN/TRP ratio correlated positively with IL-6 and $CD56^{bright}$ NK-cells and negatively with $CD56^{dim}$ NK-cells. Expression of IDO1 in PBMCs correlated positively with IL-6, regulatory T-cells and $CD56^{bright}$ NK-cells, whereas negative correlations to cytotoxic T-cells and $CD56^{dim}$ NK-cells were revealed. A significant time effect on KYN/TRP ratio was detected for RE. Regarding KA and KA/KYN ratio, an increase after exercise followed by a decrease at the follow-up measurement was revealed in EE. KAT4 expression also increased after exercise in EE. Moreover, elevated QA levels were observed after the EE session.*

Conclusion: *In contrast to chronic exercise interventions, single-bouts of endurance exercise provoke acute alterations on KYN pathway outcomes in humans. Our results indicate that EE induces stronger alterations than RE. Enhanced conversion of KYN to both, KA and QA suggest a peripheral KYN clearance, thereby preventing pathological accumulation within the CNS. Future acute and chronic exercise studies are needed to examine the role of NAD^+ synthesis starting with TRP and the interplay between KYN pathway activation and mid- to long-term immunological modulations.*

Keywords: Exercise, Kynurenine Pathway, Endurance Exercise, Resistance Exercise, Immune system

Introduction

Only one percent of the essential amino acid tryptophan (TRP) is used for protein synthesis under physiological conditions. While merely a small portion is metabolized via the serotonergic pathway, the vast majority of available TRP (over 95%) is metabolized through the kynurenine (KYN) pathway (8). During the past two decades, the pathogenesis and progression of various chronic diseases have been linked to metabolic disturbances of the KYN pathway. Dysregulations were revealed in diseases involving the central nervous system (CNS) (e.g. Alzheimer's disease, Multiple Sclerosis or Parkinson's disease (14, 68)) and several internistic pathologies (e.g. diabetes mellitus (16) and cancer (57)).

The tryptophan 2,3 dioxygenase (TDO) and its isoenzymes indoleamine 2,3 dioxygenase (IDO) 1 and 2 catalyze the degradation of TRP to KYN, thereby representing the initial step of the KYN pathway. In contrast to TDO, which is mainly expressed in hepatic tissue and primarily stimulated by TRP itself or glucocorticoids (8), IDO can be expressed in almost all types of human cells (40). IDO1 and IDO2 have demonstrated differing levels of expression, effects on peripheral

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blood metabolites, and enzyme activity in response to specific physiological conditions (58). However, knowledge about the more recently discovered IDO2 is still sparse and the differential roles of both enzymes are not yet fully understood (51, 58). Interestingly, IDO activity closely interacts with the immune system. Especially peripheral blood mononuclear cells (PBMCs) have been shown to be potent producers of IDO (35). Based on immune homeostasis, IDO mediated conversion of TRP to KYN is suspected to be constant and almost entirely mediated by TDO under basal conditions. In contrast, local and systemic inflammatory stimuli provoke a dramatic increase in IDO1 mediated conversion (57). Particularly, elevated levels of interferon-gamma (IFN-y) (66, 70), but also increases in other pro-inflammatory cytokines, such as interleukin (IL)-6 (58) and tumor necrosis factor (TNF)-α (27), seem to be decisive stimuli for IDO induction. KYN itself possesses immunomodulatory effects comprising the suppression of cytotoxic T-cell, NK-cell activity (43, 51, 52) and mediating the differentiation of regulatory T-cells (T_{regs}) (18). Evidence suggests that the IDO1-mediated conversion to KYN activates the aryl hydrocarbon receptor (AHR), which plays a key role in T-cell differentiation (46). Since IDO1 is upregulated in tumor microenvironment (58), it is not surprising that IDO1 has recently attracted extended attention as promising immunotherapeutic target in cancer research (57). Furthermore, a chronic inflammation-induced activation of the KYN pathway has been described as pathological mechanism of depression and might also be of relevance for mood disorders in general (6, 67). A consistent decrease in the bioavailability of TRP that is essential for the neurotransmitter serotonin seems to be one causal factor. If chronic inflammatory conditions are present, the degradation of available TRP through the KYN pathway is pathologically increased, which consequently leads to an impaired synthesis of the neurotransmitter serotonin (6).

Apart from its effects on immune function, KYN pathway metabolites also influence CNS homeostasis. KYN itself can either be converted to the neuroprotective kynurenic acid (KA) or (over several intermediate steps) to quinolinic acid (QA), which is closely linked to neuronal excitotoxicity. Within the CNS, KA and QA act as antagonist and agonist of N-methyl-d-aspartate (NMDA) receptor activation, respectively, thereby mediating either neuronal protection or excitotoxicity. Specifically, KA has been described to act as NMDA receptor inhibitor, either at the strychnine-insensitive glycine-binding site (at low concentrations) or at the glutamate site (at higher concentrations) (17). Furthermore, KA is also able to inhibit α7 nicotinic acetylcholine receptors (nAChRs) that are expressed by glutamatergic axon terminals and involved in increased glutamate release (68). Since NMDA receptor mediated excitotoxicity represents a common pathological mechanism in different neurodegenerative diseases (68), the anti-glutamatergic properties of KA may have beneficial effects on neurodegenerative processes. Regarding QA, the predominant and most-investigated neurotoxic effect is undoubtedly its activation of the NMDA receptor, leading to excitotoxicity. However, several other neurotoxic aspects of QA have been described. These aspects include the inhibition of glutamate uptake by astrocytes, the generation of reactive oxygen intermediates or the suppression of astroglial glutamine synthetase (30, 68), emphasizing its strong association with CNS damage. Interestingly, neurons in certain brain regions, specifically the hippocampus, striatum and neocortex, seem to be more susceptible to QA than others (30).

The homeostasis of this neuroactive branch largely depends on the activity of the enzymes kynurenine aminotransferases (KATs) and kynurenine-3-monooxydase (KMO), which catalyze the degradation of KYN to KA or QA, respectively. More precisely, the conversion to KA can be mediated by four different isoforms of KAT (KAT 1-4), whereas the conversion to QA proceeds through two metabolic intermediate products, namely the 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAA). Under non-pathological conditions, the vast majority of KYN is metabolized to QA, yielding the preferred end product nicotinamide adenine dinucleotide (NAD^+), which is highly relevant for oxidative energy production. Based on specific environmental conditions or if higher levels of TRP or KYN are present, the metabolic flux towards KA can increase (16). Considering the neurotoxic potential along the KYN pathway, it is not surprising that central and/or peripheral dysregulations in metabolites and enzyme activities are associated with various neurodegenerative disorders, such as Parkinson's disease, Huntington's disease or Multiple Sclerosis (68). Drawing a parallel between all these neurological diseases, an over-activated KYN pathway leading to an accumulation of QA in the CNS as well as a decrease in the neuroprotective KA has been ubiquitously linked to pathogenesis or disease progression (65, 68). As a consequence of pathological QA accumulation, induced neurodegenerative processes implicate neuro-inflammatory conditions, representing a hallmark of CNS damage. Accordingly, KMO-Inhibitors represent a common therapeutic drug target in various neurological disorders (68).

Exercise and the kynurenine pathway

Evidence suggests that physical exercise has beneficial effects on diseased populations such as persons with Cancer (21), Parkinson's disease (28) or Multiple Sclerosis (49). To date, our understanding of the underlying mechanisms is limited. Some studies in both, human (2, 4, 10, 11, 31, 32, 39, 41, 45, 47, 50, 60, 63, 64) and rodent (1, 38) have shown that physical exercise influences the KYN pathway, thereby representing a potential link between an active lifestyle and primary and tertiary disease prevention. Although studies in rodents investigating the impact of exercise on KYN pathway activation may represent a suitable approach to examine potential underlying mechanisms highly standardized, especially in view of enzyme expression in different tissues, it is worth mentioning that discrepancies in metabolism between humans and rodents impair the transferability of results. In contrast to humans, high output of nitric oxide by inducible nitric oxide synthase (iNOS) has been reported in rodents (71). Since nitric oxide radical is known to supress IDO expression (71), different responses in KYN pathway activation mediated by IDO in immune cells can be assumed. Therefore, among others, studies in rodents may predominantly focus on skeletal muscle and the further course of the KYN pathway. Overall, existing studies in both human and rodent strongly differ in regard to exercise modalities, outcome measures and study populations.

Training studies with humans were inspired by a promising preclinical investigation of Agudelo et al. (1). The authors have shown that endurance exercise training increases muscle expression of KATs through an activation of the transcription coactivator PGC-1α1 in a mouse model of depression. Subsequently, increased serum/plasma KYN levels, as they can also be observed in depressed humans, were degraded to KA. In contrast to KYN, KA is not able to pass the blood brain barrier. Thereby, exercise reduced neurotoxic effects of KYN and its downstream metabolite QA. In fact, exercise-induced reductions of CNS KYN levels were associated with decreased inflammatory stress, increased levels of neurotrophic/protective factors and reduced symptoms of anxiety and depressions. In another mouse model, Kim et al. (38) revealed that exercise counteracts a decline in cognitive performance in QA-induced Huntington's disease.

