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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

The effect of physical activity on muscle-derived interleukin 15 and autophagy

Lieselotte Cramer

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

dr. Helena SLAETS

MENTOR :

Mevrouw Lena FONTEYN

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The effect of physical activity on muscle-derived interleukin 15 and autophagy*

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* Running title: Acute exercise on IL-15 & autophagy

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ABSTRACT

An ageing population is one of the world's major future concerns. While our life expectancy increases due to improvements in healthcare and lifestyle, the quality of life is paradoxically declining. Over the years, stressors, including illness and ageing, cause the immune system's function to deteriorate, which is responsible for removing unnecessary or damaged cells and cellular waste—a process known as autophagy. In the elderly, autophagy diminishes, causing an accumulation of cells and pathogens, leading to susceptibility to infections and age-related disorders. Research has shown that the immune system and autophagy are regulated by cytokines. One of the cytokines highlighted in this research is interleukin 15 (IL-15). Related studies demonstrated that physical exercise stimulates the production of cytokines depending on various parameters: the type and duration of exercise and the subject's age and sex. Previous research mainly focussed on the impact of IL-15 on the immune cells' function, leaving little known about its effect on autophagy. This research therefore aims to determine whether or not acute exercise induces IL-15 production and improves autophagy. Young and old c57BL/6 mice were divided into sedentary control mice (n = 3) and exercise mice (n = 3), which perform an acute bout of treadmill running. Mice are euthanised and skeletal muscle tissue was collected for analysing the gene and protein expression of IL-15 and autophagy. In conclusion, the duration and intensity of treadmill running plays a role in the expression of IL-15 and autophagy.

INTRODUCTION

For several decades, life expectancy has been rising worldwide and is expected to continue increasing in the current century. By 2050, it is projected that 20% of the world population—which amounts to 2.1 billion people by then—will be over 60 years old. This is a threefold increase compared to the global demographics of 2021 that will be the result of current and future improvements in healthcare, nutrition, and lifestyle (1). However, when a rising average human lifespan is not accompanied by a similar advancement of its quality, an ageing population could adversely affect society by burdening healthcare systems and disrupting the economy (1, 2).

Ageing is an evolutionary process in which, over time, repair mechanisms increasingly fail to perform, causing cells to experience stress, such as DNA damage, telomere dysfunction and general cellular stress (3). As a result, cellular senescence occurs with cells recognising their dysfunction, which induces cell-cycle arrest and signals the immune system for cellular clearance (4). These senescent cells develop a senescence-associated secretory phenotype (SASP) that involves the secretion of various cytokines and chemokines in order to chronically activate the innate immune system by attracting natural killer (NK) cells, T cells and macrophages (3, 5). In general, senescence is a beneficial physiological process involved in tissue repair and regeneration (1). However, the persistent presence of senescent

cells and SASP in later life becomes detrimental to the body due to the immune system increasingly struggling to eliminate unwanted cells and pathogens (3). This process is termed immune senescence and is characterised by dysfunction and dysregulation of immune cells as well as changes in maturation and transcriptional reprogramming of individual immune cells (1, 5). Once the immune system malfunctions, senescent cells are no longer removed adequately, and an excess of SASP will accumulate, triggering low-grade inflammation, known as inflammageing (1, 6, 7). In addition, research has shown that the development and progression of immune- and age-related disorders are highly associated with autophagy deficits (1, 8, 9, 10).

Autophagy is an evolutionary catabolic recycling process within cells that decomposes dysfunctional cellular components, protein aggregates, damaged organelles, and intracellular pathogens (11, 12, 13). These components are removed by engulfing them in a double-membrane autophagosome, after which these fuse with lysosomes for degradation (12, 13). Besides cellular clearance, autophagy is also responsible for regulating the immune system by thymic T cell selection, antigen presentation, T and B cell homeostasis and survival, and cytokine production (14, 15). Under normal circumstances, a basal level of autophagy is present in all eukaryotic cells to guarantee cellular clearance and cellular and tissue homeostasis (16, 17). When the human body experiences stress such as starvation, increased oxidative stress or physical activity, autophagy is upregulated for recycling and resynthesising cellular components, thus maintaining cellular vitality and cell survival (16, 18, 19, 20). Research has demonstrated that autophagy activity declines during ageing, allowing damaged cellular components or senescent cells to accumulate. This will cause tissue dysfunction and imbalance in homeostasis (21, 22, 23, 24). The presented study examines whether acute physical activity enhances autophagy, which will stimulate the removal of senescent cells and may prevent excessive cellular ageing and age-related disorders in the long run (8, 21).

Over the last decade, research has increasingly directed its attention towards the impact of physical activity on immune health (25). Skeletal muscle tissue has been recognised to be a significant secretory organ of muscle-derived cytokines, or myokines, such as interleukin 2 (IL-2) and IL-15 (26, 27). IL-15 is a pleiotropic cytokine with pro-inflammatory properties, which modulates the innate and adaptive immune system by activating, proliferating and surviving T cells and NK cells and supports the replenishment of naïve and memory T cells (26, 28, 29). Although, in addition to skeletal muscle tissue, various cells, including dendritic cells, monocytes and macrophages, have been observed producing IL-15 (30, 31), the role of physical activity on this process through skeletal muscle remains unclear and requires further insights (32, 33). Its expression level depends on multiple parameters, such as the type and duration of the physical exercising (34). Other parameters to factor in when examining muscle-derived cytokine production are the subject's age and sex. It is well established that men and younger individuals possess more muscle mass than women or older people, thus containing higher cytokine concentrations (35).

The effect of physical activity on immune health depends on various parameters: duration, intensity, type, dose and frequency (26, 32, 36). The duration is essential in activating immune cells to induce an immune response (32, 37). More specifically, long-term exercise triggers immune adaptation, while acute exercise will cause a transient immune system activation (32). Acute physical exercising thereby provides an instantaneous supply of lymphocytes into the bloodstream. During recovery, T cells release cytokines and other hormones, and NK cells get better-killing properties for specific pathogens (26, 37). While acute exercise improves immune surveillance, regular exercise has shown relieving the detrimental effects of ageing, obesity and chronic infection on T cells (26). People who regularly exercise have known to possess low senescent/naïve T cell ratios leading to optimal immune responses (26). Long and intense exercising, on the other hand, can deteriorate the immune system by impairing the function of T cells, NK cells and neutrophils (32, 37). Contrarily, moderate-intensity exercise positively affects the immune system by reducing inflammation and increasing immune surveillance and cell turnover (32). However, studying the relationship between the various parameters that influence immune reactivity requires further attention to get hold of the long-term benefits of exercise (38).

In summary, related researches suggest that physical activity both induces myokine secretion and improves the immune system. However, the molecular mechanism behind these observations still remains unclear. In this study, the authors hypothesise that physical exercise causes IL-15 expression in skeletal muscle tissue, thereby boosting the immune cells' autophagy. If exercise stimulates autophagy within immune cells, it will ensure these cells' ability to dispose their waste and maintain their optimal functioning. This research firstly examines the baseline values of IL-15 in the skeletal muscle tissue of young and old mice of both sexes. Subsequently, the effect of acute exercise on the expression of IL-15 in the skeletal muscle tissue of a similar mice group is analysed. The outcome of this study will lay the basis for a follow-up study targeting the effects of regular exercise on murine subjects. Looking forward, it is expected that exercise could become part of preventive measures or treatment for immune-related disorders in people of different ages.

