Experimental Gerontology 181 (2023) 112280

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

High-intensity interval training reduces the induction of neutrophil extracellular traps in older men using live-neutrophil imaging as biosensor

Nicolás Vidal-Seguel [a,b,1], Camilo Cabrera [c,1], Luciano Ferrada [d], Macarena Artigas-Arias [e,f], Andrea Alegría-Molina [g], Sergio Sanhueza [c], Alejandra Flores [c], Nolberto Huard [h], Jorge Sapunar [i], Luis A. Salazar [h], Reuben McGregor [j], Estefanía Nova-Lamperti [c,*], Gabriel Nasri Marzuca-Nassr [g,**]

[a] Universidad de La Frontera, Facultad de Medicina, Departamento de Ciencias Básicas, Temuco, Chile
[b] Universidad de La Frontera, Facultad de Medicina, Programa de Doctorado en Ciencias Morfológicas, Temuco, Chile
[c] Molecular and Traslational Immunology Laboratory, Clinical Biochemistry and Immunology Department, Pharmacy Faculty, Universidad de Concepción, Concepción, Chile
[d] Centro de Microscopía Avanzada, Universidad de Concepción, Concepción, Chile
[e] Universidad de La Frontera, Doctorado en Ciencias mención Biología Celular y Molecular Aplicada, Temuco, Chile
[f] Departamento de Procesos Terapéuticos, Facultad de Ciencias de la Salud, Universidad Católica de Temuco, Temuco, Chile
[g] Universidad de La Frontera, Facultad de Medicina, Departamento de Ciencias de la Rehabilitación, Temuco, Chile
[h] Universidad de La Frontera, Facultad de Medicina, Departamento de Ciencias Básicas, Centro de Biología Molecular y Farmacogenética, Temuco, Chile
[i] Universidad de La Frontera, Facultad de Medicina, Departamento de Medicina Interna, Temuco, Chile
[j] Department of Molecular Medicine and Pathology, School of Medical Sciences, The University of Auckland, Auckland, New Zealand

ARTICLE INFO

Section Editor: Maria Cavinato

Keywords:
NETs
Neutrophil extracellular trap
High-intensity interval training
Exercise
Elderly

ABSTRACT

Neutrophil extracellular trap formation (NETosis) is a mechanism used by neutrophils to capture pathogens with their own DNA. However, the exacerbation of this immune response is related to serious inflammatory diseases. Aging is known to lead to an excessive increase in NETosis associated with various diseases. Under this scenario, the search for strategies that regulate the release of NETosis in older people becomes relevant. High-intensity interval training (HIIT) involves repeated bouts of relatively intense exercise with alternating short recovery periods. This training has shown beneficial effects on health parameters during aging and disease. However, little is known about the potential role of HIIT in the regulation of NETosis in healthy older people. The aim of this study was to evaluate the induction of NETosis by serum from healthy young and older men, before and after 12 weeks of HIIT using healthy neutrophils as a biosensor. HIIT was performed 3 times per week for 12 weeks in young (YOUNG; 21 ± 1 years, BMI 26.01 ± 2.64 kg·m^{-2}, n = 10) and older men (OLDER; 66 ± 5 years, BMI 27.43 ± 3.11 kg·m^{-2}, n = 10). Serum samples were taken before and after the HIIT program and NETosis was measured with live cell imaging in donated neutrophils cultured with serum from the participants for 30 h. Our results showed that serum from older men at baseline induced greater baseline NETosis than younger men ($p < 0.05$; effect size, ≥0.8), and 12 weeks of HIIT significantly reduced (Interaction Effect, $p < 0.05$; effect size, 0.134) the induction of NETosis in older men. In conclusion, HIIT is a feasible non-invasive training strategy modulating NETosis induction. Additionally, the use of neutrophils as a biosensor is an effective method for the quantification of NETosis induction in real time.

1. Introduction

High-intensity interval training (HIIT) is a form of physical training in which short periods of high-intensity are alternated with brief recovery periods (Robinson et al., 2017). Similar or even higher beneficial changes in adult health markers are induced by HIIT using less working

* Correspondence to: E.N. -Lamperti, Facultad de Farmacia, Universidad de Concepción, Barrio Universitario, Concepción, Chile.
** Correspondence to: G.N.M.-Nassr, Facultad de Medicina, Universidad de La Frontera, Claro solar 115, Temuco, Chile.
E-mail addresses: enovalamperti@gmail.com (E. Nova-Lamperti), gabriel.marzuca@ufrontera.cl (G.N. Marzuca-Nassr).
[1] Equal contribution.

https://doi.org/10.1016/j.exger.2023.112280
Received 24 May 2023; Received in revised form 28 August 2023; Accepted 30 August 2023
Available online 5 September 2023

time than other forms of training, such as continuous aerobic training (Gibala et al., 2012; Wewege et al., 2017). Several studies have suggested that HIIT improves body composition, physical performance, and reduces oxidative stress and markers of inflammation, generally affected by aging (Hayes et al., 2021; Boukabous et al., 2019).

The neutrophils are the most abundant circulating white blood cell population. These polymorphonuclear cells are the first subset recruited after pathogen entry (Malech et al., 2020), and exert several effector functions such as phagocytosis, enzyme secretion (defensin, myeloperoxidase, human neutrophil elastase), reactive oxygen species production, chemotaxis secretion, cytokine production and the capture of pathogens by the formation of neutrophil extracellular traps (NETs) (Papayannopoulos, 2018; Nathan, 2006). NETosis is a type of cell death with NETs release, formed by decondensed chromatin with bactericidal proteins embedded in cytoplasmic granules, which capture and slow down the spread of pathogens (Pérez-Figueroa et al., 2021; Nirmala and Lopus, 2020). In addition, NETs have been implicated in inflammatory diseases (Boukabous et al., 2019; Papayannopoulos, 2018), since NETosis can also be activated by systemic factors such as cytokines, autoantibodies, immune complexes, and bacterial toxins (Kenny et al., 2017; Keshari et al., 2012). Beside the protective immunological role of NETosis, the exacerbated formation of NETs due to sustained stimuli has been associated with respiratory, autoimmune, cardiovascular, metabolic and malignant diseases (Klopf et al., 2021; Vorobjeva and Chernyak, 2020). Moreover, aging also impairs the efficacy of NETosis by promoting NETs formation in response to unspecific inflammatory factors rather than pathogens (Sabbatini et al., 2022). Therefore, the measurement of NETosis is a promising biomarker of the innate immunity activity in response to pathogens or circulating pro inflammatory stimuli (Pokrywka et al., 2015; Hummel et al., 2018). However, there is heterogeneity in the current quantification methods (Stoimenou et al., 2022). In line with this, NETosis has been evaluated through fluorescence microscopy, flow cytometry, electron microscopy, multispectral imaging flow cytometry, among other techniques (Gupta et al., 2018; Zharkova et al., 2019). These techniques can be laborious, complex, and sometimes only a single time point of cells actively experiencing NETosis can be measured (Gupta et al., 2018; Zharkova et al., 2019). A new quantification alternative is the use of a method with donated polymorphonuclear (PMN) cells as a biosensor, demonstrating the effectiveness of this technique in the quantification of NETosis in real time from 6 h to 30 h of incubation (Nakabo et al., 2022).