In contrast to the promising results of animals studies, a clinical trial by Millischer et al. (47) comparing the impact of three non-supervised 12-week exercise interventions varying in intensity (yoga vs. moderate aerobics vs. vigorous aerobics and strength training) in depressed humans did not show any effects on KYN and KA levels. These results are in line with those of Hennings et al. (31) who found no impact of one week of non-supervised "increased physical activity" (daily fitness/stretching exercise) on KYN levels and markers of inflammation in persons suffering from major depressions. Unfortunately, Hennings et al. (31) did neither report KYN/TRP ratios nor what and how much "activity" was performed. Interestingly, both studies revealed reduced self-reported levels of depressions after the exercise interventions. Similar to Agudelo et al. (1), Allison et al. (2) described elevated KAT expression in muscle tissue of healthy older men after a 12-week supervised multimodal (resistance and endurance) exercise program. Reductions in plasma KYN and increases in KA levels did not reach statistical significance. Unfortunately, again no ratio was calculated. A recent study by Herrstedt et al. (32) investigated the impact of a chemotherapy-accompanying 12-week combined exercise in persons with gastro-esophageal junction cancer on TRP metabolites in blood plasma and enzyme expression in skeletal muscle. While the QA precursor 3-HK increased in the control group, no significant changes were observed in the exercise group. Furthermore, KMO expression in skeletal muscle was significant higher in the control group post intervention. These results indirectly suggest that regular exercise has the potential to alleviate treatment-induced alterations along the KYN pathway. However, conclusions should be drawn cautiously since strong methodological limitations exist in this study as recently pointed out by Zimmer et al. (73). Moreover, Küster et al. (39) investigated the effects of a ten-week intervention of physical training with older adults at risk of dementia on a broad range of TRP metabolites. No significant changes were revealed for any outcome in the physical training group. Absent changes might result from low training adherence or selected exercise modalities, since important information on both is insufficiently described. Finally, a study by Bansi et al. (10) has focused on the KYN/TRP ratio in persons with different subtypes of MS. Two different supervised three-week endurance training programs led to a significant increase in the KYN/TRP ratio in persons with relapsing remitting MS, whereas no effects were observed in persons suffering from secondary progressive MS (10).

Regarding acute exercise, an initial activation of the KYN pathway, indicated by a significant increase in KYN/TRP ratio has been demonstrated in healthy trained adults (63, 64) and in persons with relapsing remitting MS (10). Keeping in mind that IDO, as responsible mediator for the conversion of TRP to KYN is sensitive to inflammatory cytokines, it has been proposed that its activation is caused by inflammatory stimuli as they appear during and after acute exercise (69). Nevertheless, since high levels of cortisol were suggested to stimulate TDO activity (9) and acute exercise is known to increase peripheral cortisol levels (29, 34, 54), it cannot be ruled out that activation of the exercise-induced KYN pathway is caused exclusively by increased IDO activity. In view of the immunomodulatory properties of KYN itself, an activation of the KYN pathway is of major research interest due to two reasons. Firstly, repetitive short-term increases in KYN levels represent a potential explanation for the mechanistic interaction between acute exercise-induced responses and chronic adaptions of the immune system. Secondly, persons with inflammation-mediated diseases (e.g. MS, Parkinson's disease) could benefit from short-term elevated KYN levels regarding its long-term anti-inflammatory effects. However, this hypothesis remains to be addressed since no further KYN metabolites of the neuroactive branch, which are highly relevant in MS, were measured in the study by Bansi et al. (10). Beside an initial activation of the KYN pathway, some studies also suggest acute exercise-induced alterations on the further regulation of the KYN pathway. These studies consistently indicate, primarily in healthy populations, that acute endurance exercise upregulates the concentrations of KA and QA (41, 50, 60). Similar to IDO, increased KMO activity is suspected to be mediated by inflammatory stimuli (72), representing a potential explanation for increased QA levels after acute exercise. However, it remains unclear whether KMO activity is influenced by inflammatory stimuli directly or as a consequence of an overall metabolic activation of the KYN pathway as described above. As potential underlying mechanism of increases in KA, it was hypothesized that not only chronic training, but also acute exercise can upregulated gene expression of KATs through PGC-1α1 in skeletal muscle (50). PGC-1α represents a well-studied coactivator that plays a pivotal role in oxidative metabolism through its impact on mitochondrial biogenesis and angiogenesis and is further involved in adaptions in skeletal muscle fiber type composition (3). Especially acute endurance exercise (7, 53) but also resistance exercise is known to upregulate PGC-1α (59, 62).

In summary, current evidence (detailed overview is provided in Table 3) proposes a modifying effect of acute and chronic exercise on different sections of the KYN pathway due to distinct underlying mechanisms with promising perspectives on health benefits (see Figure 1). Nevertheless, knowledge about specific exercise modalities to impact the KYN pathway is strongly restricted by the methodological heterogeneity of existing studies. Limitations of the current state of research also comprise a lack of studies that directly compare acute effects of different exercise modalities (type, duration, inten-

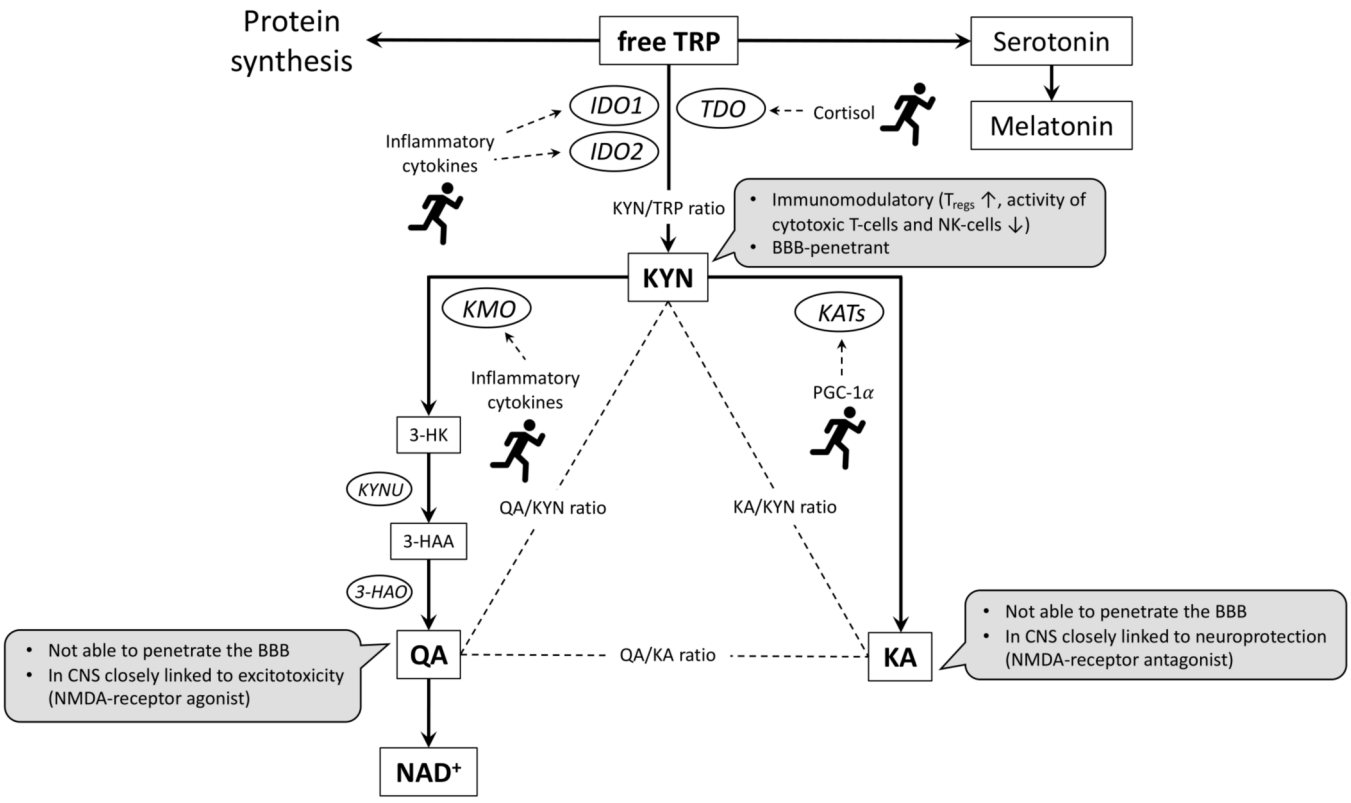


Figure 1. Potential acute exercise-induced mechanisms impacting the kynurenine pathway.
Modulatory effect of acute exercise; *TRP* tryptophan; *KYN* kynurenine; *KA* kynurenic acid; *QA* quinolinic acid; *3-HK* 3-hydroxykynurenine; *3-HAA* 3-hydroxyanthranilic acid; *NAD+* Oxidized form of nicotinamide adenine dinucleotide; *IDO* indoleamine 2,3-dioxygenase; *TDO* tryptophan 2,3-dioxygenase; *KMO* kynurenine 3-monooxygenase; *KATs*, kynurenine aminotransferases; *KYNU* kynureninase; *3-HAO* 3-hydroxyanthranilic acid oxygenase; *PGC-1α* proliferator-activated receptor-gamma coactivator-1alpha; T_{regs}, regulatory T-cells; *NK-cells* natural killer cells; *CNS* central nervous system; *BBB* blood-brain barrier; *NMDA* N-methyl-d-aspartate.

sity and frequency) under physiological conditions. Furthermore, no data on follow-up measurements exists to evaluate the kinetics of exercise-induced alterations in the KYN pathway. More detailed information about the effects of acute bouts of exercise could help to appropriately design long-term intervention studies. Future randomized controlled trials seem to be of major relevance to achieve sustainable modulation or normalization within the KYN pathway and its consequences, especially in view of populations with inflammation-mediated chronic diseases and dysregulations within the KYN pathway.

Here, we investigated the impact of acute resistance (RE) and endurance exercise (EE) on the KYN pathway. We hypothesize that KYN pathway alterations will be greater following endurance exercise compared to resistance exercise. A broad range of metabolites along the KYN pathway, IL-6 and levels of cortisol of healthy participants were determined in blood serum at pre, post and 1h follow-up measurement. Additionally, gene expression of IDO1 and KAT4 in circulating PBMCs was measured to provide further knowledge about underlying mechanisms of exercise-induced modulations of the KYN pathway. To determine potential relations between initial KYN pathway activation parameters and IL-6, cortisol and immune cell subsets during a resting condition, we performed baseline flow cytometry analysis.

Methods

Participants

A total of 24 healthy males (N=24) were recruited for participation. The inclusion criteria comprised being male, between 20 and 35 years of age, no contraindications to physical exercise and no drug intake during the last six weeks. Inclusion criteria were checked before study participation using the german version of the Physical Activity Readiness Questionnaire (PAR-Q) (22) and an additional question on drug intake during the last six weeks. All participants signed a written informed consent prior to participation. The study was approved by the local ethics committee of the German Sport University Cologne and was prospectively registered at the German clinical trial register (DRKS00014286).