EXPERIMENTAL PROCEDURES

Animal and housing – Male and female C57BL/6 mice aged 2 months, 15 months, and 24 months were purchased from Janvier-Labs (Le Genest-Saint-Isle, France). Female mice of each age and 2 months old male mice were grouped-housed, while 15 months and 24 months old male mice were solitary housed. All mice were housed in a pathogen-free controlled environment with a 12:12 light-dark cycle at room temperature (20 – 24° C), 40 – 60% humidity, and provided with a standard chow diet and water ad libitum. All animal experiments were conducted and approved by the Ethical Commission for Animal Experimentation. Mice were allowed to acclimatise to the environment and researchers for one week prior to adaptation period. Hereafter, all mice were randomly divided into following groups: sedentary control (SED, n = 6) and exercise groups (EX, n = 6 + 10% dropout 24-months old mice). At the end of the study, mice were anaesthetised with a lethal dose of dolethal, and perfused with a solution of phosphate-buffered saline (PBS) and heparin.

Exercise protocol – Mice of the exercise group were familiarised with running on a motorized treadmill (IITC Life Science, Woodland Hills, CA, USA) for 3 days. In this adaptation period, mice ran for approximately 10 min at 9 m/min for one day and 10 min at 10 m/min with 0° incline for two days. Sedentary mice remain in the same room during adaptation to ensure suffering of similar levels of stress. After treadmill adaptation, each exercise mice performed an incremental speed test. Mice start with a speed of 8 m/min with 0° incline, and every 3 min the speed increases by 1 m/min until exhaustion. Total workload was calculated as $end\ speed * 80\%$. After all mice finished the incremental speed test, the mice ran at their optimal speed until exhaustion.

Skeletal muscle dissection – Four hours after performing treadmill exercise, all mice were anesthetised as described, for removing skeletal muscle tissue and spleen. For skeletal muscle tissue, left soleus (SOL), tibialis anterior (TA), gastrocnemius (GAS), and extensor digitorum longus (EDL) muscle were rapidly removed and immediately stored in liquid nitrogen for further storage at -80 °C until further analysis. While the muscle tissue (SOL, TA, GAS, EDL) from the right hind limb was embedded in Tissue Tek O.C.T. compound and then stored in -80°C until further analysis.

Protein extraction and western blot analysis – Skeletal muscle tissue was homogenised in ice-cold radioimmunoprecipitation (RIPA) buffer supplemented with protease and phosphatase inhibitor (Hoffmann-La Roche, Basel, Switserland) and minced using a TissueRuptor II (Qiagen, Venlo, the Netherlands). Homogenates were centrifuged at 12,500 x g for 15 min at 4 °C to precipitate insoluble cell debris. Protein concentration of the supernatant were quantified performing Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

Protein aliquots (30 µg) were loaded on 4%-12% gradient bis-tris polyacrylamide gels (Invitrogen, Waltham, MA, USA) for p62 protein analysis and 4%-15% gradient for IL-15 and LC3 protein analysis. The gels were blotted onto polyvinylidene fluori (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). Once transblotted, membranes were blocked with 1% BSA in PBS-T for 1 h at room temperature, and all antibodies were diluted in this solution (Table S1). Hereafter, membranes were incubated with primary antibodies recognising IL-15, LC3-I/-II, p62, overnight at 4 °C. An-HRP-conjugated secondary antibody was incubated with the membrane for 1 h at room temperature. Positive bands were visualised using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific,

Waltham, MA, USA). For the visualisation of the reference protein, Ponceau S staining was added to the membrane, and visualised by colorimetric detection. Signal was detected using a scanner (ChemiDoc Touch Imaging System, Bio-Rad Laboratories Inc., CA, USA).

RNA isolation from murine spleen – Total RNA was isolated from frozen murine spleen tissue using RNeasy Mini kit (74106, Qiagen, Venlo, the Netherlands) according to manufacturer's instructions. RNA concentration and 260/230 and 260/280 purity ratios were assessed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

RNA isolation from murine gastrocnemius muscle – RNA from skeletal muscle tissue was isolated using RNeasy fibrous tissue mini kit (74704, Qiagen, Venlo, the Netherlands). RLT lysis buffer with β-mercapto-ethanol was added to ± 30 mg tissue and homogenised using TissueRuptor II (Qiagen) for 30 s until samples are completely homogenised. After, the samples were incubated with RNase-free water and proteinase K for 10 min at 55 °C. Samples were centrifuged for 3 min at maximum speed and supernatant was transferred to another Eppendorf tube. Ethanol was added to the RNeasy mini spin column and centrifuged for 15 s at room temperature. After this, RW1and RPE washing buffer were added to the spin column. DNase with RDD buffer are added to the column for digesting sDNA and dsDNA. RNA was eluted in 30 µl of RNAse-free water and RNA concentration and 260/230 and 260/280 purity ratios were assessed by Nanodrop. All samples were held on ice to prevent thawing and degradation of RNA.

Reverse transcriptation and qRT-PCR analysis – For analysis of IL-15 and IL-15Rα mRNA expression, RNA samples were reversed transcribed into cDNA using qScript cDNA SuperMix (Quantabio, Beverly, MA, USA). For qRT-PCR 2.5 µl of cDNA was added to SYBR Green real-time PCR master mix (Applied Biosystems/ Thermo Fisher, Waltham, MA, USA) following manufacturer's instructions. QuantStudio Real-Time PCR system was used for qRT-PCR, using recommended PCR cycling conditions. Primers of interest that were used are listed in Table S2.

IL-15/IL15Rα ELISA – Blood samples for the retro-orbital plexus were obtained before (t1) and from the heart 4h post-exercise (t2). Serum and plasma were collected by centrifugation at 13,000 rpm for 5 min and stored at -40°C until analysis. Mouse IL-15/IL-15R ELISA kit (Invitrogen, Waltham, MA, USA) was used for the quantitative detection of mouse IL-15/IL-15R, according to the manufacturer's recommendations. ELISA plates were beforehand coated with anti-mouse IL-15/IL-15R antibody. Streptavidin-HRP and substrate solution were added for the formation of coloured product to the amount of mouse IL-15/IL-15R present in the samples. Absorbance was measured for the detection of IL-15/IL-15R coloured product at the wavelength of 450 nm, using a Multimode Microplate reader (BMG Labtech, Ortenberg, Germany).

Immunohistochemical staining of IL-15 in muscle sections – Isolated muscle tissues were embedded in Tissue Tek O.C.T compound and frozen in liquid nitrogen. Cryosections with a thickness of 10 µm were cut and several sections were cut from all depths of the muscle. Sections of muscle tissue were permeabilized in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 at room temperature for 10 min. After washing, the muscle sections were blocked in blocking serum, containing PBS-T + 10% DAKO protein block (X0909, Aligent Technologies, Santa Clara, CA, USA), for 30 min at room temperature. Muscle tissue slices were incubated overnight with a primary antibody for IL-15, fiber types (MHC I, MHC IIa, MHC IIb) in blocking serum at 4°C. The next day, sections were washed (3x 5 minutes) and incubated with a secondary antibody in washing buffer. After washing, nuclei were counterstained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature Muscle sections were mounted with fluorescent medium and visualized using Leica fluorescence microscope (405137, Leica Microsystems, Wetzlar, Germany). All washing steps were performed with PBS containing 0.05% Triton. The primary and secondary antibodies used were listed in Table S3.

Statistical analyses – Statistical analysis were performed using GraphPad Prism 9 software (GraphPad software, San Diego, CA, USA). All data were presented as the means ± standard error of mean (SEM). Before data was analysed, outliers were identified using the Grubb's test at a significant level of 0.05 and excluded if needed.
The difference in age, sex and physical activity on gene and protein levels were tested using an three-way analysis of variance (ANOVA) test, followed by Tukey's multiple comparison test. If

no significant difference was shown, an ordinary two-way ANOVA test was used, followed by Tukey's multiple comparison test, for examining the difference in expression between groups differs in an age- and sex-dependent manner. $P \leq 0.05$ was considered significant.