The effect of the HIIT on systemic inflammatory of markers is known (Khalafi and Symonds, 2020). However, the effect of physical exercise upon NETosis remains under investigation. Whereas acute intense physical exercise in a single or a few sessions has shown a stimulating effect by increasing the percentage of NETosis in healthy young individuals (Da Syu et al., 2013; Orysiak et al., 2021); the effect of training as HIIT on the rate of NETosis in both healthy young and older men remain unknown. In accordance with the above, the aim of this study was to evaluate the induction of NETosis of healthy young and older men, before and after 12 weeks of HIIT using healthy neutrophils as a biosensor. We hypothesized that 12 weeks of HIIT will achieves a decrease in the induction of NETosis in both groups with a greater effect in older men.

2. Materials and methods

2.1. Study design

The present research work included 20 healthy men who completed the study, divided into two groups: young (YOUNG; 21 ± 1 years, BMI 26.01 ± 2.64 kg·m^{-2}, n = 10) and older (OLDER; 66 ± 5 years, BMI 27.43 ± 3.11 kg·m^{-2}, n = 10) men. This investigation is part of a research project aimed at determining the effect of an intervention with HIIT on health parameters between young and older men. Original study that recruited only men to achieve a homogeneous sample for the purpose of the study. This study was approved by the Scientific Ethics Committee from Universidad de La Frontera (Number 069_18, 025_18, 2018), and it was performed according to the Declaration of Helsinki, as previously described (Marzuca-Nassr et al., 2020; Artigas-Arias et al., 2021; Caparrós-Manosalva et al., 2023). Written informed consent was obtained from all subjects involved in the study. A routine medical examination and a health questionnaire were completed by all participants at the beginning of the study.

Inclusion criteria were males, between 18 and 35 years old (YOUNG) and between 55 and 75 years old (OLDER), subjects that do not perform planned and structured physical exercise more than twice a week and with body mass index (BMI) between 18.5 and 30 kg·m^{-2}. On the other hand, the exclusion criteria were recent surgery (within 12 weeks prior to the study), anticoagulation drug therapy, orthopedic or musculoskeletal injuries, uncontrolled high blood pressure, Type 2 diabetes mellitus (determined by fasting blood glucose >100 mg/dL or HbA1c-values >6.5 %), any family history of thrombosis, and/or severe cardiac problems; nutritional supplementation consumption (leucine, glutamine, casein, whey-protein, fatty acids and creatine) and smoker.

Participants underwent 12 weeks of HIIT on a stationary bicycle and they were evaluated in two separate tests (test 1, PRE and test 2, POST), 48 h before the first session and 48 h after the last training session. Two days before every test day, the participants could not drink alcohol or perform intense physical activity. A blood sample were taken between 8:00 and 10:00 a.m. in a fasting state; weight, height, waist circumference, heart rate and blood pressure were measured. Body mass index and aerobic capacity were also determined. Serum samples were taken before and after the HIIT program and NETosis was measured with live cell imaging in donated neutrophils cultured with serum from the participants for 30 h (Fig. 1).

2.2. Baseline measurements

Weight was measured on a SECA® (Madison, WI, USA) platform scale with a graduation of 0.1 kg and height was measured to a precision of 0.5 cm using a stadiometer attached to the scale, with the participant barefoot. The body mass index (BMI) was determined using weight in kilograms (kg) divided by the square of height in meters (m2) (weight in (kg)/height2 in (m)). Waist circumference was measured with a SECA® tape measure graduated in centimeters (Madison, WI, USA), the evaluation was carried out on exhalation at the midpoint between the lower rib and the iliac crest of the right half of the body.

2.3. Aerobic capacity

Aerobic capacity was measured in all participants before and after the HIIT protocol on a cycle ergometer (Lode Corival Groeningen®, The Netherlands) and with a gas analyzer (Ultima CPX Medgraphics, Minnesota®, USA), previously calibrated for volume and reference gases (Mancilla et al., 2014).

Maximal aerobic capacity (VO2max) was determined using an incremental exercise protocol. The modified Astrand volitional protocol was used as a reference, for this reason, the load was increased by 50 watts every two minutes until voluntary exhaustion.

During the test, the heart rate was continuously monitored (Polar, Finland) to obtain the maximum heart rate just at the moment the maximum aerobic capacity of the participants was recorded.