General procedure

A randomized cross-over study design including two intervention arms was applied. An initial baseline testing was conducted one week prior to the intervention. At baseline, participants' maximal endurance and strength capacity were measured in order to standardize and control exercise intensity. Subsequently, all participants performed a single bout of endurance exercise (EE) and resistance exercise (RE) with a wash-out period of at least 48 hours between both exercise

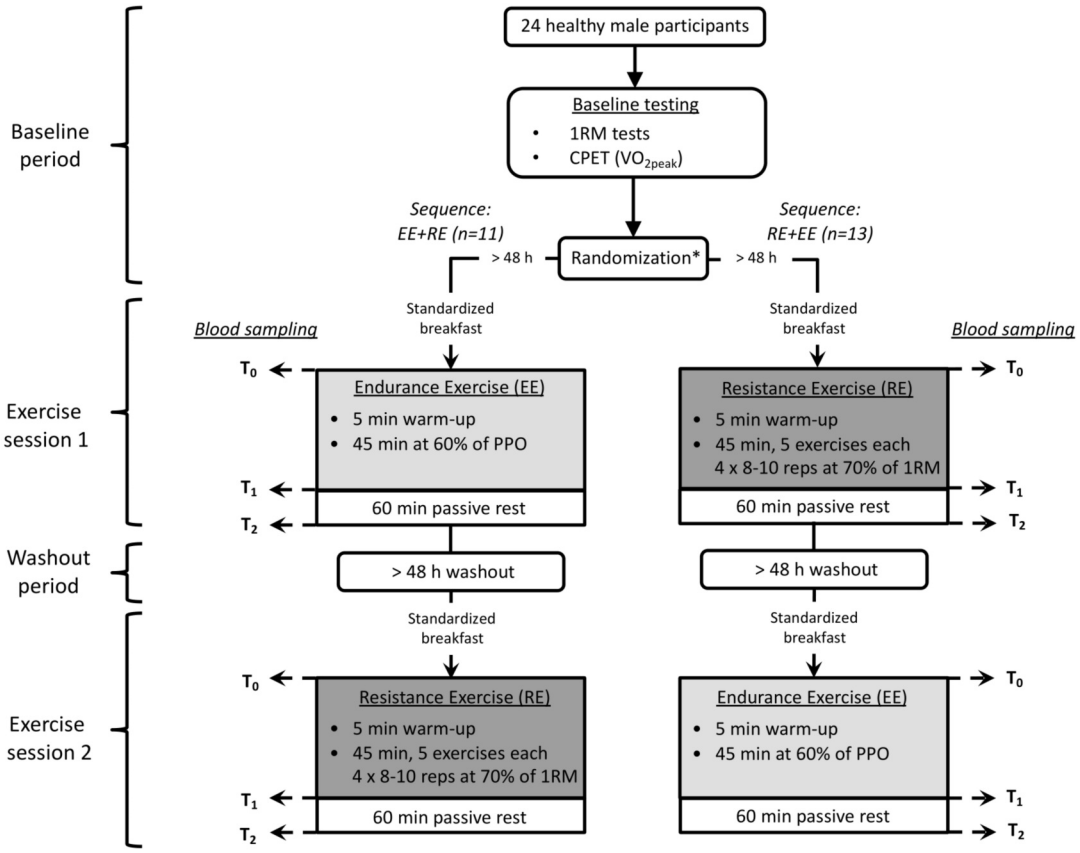


Figure 2. Schematic overview of the study design.
* Participants' smoking status, VO_{2peak}-levels and total strength values were used as stratification factors.
EE endurance exercise; *RE* resistance exercise; *1RM* one-repetition maximum; *CPET* cardiopulmonary exercise test; VO_{2peak} maximal oxygen uptake; *PPO* peak power output; *Reps* repetitions; T_0 resting condition (baseline); T_1 immediately after exercise cessation; T_2 one hour after exercise cessation.

sessions, since acute effects on cytokines and cellular immune components do usually not persist longer than 24 hours. The sequence of the interventions (EE+RE or RE+EE) was randomly allocated (see Randomization). Venous blood samples were collected immediately before (T_0), immediately after (T_1) and one hour after (T_2) both exercise sessions, respectively. The follow-up measurement time point was set at one hour after completion of the exercise sessions in order to provide outcome kinetics and to potentially define a time frame of occurring alterations. One hour prior to each session participants received a standardized breakfast. Participants were told to avoid the consumption of nicotine, supplements and alcohol and to avoid any amount of physical exercise for 48 h prior to baseline testing and each exercise session. Exercise sessions started between 8 and 11 AM. The study design is depicted in Figure 2.

Sample Size Calculation

Sample size calculation was conducted to examine different responses of EE and RE in the change of KYN/TRP ratio post intervention using G*power software (Version 3.1.9.2) (25) in a baseline-adjusted ANCOVA model. Sample size calculation was performed according to the formula $(1-\rho^2)*n$. In detail, ρ represents the estimated correlation between pre and post measurements and n represents the calculated sample size by using G*power software that would have been appropriate for an independent t-test comparing post measurements of both groups. We determined sample size calculation parameters based on the results of the study of Strasser et al. (63) investigating the acute effect of an exhaustive endurance exercise on KYN/TRP ratio in healthy athletes. The data of Strasser et al. (63) revealed an effect size (Cohen's *d* (19)) of -.804 and a correlation between pre and post measurements of r = .856. In contrast to Strasser et al. (63), our study was designed to compare acute responses of two different exercise modalities. Therefore, sample size was calculated using a more conservatively estimated effect size of d = -.65. Since we implemented two post measurement time points in our study (T_1, T_2), estimated correlation between pre and post measurements were reduced to $\rho = .7$ and alpha (α) was adjusted to $\alpha = .025$. Test power was set at 80%. Calculation revealed a total sample size of N = 48. Due to the applied cross-over design implicating that both intervention arms are completed by all participants, the required sample size resulted in N = 24. Sample size calculation was performed on the assumption that no carry-over effects exist.

Randomization

Randomization was carried out immediately after baseline testing using Randomization In Treatment Arms (RITA, Evi-

dat, Germany) software. A stratified randomization procedure according to Pocock and Simon's minimization method was used (for Review see (61)). Stratification factors comprised participants' smoking status, relative oxygen uptake maximum (VO_{2peak}) and a total strength value (see Baseline testing). Randomization was conducted by an independent research associate who was not involved in data collection.

Baseline testing

The baseline testing consisted of one-repetition maximum (1RM) tests followed by a cardiopulmonary exercise test (CPET) on a bicycle ergometer after a one hour resting break. Participants were asked to refrain from eating during the hour before the baseline testing. During the testing procedure participants were allowed to drink water ad libitum.

One-repetition maximum (1RM) tests

Following a five min warm-up on a cycle ergometer at 50 W, participants performed five machine-based (Cybex International, Medway, US) 1RM tests conducted in the following order: chest press, lat pull, leg curl, leg extension, back extension. The 1RM test at each machine started with a warm-up set consisting of 15 repetitions of self-selected resistance through a full range of motion. Following a 2-min break, participants were given five attempts to attain their 1RM at each machine. Within each attempt, the maximum amount of repetitions was conducted until exhaustion. The attempts at one machine were separated by a 2-min break, respectively. The 1RM was reached if exactly one repetition was feasible with high quality of movement execution. If the 1RM at one machine was not reached within the five attempts, the weight of the last attempt counted as maximum. Between the 1RM tests of each of the five machines a break of 1.5 min was implemented, respectively. A total strength value was calculated for each participant using the mean of all five 1RM weights. The total strength value was used for stratification.

Incremental cardiopulmonary exercise test (CPET)

An incremental CPET on a bicycle ergometer (Ergoline, Germany) with spirometry (Cortex, Meta-Lyzer 3B-R2, Germany) was conducted to determine participants' relative maximum oxygen uptake (VO_{2peak} [ml/kg/min]), maximum heart rate (HRmax [beats per minute (bpm)]) and peak power output (PPO). Participants started with a five-minute warm-up period at 25 W. Subsequently, workload was set to 100 W and was increased by 20 W/min until the participant was not able to maintain pedal cadence above 60 rounds per minute (rpm). Participants' heart rate, respiratory quotient (RQ) and rate of perceived exertion (RPE (13)) were recorded at exhaustion. PPO was defined as the average of the highest ten second interval, whereas VO_{2peak} was calculated as the average of the highest ten second interval. Additionally, lactate levels were measured at rest (La_{pre}) and at exhaustion (La_{post}).

Interventions

Participants received a standardized breakfast consisting of a cereal bar and a banana one hour prior to each exercise intervention. Each exercise session lasted 50 minutes in total and started at the same time of day. Exercise duration of 50 minutes was chosen to represent an extended and realistic training stimulus that could be transferred to clinical populations. Participants were allowed to drink water ad libitum.

Endurance Exercise (EE)

Following a five min warm-up at 50 W, participants cycled for 45 min at a power output corresponding to 60% of PPO. Participants were asked to refrain from standing during the trial and asked to maintain a cadence between 60 and 80 rpm.

Resistance Exercise (RE)

Following a five min warm-up at 50 W on a cycle ergometer, participants conducted four sets at each of the five resistance machines used in the baseline testing for 1RM determination in equal order (chest press, lat pull, leg curl, leg extension, back extension). A specific warm-up set comprising 15 repetitions at 30% of 1RM was implemented immediately before the exercise at each resistance machine. Each set consisted of 8-10 repetitions at 70% of the attained 1RM. Resting time between the sets and between the five resistance machines was set at 1 min.

Outcomes and measurements

All outcomes were assessed at each measurement time point (T_1, T_2, T_3). Concentrations of IL-6 were determined using enzyme-linked immunosorbent assay (ELISA) according to manufactures instructions (R&D Systems, Human IL-6 Quantikine ELISA Kit; detection limit: 0.70 pg/ml, coefficient of variability for intra-assay precision: 4.2%). Concentrations of cortisol were measured using chemiluminescence immunoassay (CLIA) by contract laboratory (Labor Dr. Wisplinghoff, Cologne, Germany). TRP and its metabolites KYN, KA and QA were analyzed by high performance liquid chromatography (HPLC). To obtain detailed information on changes in KYN pathway balance, ratios of KYN/TRP, KA/KYN, QA/KYN and QA/KA were calculated. Moreover, gene expression of central enzymes along the KYN pathway (IDO1 and KAT4) were measured in PBMCs. In addition to the outcomes, flow cytometry analysis was performed at baseline (T_0) to evaluate potential relationships between numbers and proportions of immune cell subsets (lymphocytes, NK-cells, NK-cell subsets (dim/bright), cytotoxic T-cells, T-helper cells and regulatory T-cells) and initial KYN pathway activation parameters (KYN, KYN/TRP, IDO1). Detailed information on the procedure of haematological analyses is provided in the following.