RESULTS

IL-15 and IL-15Rα are not upregulated in young sedentary C57BL/6 mice – In order to investigate whether exercise affects the expression of IL-15 and IL-15Rα, we first examined the expression in sedentary mice of all ages to determine the baseline values in skeletal muscle tissue, focused on gastrocnemius muscle. As shown in Fig. 1A, B, females express higher levels of IL-15 and IL-15Rα, but no significant changes were seen with increasing age.

Due to technical difficulties, western blot analysis could not be conducted to confirm mRNA expression results further. Therefore, immunohistochemical staining was conducted on gastrocnemius muscle tissue to detect the presence of IL-15. Skeletal muscles exhibit different muscle fiber types based on their various activities. The gastrocnemius muscles comprise type I and type IIa/b muscle fibers. This study investigated which muscle fiber types were responsible for the IL-15 expression by staining for IL-15, type I and type IIa/b myosin heavy chains (MHC). Here, the immunohistochemistry staining revealed predominant type IIa muscle fibers in the gastrocnemius muscle of any group (Table 1, Fig. S1). To determine the IL-15 protein concentration, the 'Corrected Total Cell Fluorescence' by IHC was analysed in gastrocnemius cryosections. The staining showed age-related differences in IL-15 expression. In younger and middle-aged mice, more IL-15-expressing muscle cells were visually observed (Fig. 2A). As shown in Fig. 2B, a sex-based significantly increase ($P \leq 0.01$) was observed between 24-month-old sedentary mice, with IL-15 significantly elevated in males (Fig. 2B). No difference in IL-15 protein levels was determined in 2-month-old and 15-month-old mice compared to 24-month-old mice. In summary, these results show that IL-15 and IL-15Rα mRNA are age- and sex-independently expressed, while sex-differences in IL-15 protein levels are evident. A higher expression is measurable in 24-month-old male mice with sedentary behaviour.

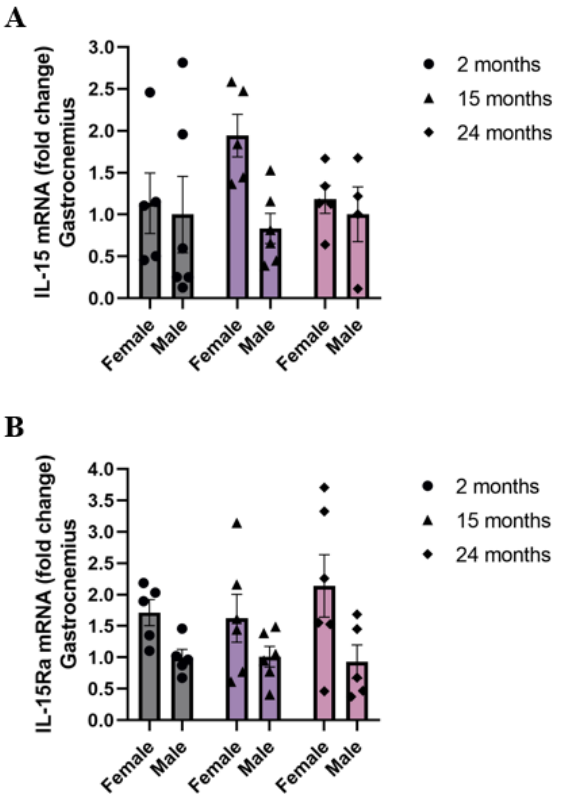

Fig. 1 – The expression of IL-15 and IL-15Rα in m. gastrocnemius of sedentary C57BL/6 mice. Quantitative analysis of IL-15 **(A)** and IL-15Rα **(B)** by qPCR in gastrocnemius muscle of sedentary mice ($n \geq 4$). Data are presented as mean ± S.E.M. Ordinary two-way ANOVA test was used for determining statistical significance in IL-15 and IL-15Rα expression between different ages and sexes.

Table 1 –Muscle fiber types responsible for the expression of IL-15 in murine gastrocnemius muscle (n = 4).

Muscle fiber type	% IL-15-expressing myocytes
Type I	0 %
Type IIa	100 %
Type IIb	0 %

IL-15, interleukin 15

Acute bout of exercise did not induce an upregulation of IL-15 and IL-15Rα in exercise C57BL/6 mice – Next, we sought to confirm that single bout of exercise induces IL-15 and IL-15Rα production in gastrocnemius muscle and spleen tissue. First, the effect of acute exercise is determined in sex- and age-different groups. The single bout of treadmill running triggered an upregulation in IL-15 mRNA content in female 15-month-old mice, while an decrease in IL-15

protein levels was present in 15-month-old mice in general compared to 2-month-old and 24-month-old mice (Fig. 3A,B). In general, there can be visually observed increased IL-15 expression at both mRNA and protein levels in female mice of any age group. Similarly, IL-15Rα mRNA expression was upregulated in female 15-month-old mice (Fig. 3C). An upregulation of IL-15 and IL15Rα gene and protein levels was present in sedentary female and male mice (Fig. 3A-C). Next, the difference in IL-15 and IL-15Rα expression in sedentary and exercise mice are compared. No significant increase in IL-15 mRNA and IL-15 protein levels as well as, IL-15Rα mRNA levels was observed in exercise mice (Fig. 3D-F). However, a significantly increase in IL-15 protein expression was measured in 24-month-old male sedentary mice compared to 15-month-old male sedentary ($P \leq 0.001$) and 2-month-old male sedentary ($P \leq 0.001$) mice (Fig. 3E). Also, an significant increase ($P \leq 0.001$) in IL-15 proteins levels was present in 24-month-old male sedentary mice compared to female. When focussing on 24-month-old male mice, a significant increase ($P \leq 0.01$) in IL-15 protein level was detected in sedentary mice compared to exercise mice (Fig. 3E). Based these observations, it can be cautiously concluded that acute bout of treadmill running does not induce an upregulation of IL-15 expression in gastrocnemius muscle in both young and old exercise mice of different sex.