2.4. High-intensity interval training protocol

The HIIT protocol was performed 3 times a week for 12 weeks using a stationary bicycle (Oxford®, BE2700). Each HIIT session consisted of 10 sets of 1 min of high-intensity exercise, followed by 2 min of inactive rest. During each work interval, the high intensity was regulated by manual adjustment of the pedaling resistance, which was carried out at a controlled speed between 30 and 40 km/h, in addition, the 90 % of

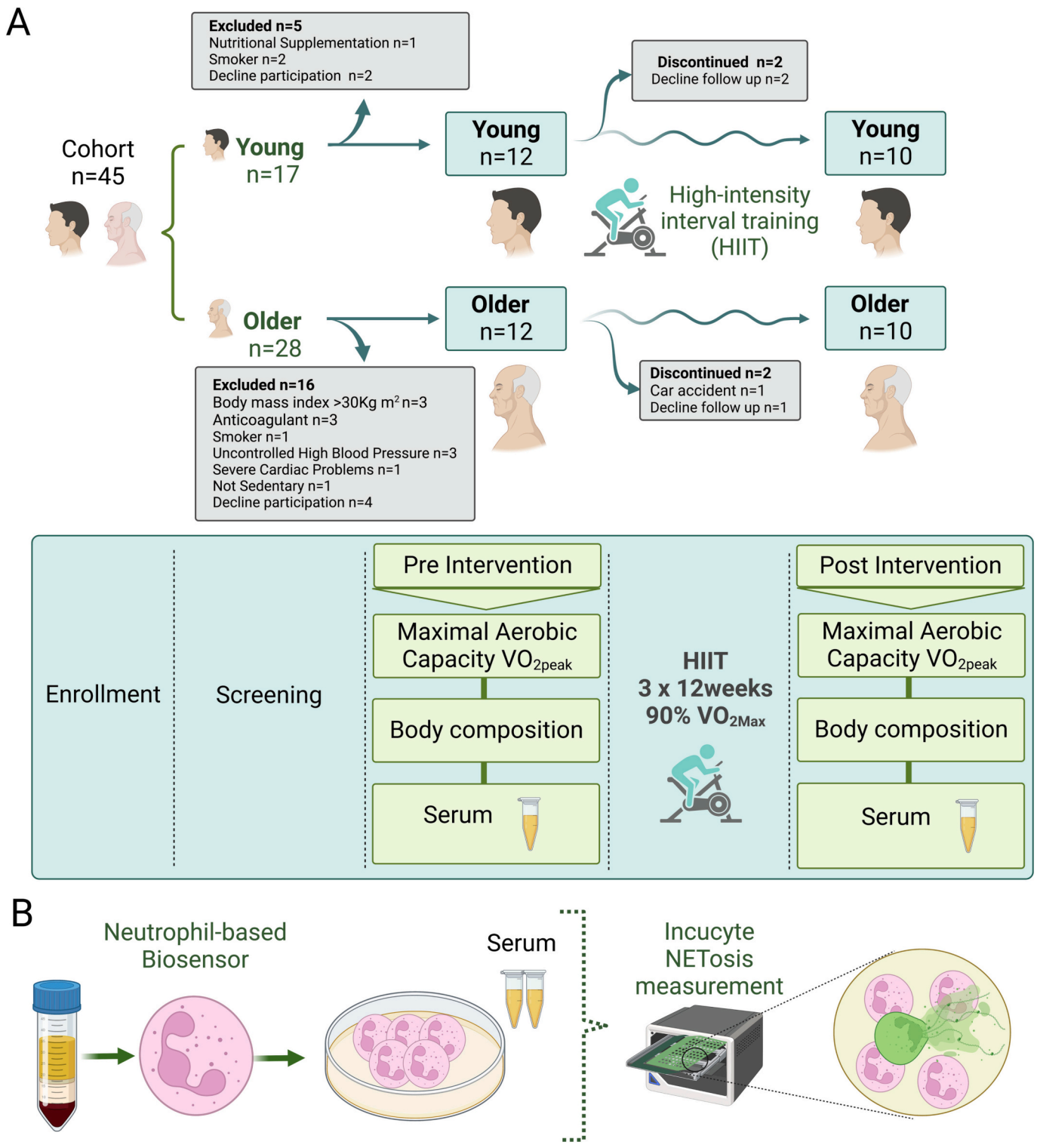

Fig. 1. Flow Chart. (A) An original cohort of 45 participants was invited to the study, from which 24 were included and 20 of them completed HIIT intervention, thus the final cohort included 10 young men, (21 ± 1 years old) and 10 older men (66 ± 5 years old). HIIT was performed 3 times a week for 12 weeks training at 90 % of maximal heart rate. Parameters such as maximal aerobic capacity and body composition were measured. (B) Serum from all participants was obtained before and after HIIT intervention and freshly isolated neutrophils from a healthy donor was used as a biosensor to measure NETosis induction by serum with live imaging (IncuCyte).

maximum heart rate based on VO2max test was used as a reference result. The participants had personalized supervision (Marzuca-Nassr et al., 2020). In addition, at the beginning and end of each training session, blood pressure was measured using an OMRON® brand automatic digital blood pressure monitor (Digital Blood Pressure Monitor HEM-7122, Japan). Measurements were made on the uncovered left arm, with the participant in a sitting position. On the other hand, the heart rate was controlled throughout the training and was recorded at the beginning and end of each work interval. This continuous heart rate monitoring was performed with a heart strap (Polar, Finland).

2.5. Serum collection

Samples were collected after a 12 h fast from one of the superficial veins located in the cubital fossa. Blood samples were taken from the participants 48 h before the first HIIT session and 48 h after the last HIIT session. From each participant, 6 mL of blood was drawn in tubes without anticoagulant. The samples were then centrifuged at 1100*g* × 15 min. The serum was distributed in microtubes and stored in a freezer at −80 °C for later analysis.

2.6. Polymorphonuclear cell isolation

PMN cell isolation was performed from peripheral blood collected by venous puncture from a healthy donor following the protocol proposed by the manufacturer (Axis-Shield). For this purpose, 5 mL of blood was collected by venipuncture using clean, sterile needles and EDTA tubes. The whole blood (5 mL) was carefully layered over 5 mL of Polymorphprep separation reagent in a 15 mL Falcon tube. The samples were centrifuged at 702*g* for 20 min at room temperature in a swinging rotor centrifuge (Changsha Xiangyi centrifuge L-550). After centrifugation, 2 leukocyte rings were obtained, the upper ring was discarded and the lower ring containing PMN was collected. PMNs were extracted and transferred into 1 mL of warm X-vivo (Lonza) with Ca2+ 2pM medium. Cells were washed twice with sterile PBS 10 mM pH 7,2 at 400 g for 15 min, and the supernatant was discarded and resuspended in 1 mL warm Ca2+ 2pM X-vivo medium (Lonza). The phenotype of PNM was analyzed with anti-CD16 (BioLegend) and anti-Siglec-8 (BioLegend) using flow cytometry (Fortessa X20, BD). Data was analyzed using FlowJo (BD). The phenotype of PNM from allergic donors was evaluated during the standardization of the assay.