Blood sampling

Blood samples were drawn at each measurement time point in supine position from a medial cubital vein. Blood samples were used (i) to isolate serum and (ii) to isolate PBMCs. Regarding serum isolation, blood was rested at room temperature for 10 min for clotting and thereafter centrifuged at 3500 rpm for 10 min. Serum samples were aliquoted and stored at -80°C until analyses with HPLC, ELISA and CLIA were per-

formed. EDTA blood was used for PBMC isolation by density gradient centrifugation using a lymphocyte separation medium (PromoCell, Heidelberg, Germany). In brief, blood was layered on top of the lymphocyte separation medium and centrifuged for 30 min at 800xg. Thereafter, the PBMC containing interphase was separated and washed with PBS. PBMCs were aliquoted and frozen at -150°C until analyses with real-time quantitative polymerase chain reaction and flow cytometry were performed.

High performance liquid chromatography (HPLC) and mass spectrometry

Chemicals and materials

For the preparation of aqueous solutions and eluents, ultrapure water obtained from a Sartorius Stedim Arium® pro UV apparatus (Guxhagen, Germany) was used. Acetonitrile (ACN), ammonium acetate, acetic acid, and ammonium hydroxide solution (25% NH3) of analytical grade were from Merck (Darmstadt, Germany). Reference compounds (purity ≥98%) including TRP, KYN, KA and QA as well as pooled human serum (male donors) were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). As internal standards (ISTDs), multiply deuterium-labelled analogues L-Tryptophan-d5 (TRP-d5), Kynurenic acid-d5 (KA-d5) and 2,3-Pydridinedicarboxylic acid-d3 (QA-d3) were obtained from Toronto Research Chemicals (North York, ON, Canada).

Sample preparation/analyte extraction

In accordance to previously published protocols (5), aliquots of 50 µL of serum were spiked with 5 µL of the ISTD [10 µg/mL TRP-d5, 1 µg/mL KA-d5 and 10 µg/mL QA-d3] and were further diluted by the addition of 25 µL of water and 70 µL of ACN. After 5 min of sonication, serum proteins were precipitated by centrifugation at 14.000xg at room temperature (RT) for another 5 min. The supernatant was separated from the obtained pellet and transferred to a fresh polypropylene vial. Subsequently, 5 µL of 25% NH3 was added and samples were ready for analysis.

Liquid chromatography (LC)

Liquid chromatographic separation was achieved by using an ACQUITY UPLC® system (Waters GmbH, Eschborn, Germany) equipped with a Gemini C6-Phenyl analytical column (100 mm x 2 mm ID, 3.0 µm particle size, 110 Å) from Phenomenex (Aschaffenburg, Germany). Gradient elution was carried out using 5 mM ammonium acetate solution acidified with 0.1% acetic acid as solvent A and acetonitrile fortified with 5% of Eluent A as solvent B. The overall gradient program lasted 20 min and the flow rate was set to 250 µL/min. Initiated by the injection of 2 µL of the sample into the LC system, the analytical run started with 1 min of constant flow of 100% A. Subsequently, the organic phase was increased to 40% within 6 min and to 100% within another 2 min. Finally, the initial conditions of 100% A were restored followed by re-equilibration for 10 min.

Mass spectrometry

The mass spectrometric analysis was performed on a Xevo® TQ-XS triple quadrupole mass spectrometer (QqQ/MS) (Waters GmbH, Eschborn, Germany) equipped with a UniSpray™ (US) ion source. The system was operated in positive ionization mode (US+) with activated soft transmission mode. The ion source was set to a desolvation temperature and cone voltage of 400°C and 45 V, respectively. Data were generated by multiple reaction monitoring (MRM) experiments after optimization of the method and individual tuning of all analytes of interest. Argon (purity grade 5.0) was used as damping gas for collision-induced dissociation (CID) experiments. Waters' Mass-Lynx software (V4.2 2016) was used for result interpretation. Further information is provided in supplement 1.

Method evaluation and result interpretation

During the evaluation of the detection method, parameters such as specificity, linearity of calibration curves, lower limit of quantification (LLOQ), recovery and intraday precision were assessed. To ensure the method's specificity, 12 blank serum samples from different female and male volunteers (n = 6 + 6) were analyzed after activated charcoal stripping as published elsewhere (48). Samples were successfully tested for the absence of potentially interfering signals at expected retention times. Moreover, analogously purified commercially available pooled human serum was used for the determination of further validation parameters. Calibration curves for quantitative result interpretation of all target analytes were prepared individually and were found to be linear within appropriate concentration ranges [TRP: 0.5, 2, 5 and 20 µg/mL; KYN: 20, 50, 200, 5000 and 2000 ng/mL; QA: 5, 20, 50, 200, 500 and 2000 ng/mL and KA: 0.5, 2, 5, 20, 50 and 200 ng/mL] with coefficients of correlation of greater than 0.98. Linear correlation was utilized to calculate concentrations of the measured analytes by their peak areas (peak area ration of analytes and corresponding ISTDs). The analytical performance between different sample batches on consecutive days was monitored by measuring quality control samples with defined concentrations. The LLOQ, defined as the lowest concentrations measurable via a signal-to-noise ratio (S/N) greater than 9 was estimated for each substance at 10 ng/mL (QA and KA), 25 ng/mL (KYN), and 50 ng/mL (TRP), and was confirmed by six sample replicates (n = 6) spiked at the aforementioned concentrations. With regard to the recovery during the sample work up, two sets of six charcoal stripped serum aliquots (n = 6 + 6) were fortified with 1 µg/mL of TRP and QA, 100 ng/mL of KYN and 50 ng/mL of KA. In the first set, the analytes were added simultaneously with the ISTDs before sample preparation while for the second set spiking was carried out just before injection to the LC-MS system. Resulting recoveries were between 66-76%. Moreover, six sample replicates at 3 concentration levels (high, medium and low, n = 6 + 6 + 6) were prepared and analyzed to determine the intraday imprecision. Values ranged between 2-18% for TRP, 2-12% for KYN, 2-15% for QA and 3-13% for KA. Further details are summarized in supplement 1.

Real-time quantitative polymerase chain reaction (qRT-PCR)

For gene expression analyses PBMCs were thawed and RNA was isolated using TRIzol reagent (Merck, Darmstadt, Germany) according to the manufacture´s protocol. In brief, phenol chloroform phase separation was used and the aqueous

phase containing the RNA was further processed. RNA was precipitated with isopropanol, washed with ethanol and resuspended in RNAse free water. cDNA synthesis and qRT-PCR were performed using the GoTaq 2-Step qRT-PCR Kit (Promega GmbH, Mannheim, Germany) according to the manufacture´s protocol. For cDNA synthesis a maximum of 1µg RNA per reaction was applied and 4 reactions per sample were performed. The cDNA of these 4 reactions per sample were pooled for further qPCR analysis. Primer for IDO1 and KAT4 were described previously by Dewi et al. (23) and Agudelo et al. (1), respectively. As reference genes HPRT1 and UBE2D2 were chosen, since Oturai et al. (55) found them to be stably expressed in PBMCs. Primers for the housekeeping genes were generated to span Exon-Exon junctions (HPRT1: forward GCGTCGTGATTAGTGATGATG, reverse GTTCAGTCCTGTCCATAATTAGTC; UBE2D2: forward ACTAACTATTTCAAAAGTACTCTTGTCCATCT, reverse CGAGCTATTCTGTTGTACTTTTCTCTA). Gene expression was analyzed by comparison of the delta Ct values of the target genes.

Flow cytometry

PBMCs were thawed for flow cytometry analyses. Two different panels were analysed. In the first panel PBMCs were stained with anti-CD3 PE-Cy7, anti-CD8 PE, anti-CD4 APC, anti-CD16 PE (BD Bioscience, Heidelberg, Germany) and anti-CD56 APC-Cy7 (BioLegend, San Diego, CA, USA). Lymphocytes were gated by size and granularity. Cytotoxic T-cells were gated as $CD3^+CD8^+$, whereas T-helper cells were gated as $CD3^+CD4^+$. NK-cells were gated as $CD3^-CD16^+$ and further divided into $CD56^{dim}$ and $CD56^{bright}$ NK-cells. In the second panel PBMCs were stained with anti-CD3 PE-Cy7, anti-CD4 APC-Cy7, anti-CD127 PE and anti-CD25 APC (BD Bioscience, Heidelberg, Germany). T_{regs} were gated as $CD3^+CD4^+CD25^+CD127^{dim}$. Flow cytometry was performed on a FACS Array (BD Bioscience, Heidelberg, Germany) and gating was performed using the BD FACS Diva Software.

Statistical analyses

To investigate relationships between IL-6, cortisol, immune cells subsets and initial KYN pathway activation outcomes (KYN, KYN/TRP, IDO1) at baseline (T_0), Pearson's correlation coefficient (r) was calculated. To examine the hypothesis of this study, within and between differences in EE and RE post intervention were tested in TRP metabolites, IL-6, cortisol and KYN pathway enzyme expression measured in PBMCs. Therefore, outcome data was first tested for potential significant interaction effects of intervention sequences (EE+RE vs. RE+EE) or intervention periods (trainings session day 1 vs. training session day 2) using a baseline-adjusted analysis of covariance model (ANCOVA) for each outcome (necessary pre testing for cross-over design). If no effects of intervention sequence or periods were revealed, data of equal training modalities were pooled for further analyses. Separated baseline-adjusted ANCOVA models were conducted for all outcomes to examine time and interaction (time*group) effects of EE and RE. Mauchly's test of Sphericity was applied to detect potential violations and Greenhouse-Geisser correction was used if necessary. In case of significant ANCOVA results Bonferroni corrected post-hoc analyses were conducted. In addition, Cohens' *d* effect sizes were calculated for significant results. For all statistical analyses parametric procedures were conducted using SPSS statistics 25 (IBM®, Armonk, NY, USA). Level of significance was set at $p \leq .05$.