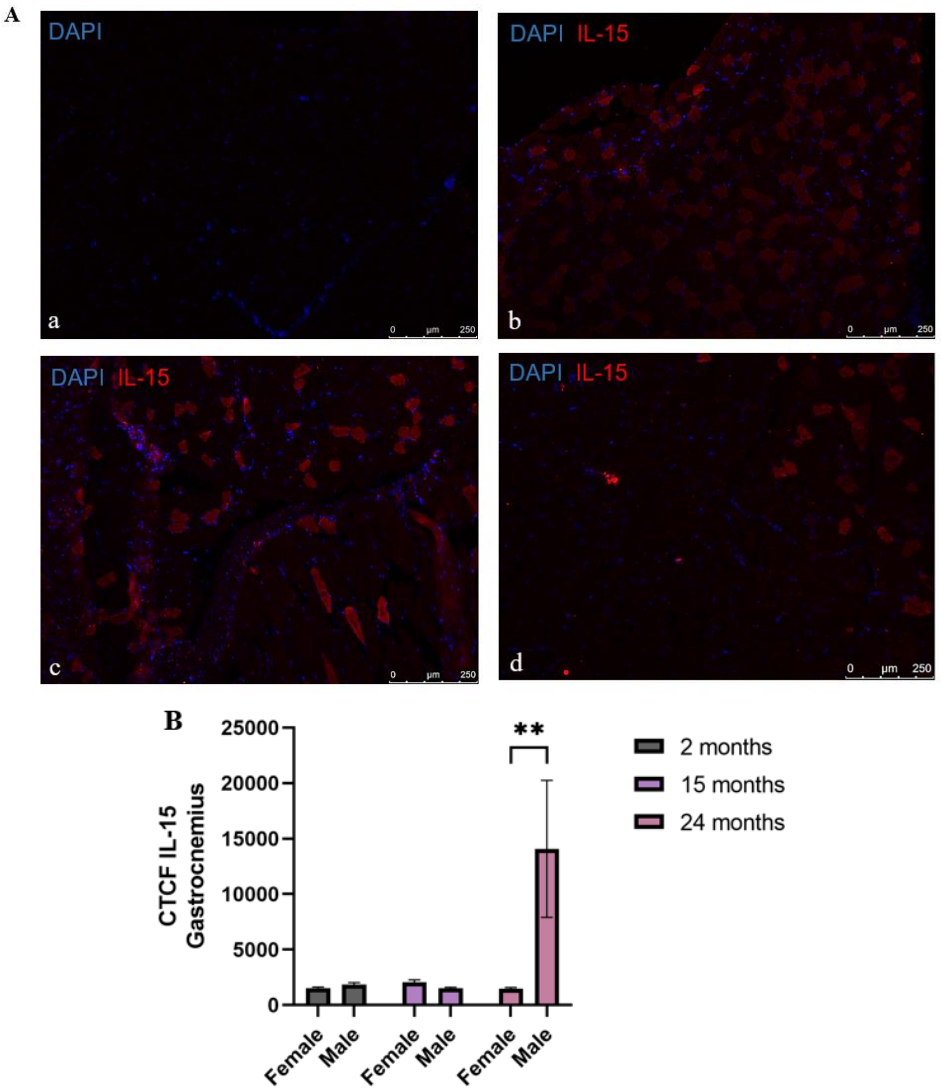

Fig. 2 – Analysis of IL-15 protein expression in gastrocnemius muscle of sedentary mice. A) Immunohistochemical staining of murine gastrocnemius muscle tissue for IL-15 (red) and DAPI (blue) in 2-month-old (a), 15-month-old (b) and 24-month-old (c) sedentary control mice. The fluorescence images are taken with objective 10x. **B)** IL-15 expression of IL-15 in gastrocnemius muscle of sedentary mice (n = 1). Data are presented as mean ± S.E.M. Ordinary two-way ANOVA test was used for analysing statistical significance in IL-15 and IL-15Rα expression between different ages and sexes.
Abbreviations: CTCF, corrected total cell fluorescence; SED, sedentary control mice; EX, exercise mice.

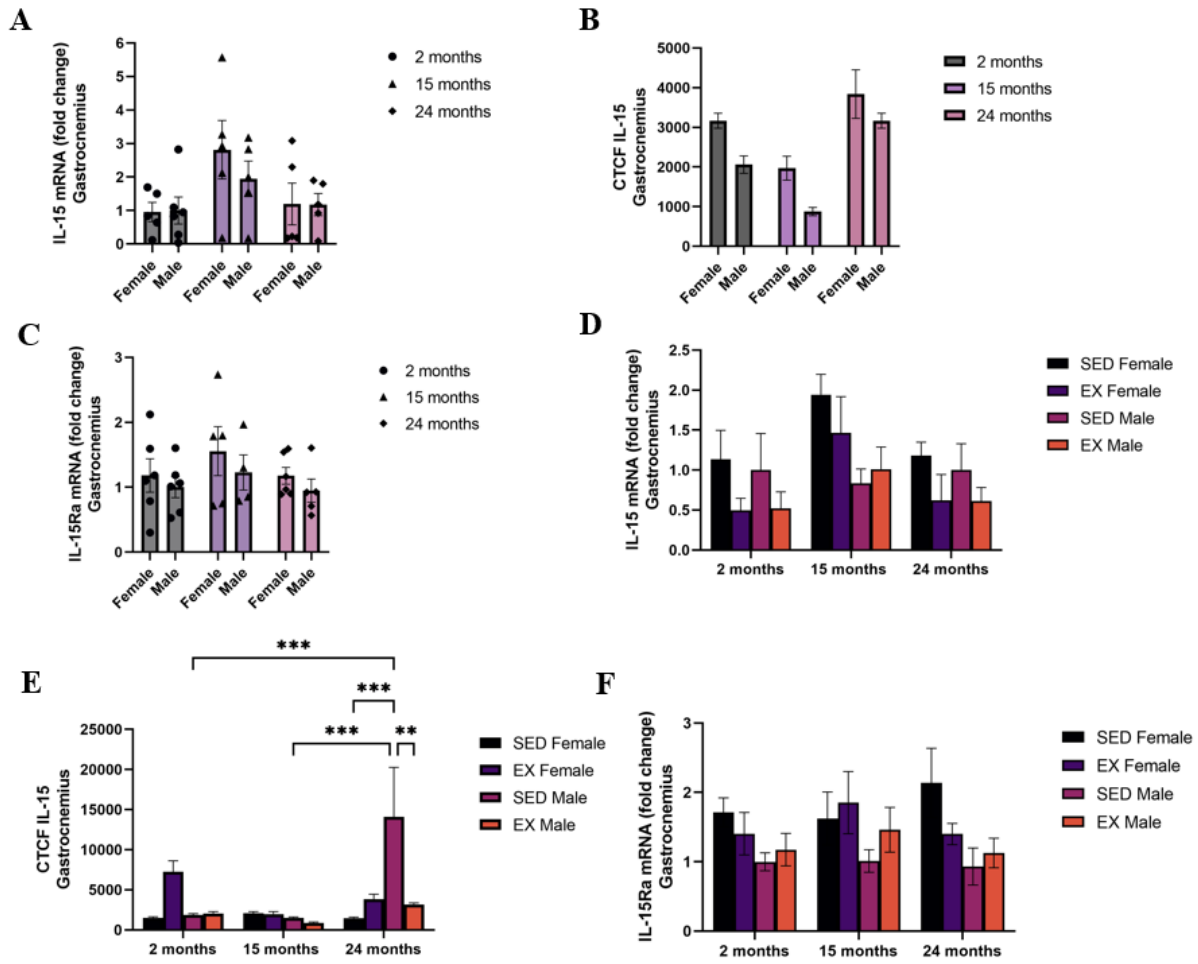

Fig. 3 – Determination of exercise-induced IL-15 and IL-15Rα in the gastrocnemius muscle of exercise mice. A-B) Quantitative analysis of exercise-induced IL-15 on gene **(A)** and protein **(B)** expression in exercise mice. **C)** Analysis of exercise-induced IL-15Rα gene expression in exercise mice. **D-F)** Comparison of IL-15 gene expression **(D)** IL-15 protein levels **(E)** and IL-15Rα mRNA levels **(F)** between sedentary control mice and exercise mice. Data are presented as mean ± S.E.M. Ordinary two-way ANOVA and three-way ANOVA tests were used for determining statistical significance in IL-15 and IL-15Rα expression between the different age, sex and physical activity in mice. Asterisks indicate statistical significance (**$P \leq 0.01$), (***$P \leq 0.001$). $n \geq 4$ for gene expression analysis, n = 1 for IHC protein analysis. Abbreviations: CTCF, corrected total cell fluorescence; SED, sedentary control mice; EX, exercise mice.

After the single bout treadmill exercise, 2-month-old male and female mice (n = 3) were kept three days longer to determine whether an extended time between exercise and sacrifice plays a role in inducing IL-15 and IL-15Rα. No significant difference in IL-15 and IL-15Rα mRNA is observed between the mice sacrificed after 4 hours and after three days (Fig. 4). Sacrificing after three days shows an increased expression of IL-15 in female mice compared to sedentary mice, while in male, the expression after three days decline. When comparing the expression of IL-15 based on the time points of sacrificing, IL-15 levels are increased three days after the last exercise bout in female and male mice (Fig. 4A). Similar effect is measured in IL-15Rα gene expression only in male the expression increased four hours after last exercise both compared to sedentary mice and sacrificing after three days (Fig. 4B).

The expression of IL-15 in spleen tissue was investigated to determine if IL-15 is solely expressed by skeletal muscle cells or immune cells. As shown in Fig. 5A, a significant increase ($P \leq 0.05$, $P \leq 0.001$) in IL-15 is present between 2-month-old and 24-month-old sedentary mice, both female and male. Likewise, a significant increase ($P \leq 0.05$) in IL-15 expression in the spleen of 24-month-old female sedentary mice is measured compared to the exercise mice of that group. Taken together, IL-15 might also be expressed by skeletal muscle cells and immune cells.