2.7. IncuCyte assay and analysis

To perform this assay, PMN were stained with Sytox Green DNA tag (Thermofisher) at 30 nM and 1 × 104/well were placed in 100 μL in a 96-flat bottom well plate. The serum was centrifuged at 12000 *g* for 5 min prior activation. Finally, 50 μL of serum from the different samples were added and the plate was placed inside the IncuCyte S3 equipment (Essen Bioscience, Sartorious) for real-time microscopic monitoring for 24 h at 37 °C and 5 % CO2. The exposure time for the acquisition of images in the green channel was 300 ms and 4 images were taken per well every 1 h. The data was processed with the basic analysis module of IncuCyte and the objects of interest were segmented based on size and fluorescence (Gupta et al., 2018) according to the following parameters: in phase contrast, the segmentation adjustment was 0.5 and the minimum area was 70 μm2, the cleanup was used by default with a value of 0. For the green channel, Top-Hat segmentation was used with a radius of 10 μm and a threshold of 1 GCU (Green Calibrate Unit), Edge sensitivity was set to −45, a minimum area filter of 40 μm2 and a maximum of 500 μm2 was used, finally cleanup was used by default with a value of 0.

2.8. NETosis induction standardization

To date "gold-standard" method to study NETosis induction has not been identified, as it requires several considerations regarding cell-type isolation, stimuli selection, time of NET liberation and specific NET formation measurement. In this study we standardize a method using freshly isolated peripheral blood PMN cells obtained from a healthy individual. Of note, neutrophils are glycolytic cells, therefore it is very important to exclude the presence of metabolic diseases, especially associated to glucose metabolism in the donor. Neutrophils are short-lived in circulation; therefore, the experiment must be performed within 1–2 h after neutrophil isolation to reduce cell death. Isolation of PMN cells was performed using gradient centrifugation and characterized using flow cytometry, obtaining 90–95 % of neutrophils and 5–10 % of eosinophils based of CD16 and Siglec-8 expression (Fig. 2A). Higher presence of eosinophils percentages is observed in allergic individuals (Fig. 2A); therefore, it is recommendable to avoid allergic donors, since other isolation methods reduce drastically neutrophil viability (cell sorting). NETs quantification was performed using IncuCyte software by measuring DNA staining outside the cell in green, due to Sytox Green staining, to avoid measurement of dead cells by other mechanism such as apoptosis (Fig. 2B). Finally, for timing, we analyzed the formation of NETs over time within the Sytox Green+ cells, and we defined that NETs formation starts after 12 h post-activation (Fig. 2C).

2.9. Statistical analysis

The results were analyzed in the statistical software SPSS (IBM SPSS Statistics, v. 21) and the figures were created using the GraphPad Prism 8.2 software (San Diego, CA). The data is expressed as mean ± standard deviation (SD). Baseline characteristics between groups were compared by means of an independent sample *t*-test. Pre- versus post-intervention data were analyzed using repeated-measures ANOVA with time (PRE versus POST) as the within-subject factor and group (YOUNG versus OLDER) as the between-subjects factor. In the case of a significant interaction, paired *t*-tests were performed to determine time effects within groups and independent t-tests for group differences in the PRE- and POST-intervention values. The baseline effect size between Group results was estimated using Cohen's d and represented as d. An effect size <0.2 indicates no effect, 0.2–0.49 indicates small effect, 0.5–0.79 indicates medium effect, and ≥0.8 indicates large effect (Cohen, 2013). *Partial eta squared* was used to estimate effect sizes of ANOVA calculation and represented as η2. Where 0.01 indicates Small Effect size, 0.06 medium Effect size, 0.134 or Higher large Effect size. The statistical significance level was set at $p < 0.05$.

3. Results

3.1. Baseline characteristics

The basal characteristics of the participants show homogeneity between the young and older men in all the variables ($p < 0.05$) with the exception of age ($p = 0.007$) (Table 1). During the development of the study, 4 participants discontinued their participation as indicated in the flow chart (Fig. 1A). During the statistical analysis, a participant from the young people group was identified as an outlier based on the 1.5× inter quartile range (IQR), where the value was >1.5 times the IQR above the third quartile of the data. This participant data was excluded from all NETosis analyses.

3.2. NETosis induction by serum from young and older men

The data showed that older men present a higher percentage of basal NETosis compared to the group of young people, with significant differences at 12 h, 18 h, 24 h and 30 h of incubation ($p > 0.05$; large effect size, ≥0.8; Table 2). The effect of 12 weeks of HIIT shows a significant reduction of the percentage of NETosis after the intervention (Factor Time, $p < 0.05$; Higher large Effect size, 0.134; Table 3) and a significant difference in the induction of NETosis between groups (Factor Group, $p < 0.05$; Higher large Effect size, 0.134; Table 3) at 12 h, 18 h, 24 h and 30 h of incubation. When the time x group effect is compared, we observe that there is an interaction at 18 h and 24 h (Interaction Effect, $p < 0.05$; Higher large Effect size, 0.134; Table 3), where only the older group exhibited a significant reduction in the percentage of NETosis after 12 weeks of HIIT (#, $p < 0.001$; Table 3). The significant difference in the induction of basal NETosis between young and older described in Table 2 is also corroborated (*, $p < 0.01$; Table 3).