Results

Baseline characteristics for all participants and each intervention sequence (RE+EE and EE+RE) for anthropometric data, CPET and 1RM tests are provided in Table 1. The investigated sample revealed an overall mean age ± standard deviation (SD) of 24.6 ± 3.9 years, an overall mean VO_{2peak} ± SD of 48.3 ± 7.4 ml/kg/min and an overall mean of the total strength value ± SD of 101.9 ± 16.2 kg.

Table 1. Baseline characteristics of study participants separated by exercise sequence.

	RE+EE (n=13)	**EE+RE** (n=11)	**Overall** (n=24)
Anthropometric and demographic characteristics			
Age (years)	24.8 ± 4.8	24.4 ± 2.7	24.6 ± 3.9
Height (cm)	182.3 ± 6.8	182.5 ± 5.4	182.4 ± 6.2
Weight (kg)	81.6 ± 10.7	86.6 ± 10.1	83.9 ± 10.5
BMI (kg/m²)	24.5 ± 2.1	26 ± 3.3	25.4 ± 2.7
Smoking status (yes / no)	2 / 11	4 / 7	6 / 18
Performance characteristics			
$\dot{V}O_{2peak}$ (ml/kg/min)	48.8 ± 8.0	47.8 ± 6.9	48.3 ± 7.4
HR_{pre} (bpm)	78.8 ± 8.0	78.8 ± 8.6	78.8 ± 8.1
HR_{max} (bpm)	186.8 ± 7.3	184.5 ± 6.6	185.6 ± 7.0
La_{pre} (mmol/l)	1.1 ± 0.6	0.8 ± 0.2	1.0 ± 0.5
La_{post} (mmol/l)	10.0 ± 2.0	10.2 ± 1.3	10.1 ± 1.7
RER_{max} ($\dot{V}CO_2/\dot{V}O_2$)	1.14 ± 0.0	1.16 ± 0.0	1.15 ± 0.0
PPO (W)	327.7 ± 68.1	336.4 ± 44.6	331.7 ± 57.5
Watt/kg	4.0 ± 0.6	4.0 ± 0.7	4.0 ± 0.6
1RM Chest press (kg)	109.7 ± 25.1	116.8 ± 17.6	113.3 ± 22.1
1RM Lat pull (kg)	99.5 ± 22.2	103.5 ± 21.4	102.0 ± 21.5
1RM Leg curl (kg)	72.5 ± 10.1	76.8 ± 16.7	74.1 ± 13.2
1RM Leg extension (kg)	115.0 ± 26.1	122.7 ± 23.8	120.9 ± 24.8
1RM Back extension (kg)	100.3 ± 21.6	100.0 ± 20.1	100.9 ± 20.4
Total strength value (kg)	99.7 ± 17.9	104.5 ± 14.2	101.9 ± 16.2

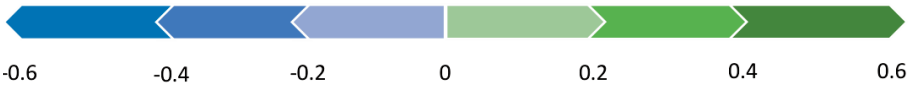
Values are presented as mean ± standard deviation. *RE* resistance exercise; *EE* endurance exercise; *BMI* body mass index; VO_{2peak} peak oxygen uptake; HR_{pre} heart rate before incremental exercise test; HR_{max} maximal heart rate; La_{pre} lactate level immediately before cardiopulmonary exercise test; La_{post} lactate level immediately after cardiopulmonary exercise test; RER_{max} maximal respiratory exchange ratio; *PPO* peak power output; *Watt/kg* relative power output; *1RM* one repetition maximum; *Total strength value* mean of all 1RM tests.

Results of bivariate correlational analyses between IL-6, cortisol, immune cell subsets and initial KYN pathway activation outcomes (KYN, KYN/TRP, IDO1) at baseline are presented in Table 2. Significant correlations were revealed between IL-6 and IDO1 (dCt HPRT1: r = -.318, p = .031; dCt UBE2D2: r = -.389, p = .008), IL-6 and KYN/TRP (r = .473, p = .001). Regarding cortisol, concentrations significantly correlated with KYN/TRP (r = -.366, p = .012). Furthermore, proportions of T_{regs} significantly correlated with IDO1 (dCT HPRT1: r = -.356, p = .014). Concerning proportions of cytotoxic T-

Table 2. Baseline correlations (Pearson's coefficient) between IL-6, cortisol, immune cell subsets and KYN pathway outcomes.

	KYN	KYN/TRP	IDO1 (dCt HPRT1)	IDO1 (dCt UBE2D2)
IL-6	.288	.473**	-.318*	-.389**
Cortisol	-.204	-.366*	-.094	-.115
T-helper cells (p)	-.070	-.180	.147	.196
T-helper cells (n)	-.121	-.231	.261	.254
T_{regs} (p)	.023	.082	-.356*	-.282
T_{regs} (n)	-.096	-.131	-.076	-.071
Cytotoxic T-cells (p)	.173	.002	.532**	.464**
Cytotoxic T-cells (n)	.071	-.069	.529**	.439**
NK-cells (p)	.199	.306*	-.193	-.220
NK-cells (n)	.098	.227	-.114	-.153
NK-cells ($CD56^{dim}$) (p)	-.209	-.357*	.353*	.282
NK-cells ($CD56^{dim}$) (n)	.068	.188	-.090	-.133
NK-cells ($CD56^{bright}$) (p)	.208	.356*	-.348*	-.277
NK-cells ($CD56^{bright}$) (n)	.248	.396**	-.234	-.230

Graphic representation of Pearson's correlation coefficient.



* p ≤ .05; ** p ≤ .01; *(p)* proportions *(n)* numbers. Proportions of T-helper cells, cytotoxic T-cells and NK-cells refer to Lymphocytes. Proportions of T_{regs} refer to $CD3^+$ lymphocytes. Proportions of NK-cells ($CD56^{dim}$) and NK-cells ($CD56^{bright}$) refer to NK-cells.

cells, significant correlations to IDO1 (dCt HPRT1: r = .532, p < .001; dCt UBE2D2: r = .464, p = .001) were revealed. In addition, numbers of cytotoxic T-cells correlated significantly with IDO1 (dCt HPRT1: r = .529, p < .001; dCt UBE2D2: r = .439, p = .002). For proportions of NK-cells, analysis indicated significant correlations to KYN/TRP (r = .306, p = .039). Moreover, proportions of $CD56^{dim}$ NK-cells significantly correlated with IDO1 (dCt HPRT1: r = .353, p = .015) and KYN/TRP (r = -.357, p = .015). Regarding proportions of $CD56^{bright}$ NK-cells, significant correlations to IDO1 (dCt HPRT1: r = -.348, p = .016) and KYN/TRP (r = .356, p=.015) were detected. Finally, analysis revealed a significant correlation between numbers of $CD56^{bright}$ NK-cells and KYN/TRP (r = .396, p = .007). A comprehensive overview of correlation coefficients is provided in Table 2.

To examine necessary assumptions considering the cross-over study design, outcome data were pretested for potential significant interaction effects of intervention sequences (EE+RE vs. RE+EE) or intervention periods (trainings session day 1 vs. training session day 2) using baseline-adjusted ANCOVAs for each outcome. Regarding KAT (HPRT1), a significant interaction effect of intervention periods was detected (p < .005, F = 7.365, df = 1.299). For all other outcomes, neither a significant effect of intervention sequences nor of intervention periods was revealed. Therefore, outcome data was pooled before conducting ANCOVA models for each parameter with training modality (EE vs. RE) as between subjects factor.

ANCOVA results of IL-6 showed a significant time (p < .001, F = 12.282, df = 1,355) and interaction (p < .001, F = 12.090, df = 1,355) effect. Bonferroni corrected post-hoc test revealed a significant increase from T_0 to T_1 (p < .001, d = 1.322), from T_0 to T_2 (p = .006, d = .549) and a significant decrease from T_1 to T_2 (p < .001, d = -.951) in EE. Significant interaction effects were detected at T_1 (p < .001) and T_2 (p = .015) with levels of EE being higher than those of RE, respectively. Concerning cortisol, a significant time (p < .001, F = 20.870, df = 1.648) and interaction effect (p < .001, F = 14.642, df = 1,648) was found. In EE, levels of cortisol were significantly increased at T_1 compared to T_0 (p < .001, d = 1.069). Furthermore, levels at T_2 were significantly decreased compared to levels at T_1 (p < .001, d = -.906). In RE, levels of cortisol were significantly decreased at T_2 compared to T_0 (p < .001, d = -.862) and T_1 (p < .001, d = -.752). Significant interaction effects were revealed at T_1 (p < .001) and at T_2 (p < .001) with levels of EE being higher than those of RE, respectively. In view of TRP, ANCOVA results showed a significant effect over time (p < .001, F = 19.894, df = 2). Bonferroni post-hoc test detected a significant decrease from T_0 to T_1 (p = .034, d = -.368) in RE. A significant effect over time was also revealed for KYN (p = .016, F = 4.359, df = 2), but Bonferroni post-hoc test did not show any significant differences between measurement time points in EE or RE. Regarding KYN/TRP ratio, ANCOVA resulted in a significant time effect (p = .041, F = 3.332, df = 2). In RE, values were significantly increased at T_1 compared to T_0 (p = .001, d = .474) and significantly decreased at T_2 compared to T_1 (p = .005, d = -.437). ANCOVA results of KA showed a significant time (p = .007, F = 5.631, df = 1.748) and interaction (p = .028, F = 3.987, df = 1.748) effect. Bonferroni post-hoc test