Acute bout of exercise did not cause an upregulation of autophagy in the gastrocnemius muscle of sedentary and control mice – To verify whether the bout of exercise was sufficient, the upregulation of autophagy in skeletal muscle tissues was investigated. Exercise-induced autophagy is analysed by measuring LC3 and p62 mRNA and protein levels. When comparing LC3 mRNA and protein levels between sedentary and exercise mice, no significant difference was observed in LC3 expression based on age, sex, and physical activity (Fig. 6A,B). However, an upregulation in LC3 mRNA was present in 15-month-old and 24-month-old mice. Considering the effect of exercise on p62 mRNA and protein expression, no significant difference was observed between sedentary control mice and exercise mice of any age and sex. (Fig. 6C,D). Upregulation of p62 mRNA was noticed in sedentary control mice of any age and sex (Fig. 6C). In summary, acute bout of treadmill running did not upregulate autophagy expression in exercise mice of any age and sex.

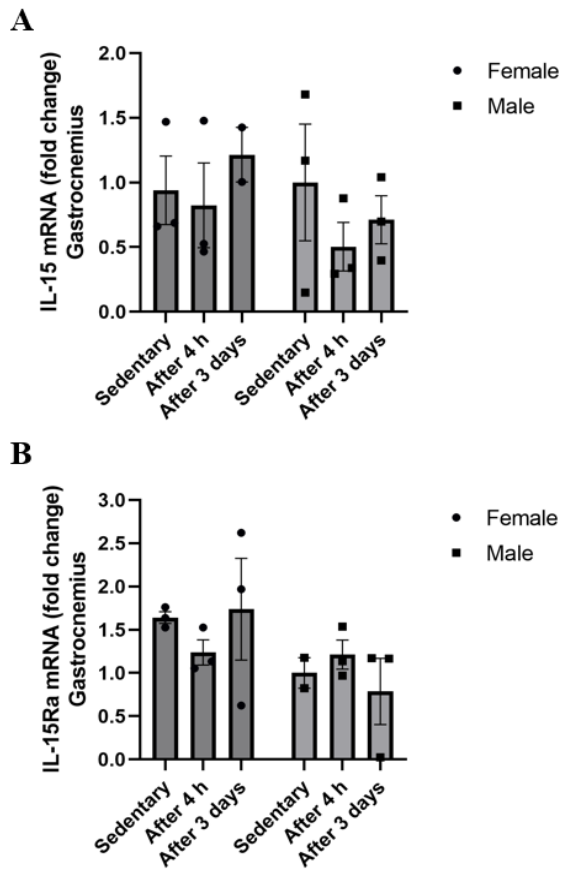

Fig. 4 – Determination of IL-15 and IL-15Rα at different time points of sacrifice after an acute bout of exercise. Quantitative analysis of IL-15 mRNA **(A)** and IL-15Rα mRNA **(B)** in exercise mice sacrificed after four hours or three days post-exercise compared to sedentary mice (n ≥ 2). Ordinary two-way ANOVA test determined the gene expression difference between sedentary control mice and sacrificed at different time points.

DISCUSSION

This study aimed to investigate the effect of an acute bout of exercise on IL-15 and autophagy expression in an age- and sex-dependent manner of healthy mice. The results demonstrated that IL-15 mRNA and protein levels did not significantly increase in the gastrocnemius muscle of mice following an acute bout of treadmill running, no upregulation of autophagy was induced during exercise. Also, IL-15 was significantly increased in spleen tissue of 24-month-old sedentary mice.

Muscle tissue consists of several fiber types which are responsible for IL-15 production. By identifying the type of fiber which is responsible for IL-15 can help with excluding a particular skeletal muscle type from further analysis. The current study detected the different fiber types in cryosection of gastrocnemius muscle by immunohistochemical staining. It was determined that murine gastrocnemius muscle mainly contains of type I and type IIa muscle fibers and no expression of type IIb muscle fibers. Those results does not support the results obtained by Mäntarri et al. (2005), which analysed the distribution of muscle fibers in murine skeletal muscle tissue, including gastrocnemius muscle, by western blotting. The results measured by Mäntarri et al. showed that gastrocnemius muscle mainly consists of type II fibers (83.3 %) than type I fibers (15.7 %) on protein levels. The type II fiber analysis was further divided into type IIA (20.6 %), type IIB (28.1 %) and type IID (35.6%). A possible reason for differences in results could be the method used (western blot versus immunofluorescent staining of cryosections) to determine the presence of the muscle fiber types. Both techniques rely on the principle of the antibody-antigen interaction. The main advantage of IHC is that the precise location of the target protein can be detected. A drawback is that IHC cannot detect low concentrations of target antigens. Western blotting has the advantage of measuring a signal

proportional to the amount of protein that exists (39). Another reason could be that the used antibodies could not properly bind to the tissue sections due to the freezing damage present. It is also possible that the location in the muscle where the measurement was made was incorrect. Fiber types may be more concentrated in certain places than in the place where the measurement was made. As a result, the expression of certain fiber types may have been lost. The findings of the current study regarding the IL-15 producing muscle fiber types are consistent with those of a human study conducted by Nielsen et al. (2007), which reported that IL-15 is mainly expressed in type II fibers (40). To the best of the authors' knowledge, limited literature on the muscle fiber composition of mice was available. Therefore, comparing the results of the present study with other studies is difficult.

In the current study, no difference in baseline values of IL-15 and IL-15Rα between age and sex was found, which is in contrast to previous studies on ageing rodents (41, 42, 43). Quinn et al. (2010) reported that IL-15 baseline values radically decreased with age (42). This study used 12-month-old, 18-month-old, 24-month old and 28-months old B6C3 male mice and collected serum and quadricep tissue. The quadriceps tissue was used as both isoforms long signal peptide (LPS)-IL-15 and short signal peptide (SSP)-IL-15 were present. No significant difference in the quadriceps IL-15 gene expression of both isoforms was observed. However, a significant decrease in IL-15 protein levels was detected in the serum and muscle tissue of 28-month-old mice compared to 18-months old mice (42). It is therefore suggested that the age-dependent reduction of IL-15 protein is not caused by a reduction in IL-15 mRNA, but rather by translational or post-translational mechanisms (42). Detecting IL-15 baseline values in rodents poses technical difficulties, and various translational and post-translational regulations of IL-15 are complex, which may explain conflicting results in rodents (42, 44). The difference between the current study and Quinn et al. (2010) could be the type of skeletal muscle tissue used in both studies. The current study used the gastrocnemius muscle, which mainly consists of type I and IIa muscle fiber types, while Quinn et al. (2010) focussed on the expression of IL-15 in quadriceps muscles. The quadriceps muscle consists of 50% type I and 50% type II muscle fibers (45). It is possible that type I muscle fibres play a role in the expression of muscle-derived IL-15, although no colocalization of IL-15 in type I fibres was observed in the current study.

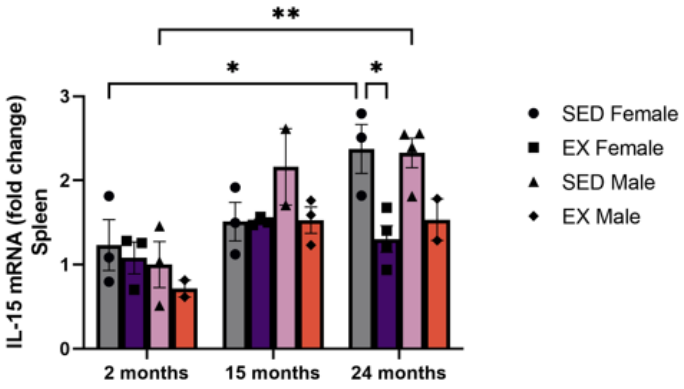

Fig. 5 – IL-15 expression in sedentary and exercise mice spleen tissue. Quantitative analysis of IL-15 by qPCR in spleen tissue of sedentary control and exercise mice ($n \geq 2$). Data are presented as mean ± S.E.M. Ordinary two-way ANOVA test was used for determining statistical significance in IL-15 expression in mice differing in physical activity, age and sexes. Asterisks indicate statistical significance (* $P \leq 0.05$), (** $P \leq 0.01$).
Abbreviations: CTCF, corrected total cell fluorescence; SED, sedentary control mice; EX, exercise mice.