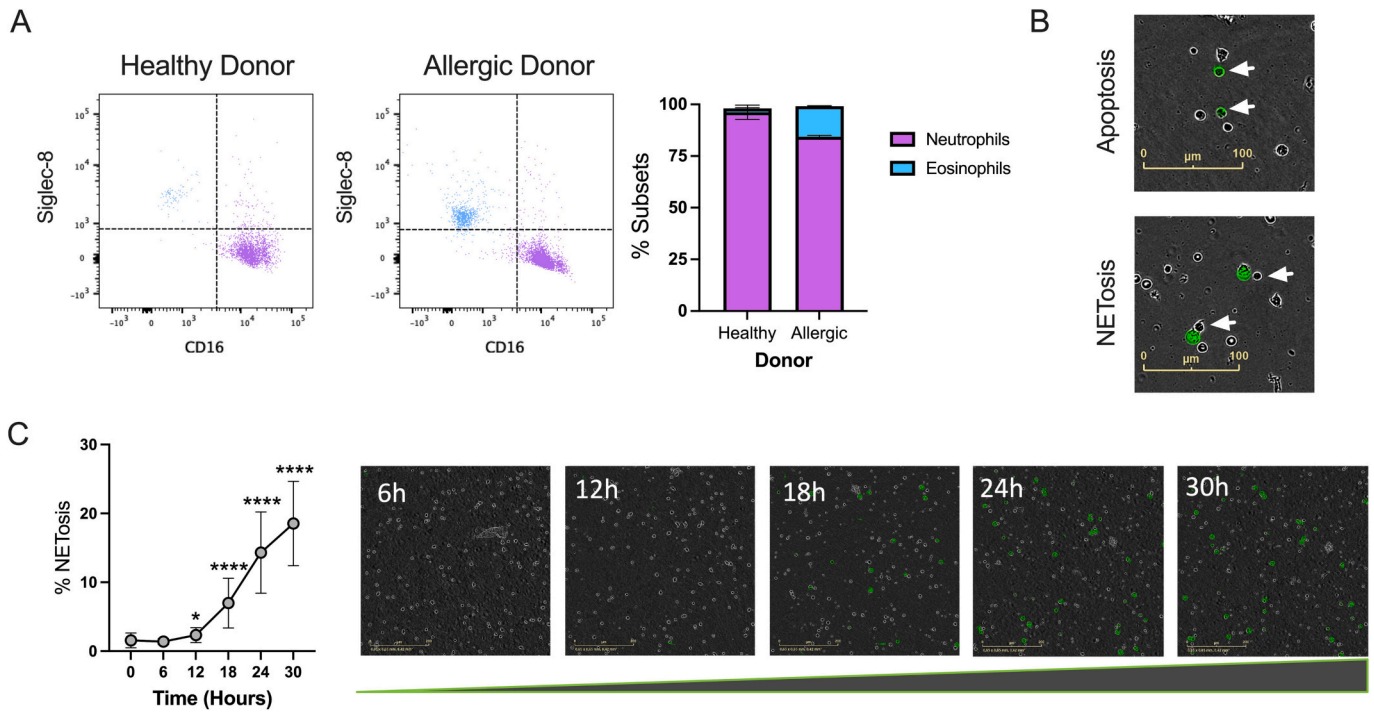

Fig. 2. Standardization of NETosis quantification. (A) Representative dot plots and bar charts of the percentages of neutrophils in donors with and without active allergy. (B) Identification of apoptotic (arrows) and NETotic (arrows) neutrophils by live-imaging using Sytox green staining and brightfield. (C) Time-plot of mean with standard deviation and representative images of NETosis percentages before and after 6 h, 12 h, 18 h, 24 h and 30 h of incubation with serum (Repeated Measures ANOVA with Dunnett's multiple comparison tests, **** $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Participants' characteristics.

	YOUNG (n = 10)	OLDER (n = 10)	P value
Age (years)	21 ± 1	66 ± 5	**0.007**
Weight (kg)	76.59 ± 10.58	78.53 ± 11.59	0.995
Height (m)	1.71 ± 0.06	1.68 ± 0.06	0.906
BMI ($kg{\cdot}m^{-2}$)	26.01 ± 2.64	27.43 ± 3.11	0.611
Waist circumference (cm)	89.17 ± 9.16	99.12 ± 9.82	**0.031**
HR ($b{\cdot}min^{-1}$)	72.90 ± 12.63	70.05 ± 10.24	0.299
SBP (mm Hg)	125.30 ± 5.10	129.30 ± 9.08	0.058
DPB (mm Hg)	78.40 ± 6.26	80.90 ± 825	0.364
VO2max (mL/kg/min)	29.87 ± 6.60	16.65 ± 4.55	**0.001**

n: number of patients. **BMI**: body mass index; **HR**: heart rate; **SBP**: systolic blood pressure; **DBP**: diastolic blood pressure. Values represent means ± SD. Bold values indicated different between YOUNG and OLDER at the $P < 0.05$ level.

4. Discussion

The present study aimed to evaluate the induction of NETosis in healthy young and older men, before and after 12 weeks of HIIT using healthy neutrophils as a biosensor. The results show that the basal percentage of NETosis was higher in older group compared to younger individuals; and 12 weeks of HIIT reduced the percentage of NETosis in older men at 18 and 24 h post activation. The use of neutrophils as a biosensor is an effective method for the quantification of NETosis induction from 6 h after the addition of the serum.

The results of our study showed that the serum of older men induce a higher percentage of baseline NETosis compared to the younger group (Table 2). During aging, both innate and adaptive immunity suffer alterations that cause greater susceptibility to disease (Montecino-Rodriguez et al., 2013; Simon et al., 2015). Neutrophils lose efficiency in the control and elimination of pathogens (Van Avondt et al., 2022), a situation that has been attributed to a lower production of NETosis both in aged animals and in older people (Simon et al., 2015; Tseng et al., 2012). However, it has now been shown that neutrophils from older men produce greater NETosis when stimulated with lipopolysaccharide compared to young adults, however with less antimicrobial activity (Sabbatini et al., 2022). On the other hand, the increment in the production of NETs can be stimulated by proinflammatory cytokines such as IL-1b, TNF-α, IL-5 and IL-8 (Keshari et al., 2012), which are increased in aging. In addition, older people develop a chronic low-grade inflammatory state associated with a higher morbidity and mortality risk due

Table 2
Basal levels of NETosis induction.