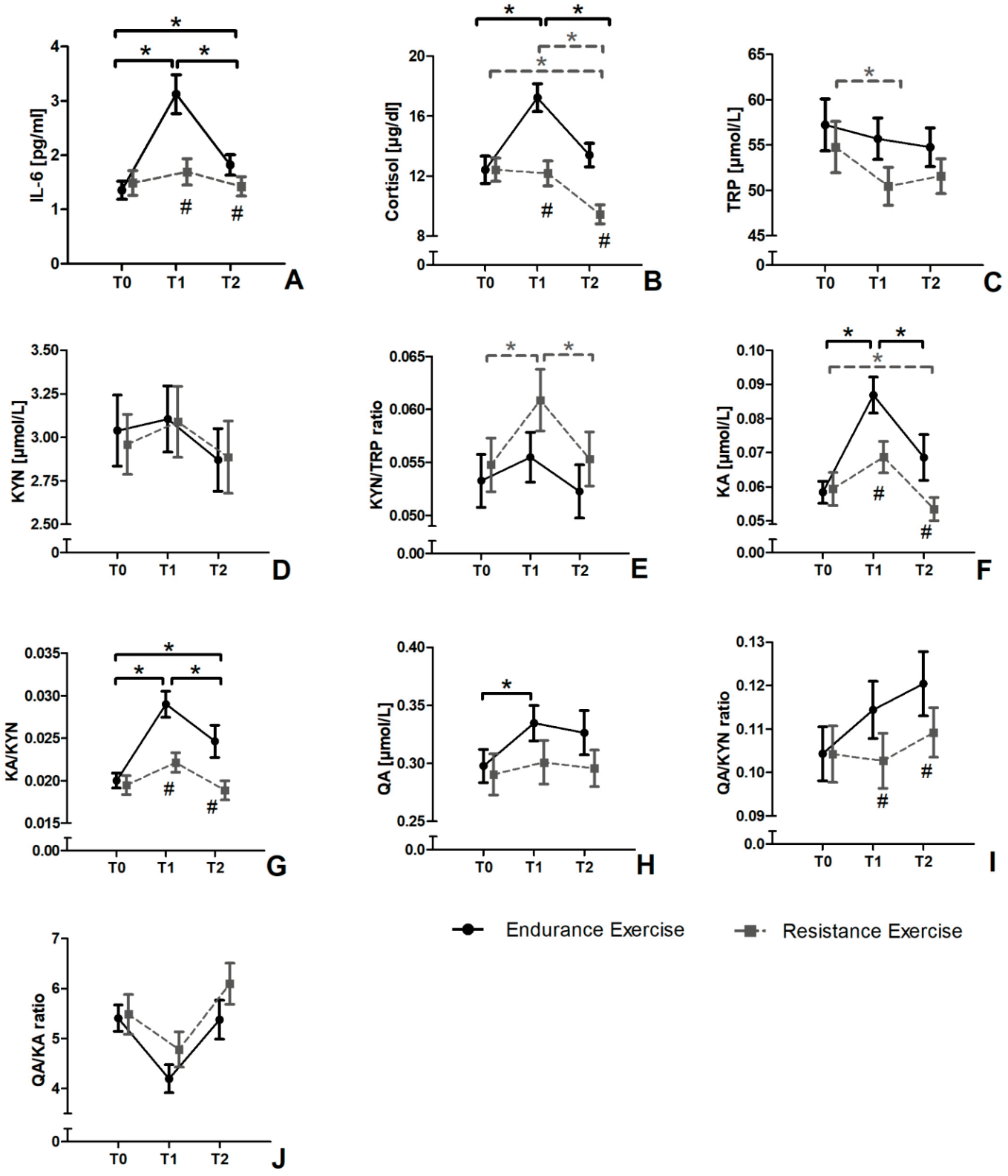


Figure 3. Kinetics of TRP metabolites separated by training intervention.
* significant time effect (p≤0.05); # significant interaction effect (p≤0.05); black shading indicates endurance exercise; grey shading indicates resistance exercise; *T0* baseline; *T1* post exercise; *T2* 1 hour follow-up; values are presented as mean ± standard deviation; *(A)* interleukin-6 concentrations; *(B)* cortisol concentrations; *(C)* tryptophan concentrations; *(D)* kynurenine concentrations; *(E)* kynurenine-tryptophan ratio; *(F)* kynurenic acid concentrations; *(G)* kynurenine-kynurenic acid ratio; *(H)* quinolinic acid concentrations; *(I)* quinolinic acid-kynurenine ratio; *(J)* quinolinic acid-kynurenic acid ratio.

revealed significantly increased levels at T_1 compared to T_0 ($p < .001$, $d = 1.384$) and significantly decreased levels at T_2 compared to T_1 ($p = .008$, $d = -.646$) in EE. Additionally, significantly decreased levels of KA were found at T_2 compared to T_1 ($p = .041$, $d = -.819$) in RE. In regard to the interaction effect, values of EE were significantly higher at T_1 ($p = .002$) and at T_2 ($p = .037$) than values of RE. Considering KA/KYN ratio, a significant time ($p = .024$, $F = 3.925$, $df = 2$) and inter-

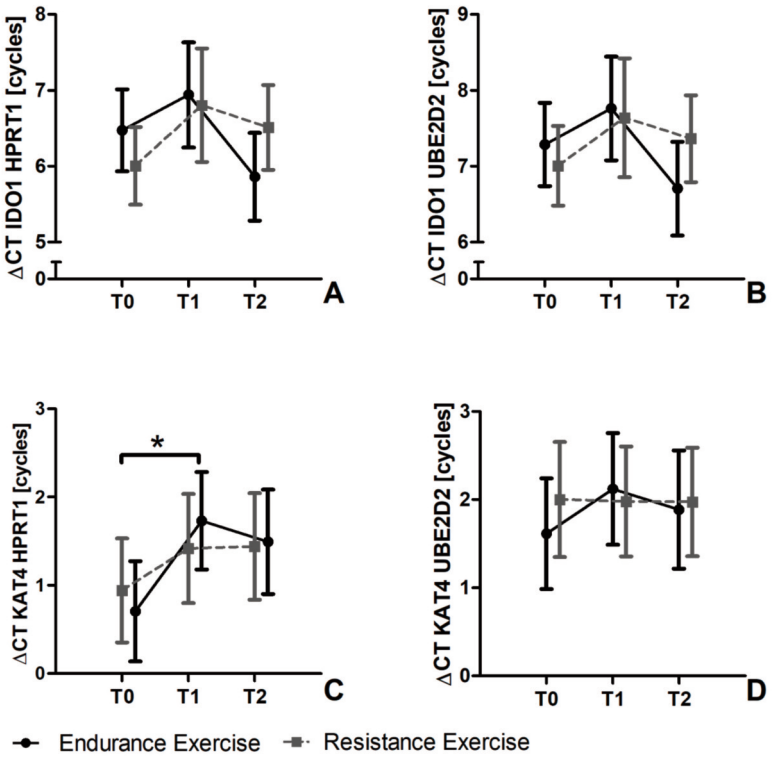


Figure 4. Kinetics of enzyme gene expression in PBMCs.
* significant time effect (p≤0.05); black shading indicates endurance training; grey shading indicates resistance training; *T0* baseline; *T1* post exercise; *T2* 1 hour follow-up; values are presented as mean ± standard deviation; *(A)* ΔCT of IDO1 expression in PBMCs referring to HPRT1 housekeeping gene; *(B)* ΔCT of IDO1 expression in PBMCs referring to UBE2D2 housekeeping gene; *(C)* ΔCT of KAT4 expression in PBMCs referring to HPRT1 housekeeping gene; *(D)* ΔCT of KAT4 expression in PBMCs referring to UBE2D2 housekeeping gene.

action effect (p = .002, F = 6.961, df = 2) was detected. In EE, values compared to T_0 were significantly increased at T_1 (p < .001, d = 1.565) as well as at T_2 (p = .004, d = .666). Moreover, values in EE were significantly decreased at T_2 compared to T_1 (p = .01, d = -.554). Significant interaction effects were found at T_1 (p < .001) and at T_2 (p = .007) with values of EE being higher than those of RE, respectively. For QA, a significant time effect was revealed (p = .014, F = 4.529, df = 2). Post-hoc test showed a significant increase from T_0 to T_1 (p = .008, d = .076) in EE. Furthermore, a significant interaction effect was detected for QA/KYN ratio (p = .016, F = 4.335, df = 2). Values of EE were significantly higher than those of RE at T_1 (p = .006) and at T_2 (p = .015), respectively. In view of QA/KA, no significant effects were revealed. Kinetics of all TRP metabolites separated by training interventions are illustrated in Figure 3.

Concerning ANCOVA results of IDO1 gene expression measured in PBMCs, no significant effects were detected, neither for HPRT1 nor for UBE2D2 calculated ΔCT values. ANCOVA results of ΔCT KAT4 HPRT1 gene expression in PBMCs revealed a significant effect over time (p = .001, F = 10.441, df = 1.254). Bonferroni post-hoc test showed significantly increased values at T_1 compared to T_0 (p = .019, d = .374) in EE. No significant effects were detected for ΔCT KAT4 UBE2D2. Kinetics of enzyme gene expression in PBMCs are shown in Figure 4.

Discussion

This is the first study investigating the acute impact of two different exercise modalities on a broad range of KYN pathway outcomes with simultaneous consideration of the potential mediators IL-6 and cortisol. Moreover, we examined associations between KYN pathway outcomes and immune cell subsets during a resting condition in humans and provide first information on outcome kinetics due to the implemented follow-up measurement time point one hour after exercise cessation. Overall, our results indicate a stronger activation along the KYN pathway induced by EE compared to RE. However, the KYN/TRP ratio was only significantly increased in RE immediately after exercise, representing an initial activation of this metabolic pathway. In line with previous investigations, our results emphasize an increased conversion of KYN to KA in response to EE (41, 50, 60).