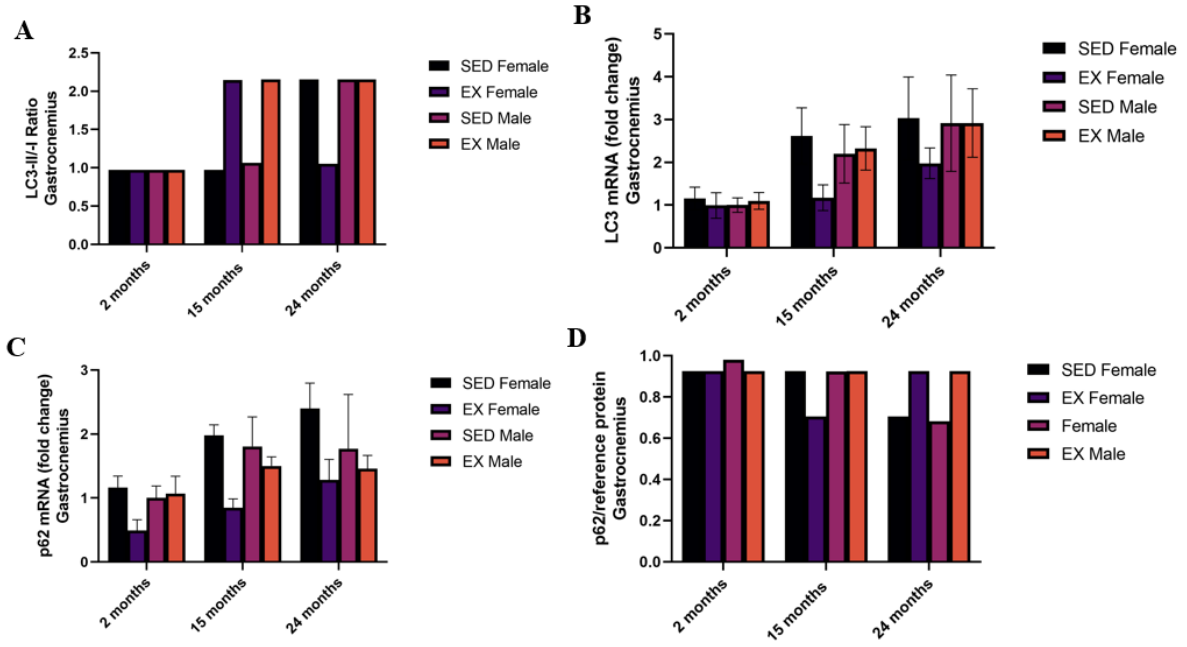

Fig. 6 – **Expression of autophagy markers LC3 and p62 in gastrocnemius muscle of sedentary and exercise mice after an acute bout of exercise.** Analysis of LC3 mRNA (A) and LC3-II/-I ratio (B) in sedentary control and exercise mice of different ages and sexes. Gene expression (C) and protein levels (D) of p62 were compared between sedentary control and exercise mice after an acute bout of exercise. A three-way ANOVA test was conducted to determine the difference in autophagy markers between mice differing in physical activity, age and sex. $n \geq 2$, for gene expression analysis. $n = 1$, for protein expression.
Abbreviation: SED, sedentary control mice; EX, exercise mice.

In the study conducted by Marzetti et al. (2009), IL-15 and IL-15Rα expressions were analysed in 8-month-old, 18-month-old, 29-month-old and 37-months old Fischer344xBrown Norway (FBN) male hybrid rats (43). These rats were divided into ad libitum-fed rats and life-long calorie-restricted rats. Gastrocnemius muscle was collected to detect IL-15 and IL-15Rα gene and protein expression to determine the effect of age and diet. IL-15 mRNA expression did not change among age groups in ad libitum rats, but protein levels decreased in an age-dependent manner. A progressive age-dependent decline in IL-15Rα gene expression was observed in ad libitum rats (43). The difference in animal models and methodologies may explain discrepancies concerning protein expression between the two studies (previously explained).

The extent to which exercise affects the expression of IL-15 has not yet been established (46). In addition, contradictory data are reported about the effect of exercise on IL-15 and IL-15Rα expression in skeletal muscle tissue and circulating IL-15 levels (40, 47, 48). Minuzzi et al. (2021) investigated the expression of IL-15 in 5-month-old and 26-months old Wistar male rats after performing a 5-day long short-term treadmill exercise program. This study indicated no significant difference in IL-15 protein levels between age classes, and an increased trend in IL-15 protein levels was found in 26-month-old exercise mice (47). However, other studies detected an increased expression of IL-15 in the skeletal muscle tissue of aged rodents (41, 47). This contradictory result could be due to the time of muscle tissue collection. The skeletal muscle tissue was collected after 4 hours post-exercise, while the samples in previous studies were collected between 12 - 48h. An alternative explanation for the conflicting results might be that short-term exercise is not strong enough to trigger a change in IL-15 expression, and thereby a longer duration or higher intensity of exercise may be required to induce alterations in muscle-derived IL-15 expression (47). It must be noted

that the analysis of sex-dependent IL-15 expression in humans and rodents is limited.

During the detection of IL-15 concentration in plasma pre- and post-exercise, the content of the certain samples got spilled during the centrifugation step, which prevented reliable results from being obtained. However, extremely low concentrations of IL-15/IL-15Rα were detected in few samples, but it was impossible to determine any differences based on sex, age and physical activity. Consequently, the analysis was terminated prematurely to focus on detecting IL-15 in gastrocnemius muscle and spleen. When the literature is further examined, it is apparent that several studies have reported that levels of circulating IL-15 in plasma of humans (49) and rodents (42, 43) with age decreases. In human subjects, significantly lower expression of circulatory IL-15 has been measured, which may be explained by the relationship between muscle mass loss, by sarcopenia, and insufficient plasma levels of IL-15 (25, 50). Performing acute exercise has been shown to increase the concentration of circulating IL-15 in both rodents (51, 52) and human subjects (53, 54). Anderson et al. (2016), compared different detection assays for measuring free IL-15 and IL-15/IL-15Rα complexes. They found higher concentrations of free IL-15 (38.9 pg/mL) compared to IL-15/IL-15Rα complex (18.0 pg/mL) is measured in sedentary male mice. The study indicated that there were conflicting results from other human (55, 56) and rodent (57) studies, in which the opposite was demonstrated. Anderson et al. indicated that the possible explanation for this discrepancy is their use of a higher sensitivity of 6.6 ng/mL, while other studies use an lower sensitivity. Additionally, Anderson et al. concluded that IL-15 may be initially released as IL-15/IL-15Rα complexes, which later resolve in free IL-15 (58). According to Riechmann et al. (2004), IL-15 plasma levels of young human subjects were found to increase immediately after an acute bout of intense resistance exercise, indicating that muscular activity may have triggered the release of muscle-derived IL-15 into the bloodstream (53). However, other studies by Quinn et al. (2014) and Tamura et al. (2008) found that circulatory IL-15 concentrations decreases 3h after exercise (51, 54). The short-lived increase in circulatory IL-15, is consistent with the release of free IL-15 rather than IL-15/IL-15Rα complexes (51, 52, 54). These observations suggest that the post-exercise release of IL-15 into the bloodstream may involve a mechanism independent of the IL-15Rα (58). The current study used an IL-15/IL-15Rα Complex ELISA kit (Invitrogen) to detect the IL-15/IL-15Rα complexes in murine serum, and although no concentration could be determined due to technical issues, a future study could repeat this analysis to detect not only the complex but also the free form of IL-15. Since other studies have reported a decrease in IL-15 at three hours post-exercise, it may be useful to collect blood samples at this time point. However, it first needs to be established whether IL-15 production occurs in the gastrocnemius muscle at this time point. The current study showed that IL-15 expression is reduced after 4 hours, but increases again after 3 days. As a follow-up study, exercise mice could be sacrificed at different time points to determine the most effective time point for both circulating IL-15 and IL-15 present in the gastrocnemius muscle. However, the current study needs to be redone using a larger sample size to draw a more reliable conclusion.