	YOUNG n = 9	OLDER n = 10	F value	P value	Effect size	95 % CI for mean Difference		
						Mean difference	Lower	Upper
%NETosis 6 h	1.33 ± 0.34	1.44 ± 0.53	0.654	0.608	0.247	−0.109	−0.548	0.330
%NETosis 12 h	2.22 ± 0.90	3.40 ± 1.29	1.277	**0.035**	1.061	−1.180	−2.265	−0.096
%NETosis 18 h	5.90 ± 2.11	11.46 ± 3.81	4.976	**0.001**	1.805	−5.562	−8.596	−2.529
%NETosis 24 h	14.07 ± 3.41	21.42 ± 6.51	9.249	**0.008**	1.414	−7.346	−12.465	−2.228
%NETosis 30 h	19.59 ± 4.53	25.29 ± 6.42	1.139	**0.041**	1.026	−5.707	−11.147	−0.268

n: number of patients; Values represent means ± SD. Bold values indicate difference between YOUNG and OLDER at the $P < 0.05$ level. d. An effect size <0.2 indicates no effect, 0.2–0.49 indicates small effect, 0.5–0.79 indicates medium effect, and ≥ 0.8 indicates large effect.

Table 3
Effect of HIIT upon NETosis induction.

	YOUNG n = 9			OLDER n = 10			Whithin-subjects effects		Between-subjets effects	*Partial eta square* η2		
	Pre	Post	%pre-post	Pre	Post	%pre-post	Time	Time × group	Group	Time	Time × group	Group
%NETosis 6 h	1.33 ± 0.34	1.33 ± 0.47	7,01 ± 52.82	1.44 ± 0.53	1.42 ± 0.47	26.21 ± 109.51	0.917	0.952	0.512	0.001	0.000	0.026
%NETosis 12 h	2.22 ± 0.90	1.57 ± 0.46	−14.12 ± 51.73	3.40 ± 1.29	2.02 ± 0.57	−32.31 ± 35.67	**0.001**	0.155	**0.020**	0.501	0.115	0.280
%NETosis 18 h	5.90 ± 2.11	4.90 ± 1.36	−4.81 ± 46.20	11.46 ± 3.81*	5.32 ± 1.61#	−48.70 ± 23.94	**0.0001**	**0.005**	**0.001**	0.537	0.376	0.457
%NETosis 24 h	14.07 ± 3.41	10.04 ± 1.99	−24.32 ± 24.44	21.42 ± 6.51*	11.29 ± 1.45#	−42.38 ± 19.42	**0.0001**	**0.034**	**0.003**	0.624	0.239	0.418
%NETosis 30 h	19.59 ± 4.53	13.67 ± 2.11	−26.66 ± 20.37	25.29 ± 6.42	15.23 ± 1.75	−36.21 ± 17.38	**0.0001**	0.145	**0.017**	0.670	0.120	0.293

PRE: before HIIT; **POST:** after HIIT; **n:** number of patients; Values represent means ± SD; Bold values indicate difference at the $P < 0.05$; paired t-tests: # ($P < 0.001$) between PRE vs POST; independent t-tests: * ($P < 0.01$) between PRE YOUNG vs PRE OLDER; Partial eta square n2 = (0.01 Small Effect size, 0.06 medium Effect size, 0.134 or Higher large Effect size).

to several factors, including an intense inflammatory response associated with a dysregulated NETosis. For example, it has been shown that the presence of NETs in plasma predicts the occurrence of multiple organ failure, sepsis, and tissue damage in the critically ill older patient (Custodero et al., 2020). The excess of NETosis is related to several pathological entities as previously described (Hidalgo et al., 2022), for this reason the study of strategies that modulate NETosis in humans is of particular interest (Chamardani and Amiritavassoli, 2022).

HIIT is an effective non-invasive practice to reduce or reverse disease progression (Valeria Oliveira de Sousa et al., 2021; Soriano-Maldonado et al., 2019). In addition, it has a positive effect over inflammatory parameters, oxidative stress, body composition and physical capacity parameters (Gonzalo-Encabo et al., 2021; Abd El-Kader and Al-Shreef, 2018). However, the relationship between training and NETosis is poorly described and remains controversial. NETosis can be stimulated after the practice of exercise (Valeria Oliveira de Sousa et al., 2021), which could be related to the proinflammatory response developed in the body after an intense exercise session (Pillon Barcelos et al., 2017). A study reported by Da Syu et al. (2013), states that intense physical exercise in a single session stimulates the production of NETosis in sedentary young people, but not in physically active ones. Contrary to the study by Orysiak et al. (2021), who describe that short period of intense exercise in young athletes is accompanied by an increase in NETosis. Unlike the previous results, our data show that chronic 12-week HIIT program induces a lower percentage of NETosis induction (Table 3). To the best of our knowledge, this is the first study that compares the effect of HIIT in young and older men on the percentage of NETosis induction. Showing a greater decrease in the older compared to the young men, this for a greater basal induction of NETosis in older men (Table 2). Ondracek et al. (2022) reported the effect of training on NETosis by measuring circulating cell-free deoxyribonucleic acid (cfDNA), demonstrating that 8 months of endurance training in people between 30 and 65 years old reduced the load of NETs and proinflammatory signaling. Overall, NETosis measurement and the effect of HIIT on this process is still evolving, and more research is required to understand whether HIIT is beneficial in the modulation of the innate immune response.

The decrease in NETosis after 12 weeks of HIIT observed by this study could be a consequence of the modulation of anti-inflammatory interleukins that has been described for this type of training. As reported by Alizadeh and Safarzade (2019), who intervened with HIIT on young overweight men (average age 18 ± 1.5 years) and obtained a significant increase in anti-inflammatory cytokines such as IL-4 (+43.8 ± 0.5 % pg/ml; P = 0.022) and IL-13 (+43.9 ± 2 % pg/ml; p = 0.014) after 12 weeks of intervention. Therefore, these findings suggest that the HIIT offers protection against chronic low-grade inflammation through the increase of anti-inflammatory mediators.

Anti-inflammatory effects have also been reported after 3 weeks of a variant of HIIT called short-term sprint interval training (SIT) in healthy older people, with significant decreases ($p < 0.05$) in cytokine concentrations described as pro-inflammatory and promoters of NETosis, as is the case of IL-6 (from 1.26 ± 0.44 to 0.87 ± 0.44 pg/mL) and TNF-α (from 5.10 ± 1.23 to 4.31 ± 1.20 pg/mL) (Jürimäe et al., 2023).