Kynurenine pathway activation (TRP → KYN)

IL-6 serum concentrations positively correlated with both, the KYN/TRP ratio and IDO1 expression in PBMCs at baseline. These results support findings from previous studies reporting a stimulating role of IL-6 in the activation of the KYN pathway (20). Moreover, proportions of total NK-cells as well as numbers and proportions of cytokine producing $CD56^{bright}$ NK-cells positively correlated with the KYN/TRP ratio. In contrast, cytotoxic $CD56^{dim}$ NK-cell proportions negatively correlated with the KYN/TRP ratio. These associations between KYN pathway metabolites and NK-cell phenotypes support previously described immunomodulatory properties of KYN pathway activation (16). Frumento et al. (26) already showed that IDO activity inhibits NK-cell proliferation. However, NK-cell subsets were not investigated. In addition to KYN pathway metabolites, our results suggest a positive association between IDO1 expression in PBMCs and cytokine producing $CD56^{bright}$ NK-cells and a negative association with proportions of cytotoxic $CD56^{dim}$ NK-cells. These results give a first hint that NK-cell subsets may be differentially regulated by IDO1. In line with Frumento et al. (26) IDO1 expression showed a negative correlation to numbers and proportions of cytotoxic T-cells. Apart from an inhibition of the cytotoxic potential of different lymphocyte subsets, we also found an association between IDO1 expression and proportions of T_{regs} supporting earlier results (18, 33). In brief, the baseline correlations of KYN/TRP ratio and IDO1 gene expression in PBMCs with different NK- and T-cell subsets strongly emphasize the

Table 3a. Overview of existing studies investigating the impact of acute exercise on kynurenine pathway outcomes.

Authors	Study type	Study population	Exercise intervention	Outcome Measurements		
				Metabolites in blood serum/ plasma	Gene expression of enzymes in	
					Skeletal muscle	PBMCs
Lewis et al. (2010)	longitudinal study	n = 25 (19♂; 6♀) healthy	**Marathon race** (42.2 km)	TRP ↓ KA, AA, QA ↑	–	–
Melancon et al. (2014)	longitudinal study	n = 16 (16♂) elderly	**60 min continuous treadmill exercise** 67-70% $\dot{V}O_{2peak}$	TRP ↑	–	–
Areces et al. (2015)	longitudinal study	n = 26 (26♂) experienced triathletes	**Half-ironman triathlon** (1.9 km swimming, 75 km cycling, 21.1 km running)	TRP ↔	–	–
Mudry et al. (2016)	controlled longitudinal study	n = 12 (12♂) healthy n = 12 (12♂) Type 2 diabetes	**30 min cycling exercise** 85% HR_{max} **30 min cycling exercise** 85% HR_{max}	TRP, KYN ↓ KA, KA/KYN ↑ TRP, KYN ↓ KA, KA/KYN ↑	KAT 1-4 ↔ KAT 1-4 ↔	–
Schlittler et al. (2016)	longitudinal study (results of 3 different sub-studies)	n = 9 (9♂) endurance trained n = 11 (10♂; 1♀) recreationally active n = 9 (9♂) healthy	**150 km road cycling time trial** **Half marathon race** (21.1 km) **100 drop jumps**	KA, QA ↑ QA/KA ↓ KA ↑ KA, QA, QA/KA ↔	–	–
Strasser et al. (2016a)	longitudinal study	n = 33 (16♂; 17♀) trained athletes	**Incremental cycle ergometer exercise** until exhaustion	TRP ↓ KYN, KYN/TRP ↑	–	–
Strasser et al. (2016b)	randomized controlled study[#]	n = 29 (13♂; 16♀) trained athletes	**Incremental cycle ergometer exercise** until exhaustion	TRP ↓ KYN ↔ KYN/TRP ↑	–	–
Bansi et al. (2018)	longitudinal study (with subgroup analysis of MS phenotype)	n = 24 (9♂; 15♀) Secondary Progressive Multiple Sclerosis n = 33 (10♂; 23♀) Relapsing Remitting Multiple Sclerosis	**Incremental cycle ergometer exercise** until symptom limited maximum	TRP, KYN, KYN/TRP ↔ TRP ↓ KYN ↔ KYN/TRP ↑	– –	– –
Baxter-Parker et al. (2019)	randomized controlled crossover study	n = 43 (20♂; 23♀)	**Incremental cycle ergometer exercise vs. passive control**	TRP, KYN, KYN/TRP ↔	–	–
Joisten et al. (2019)	randomized crossover study	n = 24 (24♂) healthy	**Resistance vs. endurance exercise** *Resistance exercise:* 5 exercises, each 4 x 8-10 repetitions at 70% 1RM *Endurance exercise:* 45 min cycling exercise at 60% PPO	TRP ↓ KYN/TRP ↑ KYN, KA, QA, KA/KYN, QA/KYN, QA/KA ↔ KA, KA/KYN, QA ↑ TRP, KYN, KYN/TRP, QA/KYN, QA/KA ↔	– –	IDO1, KAT 4 ↔ IDO1 ↔ KAT 4 ↑

Table 3b. Overview of existing studies investigating the impact of chronic exercise training on kynurenine pathway outcomes.

Authors	Study type	Study population	Exercise intervention	Outcome Measurements		
				Metabolites in blood serum/ plasma	Gene expression of enzymes in	
					Skeletal muscle	PBMCs
Hennings et al. (2013)	controlled longitudinal study	n = 38 (15♂; 23♀) major depressive disorder	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
		n = 27 (7♂; 20♀) somatization syndrome	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
		n = 48 (16♂; 32♀) healthy	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
Agudelo et al. (2014)	longitudinal study	n = 8-10 (N/A♂; N/A♀) healthy	**3 weeks of intensive endurance training (6x/week; 2x/day)** *Session 1:* 30 min of cycling at a HR corresponding to lactate threshold *Session 2:* 25 min of running at a HR corresponding to lactate threshold	–	KAT 1-4 ↑	–
Melancon et al. (2014)	longitudinal study	n = 16 (16♂) elderly	**16 weeks of treadmill training** 3x/week; 45 min at 80% HR_{max}	TRP ↔	–	–
Küster et al. (2017)	non-randomized controlled study#	n = 47 (20♂; 27♀) elderly at risk of dementia n = 17 (6♂; 11♀)	**10 weeks of physical training vs. cognitive training vs. waitlist control** *Physical training (5x/week):* endurance, coordination, balance, flexibility and strength exercises; 2x/week 60 min supervised and 3x/week 20 min home-based	KYN, KA, 3-HK, QA ↔	–	–
Millischer et al. (2017)	randomized longitudinal study	n = 117 (42♂; 75♀) mild-to-moderate depression	**12 weeks of yoga/aerobic training; 3 groups** *1. light exercise (3x/week):* 60 min; mean intensity: 54.1% HR_{max}	KYN, KA ↔	–	–
			2. moderate exercise (3x/week): 60 min; mean intensity: 70.3% HR_{max}	KYN, KA ↔	–	–
			3. vigorous exercise (3x/week): 60 min; mean intensity: 76.2% of HR_{max}	KYN, KA ↔	–	–
Bansi et al. (2018)	randomized controlled study (with subgroup analysis of MS phenotype)	n = 24 (9♂; 15♀) Secondary Progressive Multiple Sclerosis n = 33 (10♂; 23♀) Relapsing Remitting Multiple Sclerosis	**3 weeks of cycling; 2 groups** *1. HIT (3x/week):* 5 x 3 min at 80% $\dot{V}O_{2peak}$; 1.5 min active recovery between intervals	TRP ↓ (RRMS only) KYN ↔ KYN/TRP ↑ (RRMS only)	–	–
			2. moderate continuous training (5x/week): 30 min at 65% $\dot{V}O_{2peak}$	TRP ↓ (RRMS only) KYN ↔ KYN/TRP ↑ (RRMS only)	–	–
Allison et al. (2019)	longitudinal study	n = 25 (25♂) elderly	**12 weeks of combined HIT and RT** *HIT (1x/week):* 30 min cycling; 10 x 1 min at 90% HR_{max}; 1 min active recovery between intervals *RT (2x/week):* progressive; 3 x 6-12 repetitions at 65-80% 1RM; 4 resistance exercises	KYN ↔ KA, QA ↔ QA/KA ↔	KAT 1-4 ↑	–

Herrstedt et al. (2019)	non-randomized controlled study	n = 43 (38♂; 5♀) gastro-esophageal junction cancer receiving chemotherapy	**12 weeks of combined HIT and RT (2x/week) vs. usual care**			
		n = 18 (15♂; 3♀)	*HIT:* 30-45 min cycling; 4 x 4 min at 85-95% HR_{max}; 3 min recovery between intervals *RT:* following HIT; progressive; 3 x 8-12 repetitions at 50-80% 1RM	TRP ↓ AA ↑ KYN, KA, 3-HK, XA, HAA, QA, 3-HK/KYN ↔	KAT 1-3 ↔	–
		n = 25 (23♂; 2♀)	*Usual care*	TRP ↓ 3-HK, AA, QA, 3-HK/KYN ↑ KYN, KA, XA, HAA ↔	KAT 1-3 ↔	–

study was designed to investigate a different research question. For the purpose of this overview data was extracted from the original study.
Outcome measurements are separated by intervention group. ↑ significant increase (p≤.05); ↓ significant decrease (p≤.05); ↔ no significant change; *PBMCs* peripheral blood mononuclear cells; *TRP* tryptophan; *KYN* kynurenine; *KA* kynurenic acid; *3-HK* 3-hydroxykynurenine; *XA* xanthurenic acid; *HAA* 3-hydroxyanthranilic acid; *QA* quinolinic acid; *KYN/TRP* kynurenine-tryptophan ratio; *KA/KYN* kynurenic acid-kynurenine ratio; *QA/KYN* quinolinic acid-kynurenine ratio; *3-HK/KYN* 3-hydroxykynurenine-kynurenine ratio; *QA/KA* quinolinic acid-kynurenic acid ratio; *KAT* kynurenine-aminotransferase; *IDO* indoleamine 2,3-dioxygenase; *HIT* high intensity training; *RT* resistance training; VO_{2peak} maximal oxygen uptake; *PPO* peak power output; *HR* heart rate; HR_{max} maximal heart rate; *1RM* one-repetition maximum; *MS* Multiple Sclerosis; *RRMS* Relapsing Remitting Multiple Sclerosis.

suppressive effects of IDO-mediated KYN pathway activation on the immune system in humans.