In order to confirm that myocytes are the source of IL-15 production rather than immune cells, Cui et al. (2004) examined the expression of IL-15 and IL-15Rα in the spleen of sedentary mice. Their findings showed increased IL-15 expression in splenic stromal cells dependent on age, which is in line with the results reported in the current study (11). Due to limited research available on the expression of IL-15 in spleen tissue to the author's knowledge, and given the structural and functional similarities between IL-15 and IL-2, it was decided to explore the impact of physical activity on IL-2 expression in the spleen. In a study by Kohut et al. (2001), it was observed that mice subjected to acute strenuous treadmill running, which gradually increased speed until exhaustion, exhibited a decrease in IL-2-producing splenocytes compared to control mice. The decrease was observed 2 days after

exercise, while at later time points (1-7 days later), no significant difference in IL-2 production was detected (59). The current study found similar results, with a reduction in IL-15 levels observed in exercise mice, with the decrease being evident 4 hours after the last exercise performance.

Numerous studies have shown exercise-induced autophagy in skeletal muscle tissue of humans (60, 61, 62) and rodents (63, 64). LC3 marker is considered an important marker that positively correlates with autophagosome formation (65), while an increase in LC3 expression is associated with a decrease in p62 protein levels, strengthening the hypothesis of autophagosome formation (64). However, it is unclear whether autophagosome formation causes this increase or whether it is a result of a defect in lysosomal degradation (65). The LC3-II/-I ratio and p62 are widely used markers for autophagy induction and autophagy flux (66). Marker p62 interacts with LC3-II to facilitate the incorporation of autophagy substrates into autophagosomes. Our findings are in contradict with previous studies, which have suggested that LC3 and autophagosome content increase after short-term treadmill running (67, 68). Kim et al. (2012) demonstrated that LC3 protein levels significantly decreased during recovery in 3-month-old male ICR mice (69). The conflicting results may be demonstrated that the mice had to run longer and faster than in our study (50 minutes for 5 days compared to 30 minutes for 1 day). A significant decrease in LC3-II and LC3-I protein levels was present at all time points (0h, 6h, 12h and 18h) post-exercise compared to the control group and zero hours post-exercise. The lowest level of LC3-II and LC3-I protein was measured 3 hours after treadmill running, while LC3-I slightly increase at 6 hours post-exercise, but then gradually declines (69). A follow-up study could determine autophagy markers, specifically LC3, immediately after exercise, six hours later, and three days after exercise to establish whether LC3 occurs higher immediately after exercise or at a later time point, as was observed with IL-15 mRNA levels. Another study found that autophagy was induced in skeletal and cardiac muscle in young mice after treadmill running for 30 minutes and reached a plateau at 80 minutes. Besides a measurable increase in LC3, conversion of LC3-I to LC3-II and degradation of p62 were also observed. The study concluded that exercise serves as a type of stimulus for the induction of autophagy in vivo (68). Similarly, Halling et al. (2017) found that p62 expression decreased in mice and humans after an acute bout of endurance exercise (66), which is in line with the results of the current study. However, contrary results revealed no change in muscle p62 expression after short-term endurance exercise (62, 70, 71, 72, 73, 74). In human studies, difficulties have been encountered in monitoring autophagy induction through exercise as specific parameters such as intensity, duration, and type of exercise are involved (66). Those parameters also play a role in inducing autophagy in murine skeletal muscle tissue. By changing those parameters, the induction of autophagy can be enhanced. Additionally, the timing of muscle sampling can also influence the detection of autophagy (60, 72). Due to time constraints, the effect of exercise-induced IL-15 on autophagy was not investigated, but it will be addressed in a follow-up study.

When interpreting the results of this study, it is important to consider the next three limitations. The first limitation is the small sample size, which is common in pilot studies that aim to evaluate a study's feasibility, duration, costs, and potential improvements before conducting a full-scale research project. During the study, some older mice died, possibly due to stress experienced during husbandry, despite not suffering from any visible disease or injuries at sacrifice. The second limitation relates to the speed at which treadmill training was performed, which failed to exhaust the 2-month-old and 15-month-old mice. Consequently, insufficient optimal induction of IL-15 and autophagy occurred in the gastrocnemius muscle. Furthermore, it was found that limited contemporary literature was available on specific topics discussed in this study, which sometimes made it challenging to draw definitive conclusions. Additionally, due to time constraints, the effect of exercise-induced IL-15 on autophagy was not investigated, and this research objective will be addressed in a follow-up study. Despite these limitations, this study has

several strengths, including its investigation of the effect of age and sex on the production of IL-15 in sedentary and exercise mice, as most studies have solely focused on male subjects. Additionally, this study examined the source of IL-15 production by investigating whether IL-15 is mainly produced by myocytes or also immune cells before and during exercise, which is another significant strength.

Future studies should address the limitations of this study by exploring alternative methods to allow young mice to experience exhaustion during treadmill training. One approach might involve increasing the intensity of the speed-determination test or implementing a regimen of intensive running followed by a short rest period and another intensive run to induce fatigue. Additionally, to ensure accurate interpretation of autophagy induction, future studies should assess autophagy flux and other autophagosome component proteins, such as Atg 7, Atg 12, Atg 53, Beclin-1, ULK-1, and Lamp2a. Furthermore, in the future, the effect of regular exercise on IL-15 production will be investigated and with this, the effect on autophagy.

CONCLUSION

The current research aimed to determine the effect of acute bout of treadmill running on the expression of IL-15 and autophagy in ageing mice. The results of this study showed that there was no significant increase in IL-15 and IL-15Rα mRNA and protein levels, as well as autophagy markers LC3 and p62, after an acute bout of treadmill running in mice and any age and sex. Furthermore, we investigated whether the gastrocnemius muscle was the only source of IL-15 expression or if splenocytes also played a role and found that IL-15 was also expressed in spleen tissue in sedentary mice. This study suggests that the duration Our study suggests that the duration and intensity of acute exercise may play a critical role in upregulating IL-15, IL-15Rα, and autophagy in the gastrocnemius muscle. To explore this further, future research will increase the duration and intensity of treadmill running to determine the expression of IL-15 in both young and old mice. Finally, the study ultimately will be expanded to focus on the effect of chronic exercise on IL-15, IL-15Rα and autophagy expression in both mice and humans.

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Author contributions – LF and LS conceived and designed the research. LC performed experiments and data analysis under the supervision of LF. LC wrote the paper and LF and LS carefully edited the manuscript and provided feedback.

SUPPLEMENTAL FIGURES AND TABLES

Supplementary table S2 – Antibodies used for western blotting of IL-15 and autophagy markers in gastrocnemius muscle.