Although continuous moderate-intensity aerobic training also generates a modulating response on anti/pro-inflammatory cytokines, the size of the effect is dependent on the intensity and modality of exercise. The study by Dorneles et al. (2016), indicated that higher intensity training is superior to moderate intensity training in generating a systemic anti-inflammatory cytokine response in overweight individuals. Likewise, the study by Nimmo et al. (2013), stated that inducing an increase in circulating concentrations of anti-inflammatory cytokines, including IL-4, is proportional to exercise intensity. On the other hand, Zwetsloot et al. (2014), proposed that rest intervals in HIIT have a profound attenuating effect on the inflammatory response.

The measurement of NETosis is a promising biomarker of the innate immunity activity in response to pathogens or circulating pro

inflammatory stimuli that needs to be investigated. The present study manages to standardize the use of a method with donated PMN cells as a biosensor, demonstrating the effectiveness of this technique in the quantification of NETosis in real time from 6 h to 30 h of incubation. In line with data reported by Nakabo et al. (2022) who described the utility of the IncuCyte™S3 system (Essen BioScience, Inc.) in the physiological evaluation of neutrophils. We can observe that the interaction effect (Time x Group) is shown at 18 h and 24 h from incubation (Table 3). This result is relevant since the techniques currently used are only capable of quantifying cells that actively experience NETosis at a single point in time (Gupta et al., 2018; Zharkova et al., 2019). Losing so the ability to study the induction of NETosis in real time.

Among the limitations of this study, we can mention that these results cannot be extrapolated to women; and a larger future sample is required to generalize the results to a broader healthy population. On the other hand, it is not possible to determine if the effect of HIIT is superior to other types of training in reducing the induction of NETosis.

In conclusion, HIIT is a feasible non-invasive strategy modulating NETosis induction, being able to play a relevant role in the prevention and/or treatment of a variety of diseases associated with the excessive increase of NETosis during aging. On the other hand, the use of neutrophils as a biosensor is an effective method for the quantification of NETosis induction in real time.

CRediT authorship contribution statement

Conceptualization, E.N.-L. and G.N.M.-N.; methodology, N·V.-S., C. C., L.F., M.A.-A., A.A.-M., S.S., A.F., N.H., J.S., L.S., resources, E.N.-L, L. F. and G.N.M.-N.; data curation, N.V.-S.; writing—original draft preparation, N.V-S., E.N.-L. and G.N.M.-N.; writing—review and editing, R. M.; supervision, E.N.-L. and G.N.M.-N.; Project administration, E.N.-L. and G.N.M.-N.; funding acquisition, E.N.-L. and G.N.M.-N. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

This research was funded by ANID - FONDECYT - Chile (11180949) and the Dirección de Investigación (DIUFRO) of Universidad de La Frontera (DI18-0068 & DI20-2017). E.N.-L., C.C., A.F. and S.S. were funded by ANID-CONICYT Fondecyt Inicio 11170610, Fondecyt Regular 1211480, PAI79170073 and Anillo COVID-19 Genomics Network (C19-GenoNet) ACT210085. A.F., C.C. and S.S. were funded by Articulating Scholarship from Postgraduate Unit at Universidad de Concepcion. N.V.-S. and M.A.-A. were funded by National Research and Development Agency (ANID)/Human Capital Sub-directorate/National Doctorate Scholarships 2021 – 21211236 and 2022 – 21220848, respectively. The authors wish to thank María Angélica Olead, Yuri San Martín-Calísto, Fernanda Durán-Vejar, Francisca Beltrán-Fuentes, and Aris Muñoz-Fernández for the work done.

References

Abd El-Kader, S.M., Al-Shreef, F.M., 2018. Inflammatory cytokines and immune system modulation by aerobic versus resisted exercise training for elderly. Afr. Health Sci. 18 (1), 120–131. https://doi.org/10.4314/AHS.V18I1.16.

Alizadeh, H., Safarzade, A., 2019. High intensity intermittent training induces anti-inflammatory cytokine responses and improves body composition in overweight adolescent boys. Horm. Mol. Biol. Clin. Investig. 39 (3) https://doi.org/10.1515/HMBCI-2019-0004/MACHINEREADABLECITATION/RIS.

Artigas-Arias, M., Olea, M.A., Sanmartín-Calísto, Y., et al., 2021. Anthropometric parameters, lower limb functionality and quality of life after high-intensity interval training in healthy young people versus older adults. Int. J. Morphol. 39 (5), 1337–1344. https://doi.org/10.4067/S0717-95022021000501337.

Boukabous, I., Marcotte-Chénard, A., Amamou, T., et al., 2019. Low-volume high-intensity interval training (HIIT) versus moderate-intensity continuous training on body composition, cardiometabolic profile and physical capacity in older women. J. Aging Phys. Act. 27 (4), 879–889. https://doi.org/10.1123/JAPA.2018-0309.

Caparrós-Manosalva, C., Marzuca-Nassr, G.N., Garrido-Muñoz, N., et al., 2023. Effects of high-intensity interval training on lean mass, strength, and power of the lower limbs in healthy old and young people. Front. Physiol. 14, 1223069 https://doi.org/10.3389/FPHYS.2023.1223069.

Chamardani, T.M., Amiritavassoli, S., 2022. Inhibition of NETosis for treatment purposes: friend or foe? Mol. Cell. Biochem. 477 (3), 673–688. https://doi.org/10.1007/S11010-021-04315-X/TABLES/1.

Cohen, J., 2013. Statistical power analysis for the behavioral sciences. In: Statistical Power Analysis for the Behavioral Sciences. https://doi.org/10.4324/9780203771587. Published online May 13.

Custodero, C., Anton, S.D., Beavers, D.P., et al., 2020. The relationship between interleukin-6 levels and physical performance in mobility-limited older adults with chronic low-grade inflammation: the ENRGISE pilot study. Arch. Gerontol. Geriatr. 90, 104131. https://doi.org/10.1016/J.ARCHGER.2020.104131.

Da Syu, G., Chen, H.I., Jen, C.J., 2013. Acute severe exercise facilitates neutrophil extracellular trap formation in sedentary but not active subjects. Med. Sci. Sports Exerc. 45 (2), 238–244. https://doi.org/10.1249/MSS.0B013E31826DF4A1.