In view of the kinetics of KYN pathway activation mediators IL-6 and cortisol, EE provoked a significant increase from baseline (T_0) levels to post exercise (T_1) followed by a significant decrease from post exercise to the 1h follow-up measurement (T_2) (Figure 3). These results are in agreement with several previous studies (34, 56, 69). In contrast, RE had no effects on IL-6 and reduced cortisol serum concentrations at the 1h follow-up measurement. Overall, EE induced a stronger effect compared with RE on both outcomes not only immediately after exercise but also at 1h follow-up measurement. Thus, EE appears to be the more appropriate stimuli to impact two potential mediators of KYN pathway activation. However, serum levels of TRP, KYN and KYN/TRP ratio do not support the hypothesis of an IL-6 / cortisol mediated activation of the KYN pathway. In regard to TRP, KYN and KYN/TRP ratio, EE did not show any significant changes whereas RE provoked significant changes over time. To our knowledge, this is the first study demonstrating a transient KYN pathway activation in response to acute RE as indicated by an increased KYN/TRP ratio immediately after cessation that is followed by a decrease to baseline level at the 1h follow-up measurement. To date, most studies on exercise-induced KYN pathway activation only investigated the effects of EE. These studies revealed an increased KYN/TRP ratio following exercise, if calculated (10, 63, 64). Interestingly, all these studies used an incremental exercise protocol until exhaustion as intervention. Therefore, the absent increase of the KYN/TRP ratio provoked by EE in the present study could be due to longer exercise duration or rather the time between pre and post exercise measurements. The previously described increases in KYN/TRP ratio after EE may also be caused by the incremental nature of the applied exercise protocols implicating the highest and most exhaustive load immediately before post-measurements. Hence, future studies should examine dose-/time-response relationships of varying EE modalities with consideration of different energy supply systems.

Similar to KYN/TRP ratio, IDO1 expression in PBMCs was unaffected by EE. Since RE resulted in an upregulated KYN/TRP ratio but did not alter IDO1 expression in PBMCs, the underlying mechanisms remain to be elucidated. IDO1 expression may also occur in other cell types or tissues. Furthermore, it is worth mentioning that only one isoform of IDO was investigated in the present study. The physiological roles of IDO2 are by far not as well examined as for IDO1. However, and especially considering the significant increase in KYN/TRP ratio after RE, it cannot be ruled out that elevated IDO2 expression represents an underlying mechanism. Alternatively, the observed increase in KYN/TRP ratio after RE could be driven by the augmented demand of amino acids, as shown by a decrease of serum TRP over time in response to this anabolic exercise stimulus (12, 44).

Kynurenine pathway branch yielding KA

In line with previous studies, KA and KA/KYN ratio increased following EE (41, 50, 60). Here we have shown for the first time, that these increases are only of transient nature, since both outcomes decrease after cessation. In contrast, RE leads to a significant decrease in KA at follow-up. Taken together, EE provokes a more pronounced activation of the KA producing branch of the KYN pathway.

In parallel to the different effects of both exercise interventions on KA and the KA/KYN ratio, KAT4 expression was elevated immediately after exercise only in EE (Figure 4). To our knowledge, it was shown for the first time that KAT4 gene expression is upregulated in PBMCs after an acute bout of EE. To date, increased KAT expression was only shown in longer EE training interventions in muscle tissue in both, humans and rodents (1, 2). It remains to be investigated to what extend acute exercise-induced changes in KA/KYN ratio are driven by PGC-1α transcription coactivator signaling in different tissues (muscle and PBMCs) and how strong these tissues contribute to the conversion from KYN to KA. In greater detail, PGC-1α represents a pan-coactivator of peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcriptional factors and involved in the regulation of different immunomodulatory processes (24). As potential mechanistic insight underlying the observed effects of EE on the KYN pathway yielding KA, the increased KAT4 gene expression in PBMCs may results from a PGC-1α mediated alterations in PPARs. Agudelo et al. emphasized the regulatory role of PPARα and PPARδ together with PGC1-α1 in KAT gene expression in skeletal muscle (1). Indeed, especially PPARγ has been described to be expressed in different immune cells (24). However, a direct link between PGC-1α mediated PPARs signalling and increased KAT gene expression in PBMCs needs to be further investigated.

Kynurenine pathway branch yielding QA

In line with previous studies (41, 60), QA was significantly increased immediately after cessation in EE. In contrast, RE did not show any effects on QA serum concentrations. The different degradation of KYN yielding QA is strongly underlined by the significant interaction effects of the QA/KYN ratio with values in EE being greater than those of RE. So far, studies implicated beneficial effects of exercise for brain health, due to a reduced KYN flux to the CNS (1, 42). These effects have been interpreted by an increased peripheral degradation from KYN to KA, which is not able to cross the BBB. Interestingly, QA which is widely known to have neurotoxic properties, is also not able to pass the BBB. Therefore, an enhanced peripheral clearance of KYN towards QA through exercise might also prevent a pathological accumulation of KYN within the CNS.

Finally, little attention has been focused on another potential reason for commonly observed increases in peripheral QA levels after acute EE (41, 60). QA serves as precursor of de novo NAD^+ synthesis starting with TRP. TRP may not be considered as the most relevant precursor of NAD^+. However, especially EE represents a strong metabolic stimulus, increasing the demand for substrates which are involved in energy supply such as NAD^+. In fact, NAD^+ is known to increase in response to exercise in mammalian cells. The maintenance of consistent or even increased tissue levels of NAD^+ represent a

current research topic in the context of protection against aging and treatment of several diseases (36, 37). Moreover, increased levels of NAD^+ have been associated with enhanced mitochondrial function under metabolic stress (15). Against this backdrop, following exercise trials may also shed light on this important aspect of the KYN pathway. Importantly, exercise-induced increases of peripheral QA concentrations should not be linked to its negative properties within the CNS. Future research should investigate more intermediate metabolites of this branch and should especially focus on the expression of the rate-limiting enzyme KMO, that is potentially sensitive for inflammatory stimuli.

Strengths and Limitations

Strengths of this study comprise the first comparison of two prevalent exercise modalities that were designed as application-orientated sessions on KYN pathway outcomes. Besides commonly assessed pre- and post- exercise measurements, we additionally included a 1h follow-up measurement to gain knowledge on outcome kinetics. Regarding outcome measures, this trial covers a broad range of KYN pathway metabolites, enzymes and mediators as well as their relation to immune cell proportions and numbers. In contrast to previous studies, focusing on KAT expression in muscle, gene expression of IDO1 and KAT4 was assessed in PBMCs for the first time in the context of exercise. Limitations include the constrained transferability of the results to other populations (e. g. females, older or diseased subjects), since a homogenous sample of young, healthy, male adults was investigated. Moreover, future studies may add KMO expression as outcome and include both, gene expression in muscle and PBMCs to determine which tissue/cell type mainly contributes to exercise-induced alterations of the KYN pathway. Finally, different isoforms of IDO and KAT enzyme expression were not measured and remain to be investigated.

Conclusion

In this article, the reviewed literature indicated only restricted evidence for chronic effects of exercise interventions on KYN pathway outcomes. However, long-term intervention trials strongly differ in view of study populations and applied exercise modalities. In contrast, single-bouts of EE consistently provoke acute alterations in KYN pathway outcomes. Here, we directly compared two exercise modalities and revealed that acute resistance and endurance exercise induce different effects on KYN pathway outcomes. Elevated levels after endurance exercise of both KA and QA may lead to neuroprotection by preventing a pathological KYN accumulation within the CNS. Furthermore, the increased level of QA raises the question of an involvement in the compensation of enhanced NAD+ demand as a consequence of augmented energy supply during and/or immediately after endurance exercise. Moreover, the revealed associations between immune cell subsets and KYN pathway outcomes emphasize the hypothesis that repeated acute exercise-induced alterations of the KYN pathway provoke long-term adaptions of the immune system. Especially in view of clinical settings with inflammation-mediated diseases, future studies investigating the interplay between acute and chronic exercise stimuli, the KYN pathway and modulations of the immune system might be of major relevance.

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Supplements

Supplement 1.

Table 1. Mass spectrometric properties of all target analytes: Most intense precursor/product ion pairs were used for quantification (and qualification/identification).

Substance	Precursor ion [*m/z*]	Product ion [*m/z*]	Cone voltage [V]	Collision energy [eV]
QA	168	78 (150)	22	20
QA-d3	171	81 (153)	22	20
KA	190	144 (116)	24	18
KA-d5	195	149 (121)	24	18
TRP	205	146 (188)	24	18
KYN	209	94 (146)	20	12
TRP-d5	210	150 (192)	24	18

Table 2. Assay characteristics.

Parameter		TRP	KYN	QA	KA
Calibration curve	Concentration levels	0.5, 2, 5, 20, (50) µg/mL	20, 50, 200, 500, 2000 ng/mL	5, 20, 50, 200, 500, 2000 ng/mL	0.5, 2, 5, 20, 50, 200 ng/mL
	Linear equation	y=1.4756x-0.4224	y=0.0007x+0.0069	y=0.0008x+0.0014	y=0.0086x+0.0072
	R^2	0.9993	0.9999	0.9999	0.9997
Specificity		√	√	√	√
Recovery [%]		70	76	67	73
Imprecision	low:	400 ng/mL: 8%	40 ng/mL: 10%	400 ng/mL: 9%	20 ng/mL: 11%
	medium:	1 µg/mL: 14%	100 ng/mL: 12%	1 µg/mL: 15%	50 ng/mL: 13%
	high:	2.5 µg/mL: 2%	250 ng/mL: 2%	2.5 µg/mL: 2%	125 ng/mL: 3%
LLOQ (S/N > 9:1) [ng/mL]		50	25	10	10

Supplement 2.

Table 1. Documentation of exercise intervention modalities.

	Overall mean exercise intensity (n=24)
Resistance Exercise	
Chest press (% of 1RM ± SD)	68.8 ± 1.7
Lat pull (% of 1RM ± SD)	67.5 ± 5.0
Leg curl (% of 1RM ± SD)	65.3 ± 5.0
Leg extension (% of 1RM ± SD)	70.5 ± 6.0
Back extension (% of 1RM ± SD)	67.5 ± 7.0
Endurance exercise	
Heart rate (% of HR_{max} ± SD)	88.2 ± 5.3
Power output (% of $Watt_{max}$ ± SD)	57.0 ± 5.6

1RM One-repetition maximum (kg); *SD* Standard deviation; HR_{max} Maximal heart rate (bpm); $Watt_{max}$ Peak power output (W)