Stain	Primary antibody	Dilution	Secondary antibody	Dilution
IL-15	Polyclonal goat anti-mouse IL-15[a] (PA547014)	1:100	Polyclonal rabbit anti-goat Ig/HRP[b] (P0449)	1:1000
LC3	Polyclonal rabbit anti-mouse LC3A/B [c] (4108)	1:1000	Polyclonal goat anti-rabbit Ig/HRP[b] (P0448)	1:5000
p62	Monoclonal mouse anti-human p62 [d] (Ab56416)	1:1000	Polyclonal rabbit anti-mouse Ig/HRP[b] (P0260)	1:5000
β-actin	Monoclonal mouse anti-mouse[e] (C3022)	1:1000	Polyclonal rabbit anti-mouse Ig/HRP[b] (P0260)	1:5000

[a] Thermo Fisher Scientific, Waltham, MA, USA
[b] Agilent technologies, Santa Clara, CA, USA
[c] Cell Signaling Technology, Danvers, MA, USA
[d] Abcam, Cambridge, UK
[e]Santa Cruz Biotechnology Inc., Dallas, TX, USA

Supplementary table S2 – Primers used for the analysis of IL-15 and autophagy gene expression in gastrocnemius muscle and spleen tissue.

Gene name		Sequences (5'-3')
GAPDH	Forward primer	5'-GGC CTT CCG TGT TCC TAC-3'
	Reverse primer	5'-TGT CAT ATC TGG CAG GTT-3'
18S RNA	Forward primer	5'-ACG GAC CAG AGC GAA AGC-3'
	Reverse primer	5'-TGT CAA TCC TGT CCG TGT-3'
IL-15	Forward primer	5'-TCT CCC TAA AAC AGA GGC CAA-3'
	Reverse primer	5'-TGC AAC TGG GAT GAA AGT CAC-3'
IL-15Rα	Forward primer	5'-TGA ACT CCA GGG AGA GGT ATG-3'
	Reverse primer	5'-CTA GGG AGG GGT CTC TGA TGC-3'
LC3	Forward primer	5'-GAT AAT CAG ACG GCG CTT GC-3'
	Reverse primer	5'-ACT TCG GAG ATG GGA GTG GA-3'
P62	Forward primer	5'-GCT GCC CTG TAC CCA CAT CT-3'
	Reverse primer	5'-CGC CTT CAT CCG AGA AAC GT-3'

Supplementary table S3 – Antibodies used for immunohistochemical staining of IL-15 and muscle fiber types in gastrocnemius muscle.

Stain	Primary antibody	Dilution	Secondary antibody	Dilution
IL-15	Polyclonal goat anti-mouse IL-15[a] (PA547014)	1:250	Polyclonal rabbit-anti goat IgG[a] (A21431)	1:250
MHC I (IgG2b)	Monoclonal mouse anti-bovine IgGb [c] (BA-F8)	1:50	Polyclonal donkey anti-mouse IgG[a] (A32766)	1:250
MHC IIa (IgG1)	Monoclonal mouse anti-bovine IgG1[c] (SC-71)	1:200	Polyclonal donkey anti-mouse IgG[a] (A32766)	1:300
MHC IIb (IgM)	Monoclonal mouse anti-bovine IgM[c] (BF-F3)	1:100	Polyclonal donkey anti-mouse IgG[a] (A32766)	1:250
Laminin (IgG)	Polyclonal rabbit anti-mouse laminin [a] (PA1-16730)	1:200	Polyclonal donkey anti-mouse IgG[a] (A32766)	1:250

[a] Thermo Fisher Scientific, Waltham, MA, USA
[b] Bio-Rad Laboratories Inc., Hercules, CA, USA
[c] Developmental Studies Hybridoma Bank (DSHB), Iowa city, IA, USA
Abbreviation: MHC, myosin heavy chain

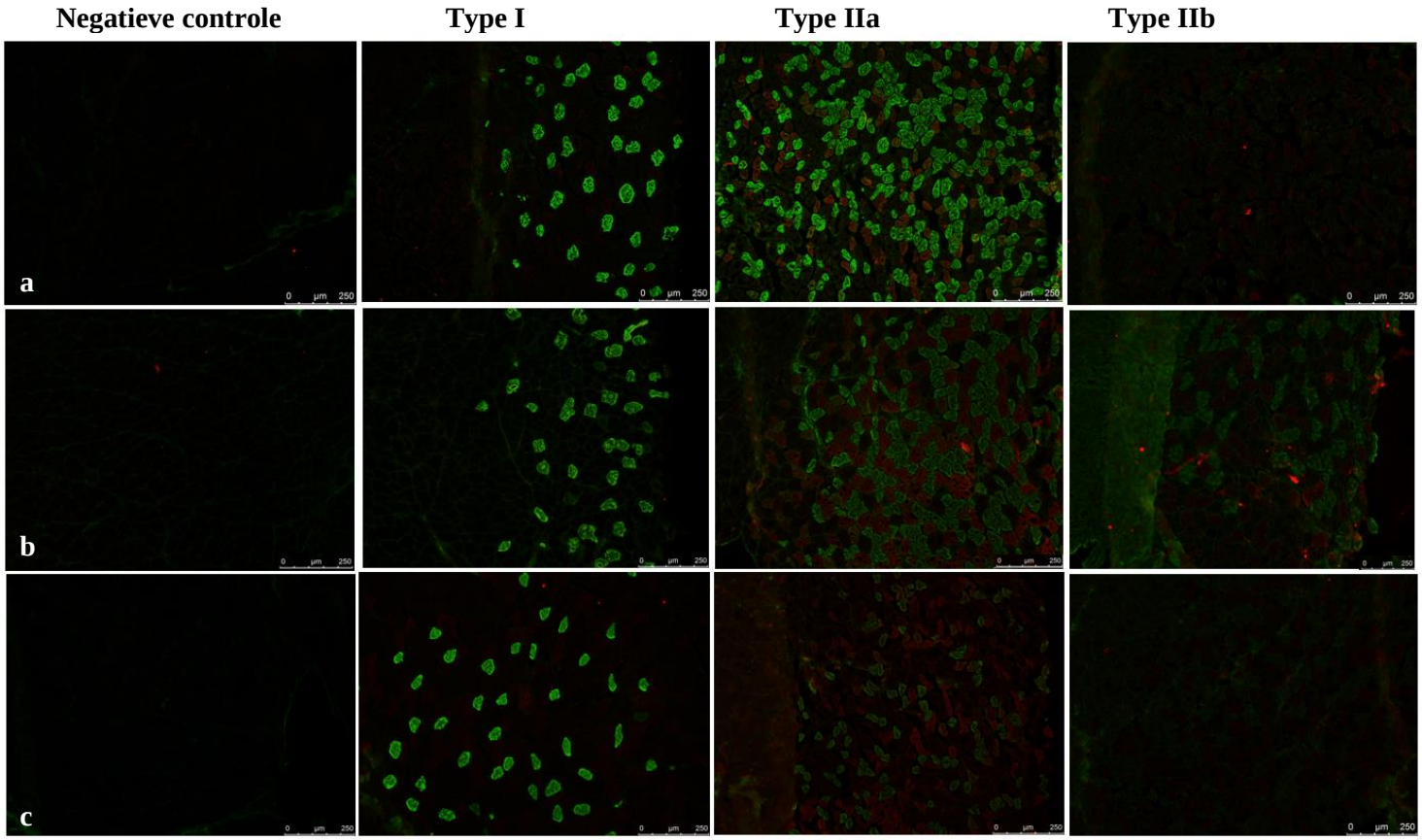

Fig. S1 – **Expression of exercise-induced IL-15 in m. gastrocnemius in C57BL/6 mice.** Immunohistochemical staining of murine skeletal muscle tissue for IL-15 (red) expression by MHC (green) proteins: MHC I, MHC IIa and MHC IIb proteins in gastrocnemius muscle are shown. The fluorescence images are taken with objective 10x.
Abbreviations: IL-15, interleukin 15; MHC, major histocompatibility complex.