Dorneles, G.P., Haddad, D.O., Fagundes, V.O., et al., 2016. High intensity interval exercise decreases IL-8 and enhances the immunomodulatory cytokine interleukin-10 in lean and overweight-obese individuals. Cytokine 77, 1–9. https://doi.org/10.1016/J.CYTO.2015.10.003.

Gibala, M.J., Little, J.P., Macdonald, M.J., Hawley, J.A., 2012. Physiological adaptations to low-volume, high-intensity interval training in health and disease. J. Physiol. 590 (5), 1077–1084. https://doi.org/10.1113/JPHYSIOL.2011.224725.

Gonzalo-Encabo, P., Maldonado, G., Valadés, D., Ferragut, C., Pérez-López, A., 2021. The role of exercise training on low-grade systemic inflammation in adults with overweight and obesity: a systematic review. Int. J. Environ. Res. Public Health 18 (24). https://doi.org/10.3390/IJERPH182413258.

Gupta, S., Chan, D.W., Zaal, K.J., Kaplan, M.J., 2018. A high-throughput real-time imaging technique to quantify NETosis and distinguish mechanisms of cell death in human neutrophils. J. Immunol. 200 (2), 869–879. https://doi.org/10.4049/JIMMUNOL.1700905.

Hayes, L.D., Herbert, P., Sculthorpe, N.F., Grace, F.M., 2021. Short-term and lifelong exercise training lowers inflammatory mediators in older men. Front. Physiol. 12 https://doi.org/10.3389/FPHYS.2021.702248.

Hidalgo, A., Libby, P., Soehnlein, O., Aramburu, I.V., Papayannopoulos, V., Silvestre-Roig, C., 2022. Neutrophil extracellular traps: from physiology to pathology. Cardiovasc. Res. 118 (13), 2737–2753. https://doi.org/10.1093/CVR/CVAB329.

Hummel, E.M., Hessas, E., Müller, S., et al., 2018. Cell-free DNA release under psychosocial and physical stress conditions. Transl. Psychiatry 8 (1), 1–10. https://doi.org/10.1038/s41398-018-0264-x, 2018 8:1.

Jürimäe, J., Purge, P., Remmel, L., et al., 2023. Changes in irisin, inflammatory cytokines and aerobic capacity in response to three weeks of supervised sprint interval training in older men. J. Sports Med. Phys. Fit. 63 (1) https://doi.org/10.23736/S0022-4707.22.13949-6.

Kenny, E.F., Herzig, A., Krüger, R., et al., 2017. Diverse stimuli engage different neutrophil extracellular trap pathways. Elife 6. https://doi.org/10.7554/ELIFE.24437.

Keshari, R.S., Jyoti, A., Dubey, M., et al., 2012. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One 7 (10), e48111. https://doi.org/10.1371/JOURNAL.PONE.0048111.

Khalafi, M., Symonds, M.E., 2020. The impact of high-intensity interval training on inflammatory markers in metabolic disorders: a meta-analysis. Scand. J. Med. Sci. Sports 30 (11), 2020–2036. https://doi.org/10.1111/SMS.13754.

Klopf, J., Brostjan, C., Eilenberg, W., Neumayer, C., 2021. Neutrophil extracellular traps and their implications in cardiovascular and inflammatory disease. Int. J. Mol. Sci. 22 (2), 1–17. https://doi.org/10.3390/IJMS22020559.

Malech, H.L., DeLeo, F.R., Quinn, M.T., 2020. The role of neutrophils in the immune system: an overview. Methods Mol. Biol. 2087, 3–10. https://doi.org/10.1007/978-1-0716-0154-9_1/COVER.

Mancilla, R., Torres, P., Álvarez, C., Schifferli, I., Sapunar, J., Bustos, E.D., 2014. Ejercicio físico interválico de alta intensidad mejora el control glicémico y la capacidad aeróbica en pacientes con intolerancia a la glucosa. Rev. Med. Chil. 142 (1), 34–39. https://doi.org/10.4067/S0034-98872014000100006.

Marzuca-Nassr, G.N., Artigas-Arias, M., Olea, M.A., et al., 2020. High-intensity interval training on body composition, functional capacity and biochemical markers in healthy young versus older people. Exp. Gerontol. 141, 111096. https://doi.org/10.1016/J.EXGER.2020.111096.

Montecino-Rodriguez, E., Berent-Maoz, B., Dorshkind, K., 2013. Causes, consequences, and reversal of immune system aging. J. Clin. Invest. 123 (3), 958–965. https://doi.org/10.1172/JCI64096.

Nakabo, S., Kaplan, M.J., Gupta, S., 2022. Quantification of neutrophils undergoing NET formation and distinguishing mechanisms of neutrophil cell death by use of a high-throughput method. Methods Mol. Biol. 2543, 129–140. https://doi.org/10.1007/978-1-0716-2553-8_11/COVER.

Nathan, C., 2006. Neutrophils and immunity: challenges and opportunities. Nat. Rev. Immunol. 6 (3), 173–182. https://doi.org/10.1038/nri1785. *2006 6:3*.

Nimmo, M.A., Leggate, M., Viana, J.L., King, J.A., 2013. The effect of physical activity on mediators of inflammation. Diabetes Obes. Metab. 15 (s3), 51–60. https://doi.org/10.1111/DOM.12156.

Nirmala, J.G., Lopus, M., 2020. Cell death mechanisms in eukaryotes. Cell Biol. Toxicol. 36 (2), 145–164. https://doi.org/10.1007/S10565-019-09496-2.

Ondracek, A.S., Aszlan, A., Schmid, M., et al., 2022. Physical exercise promotes dnase activity enhancing the capacity to degrade neutrophil extracellular traps. Biomedicines 10 (11), 2849. https://doi.org/10.3390/BIOMEDICINES10112849, 2022, Vol 10, Page 2849.

Orysiak, J., Tripathi, J.K., Brodaczewska, K., et al., 2021. The impact of physical training on neutrophil extracellular traps in young male athletes - a pilot study. Biol. Sport 38 (3), 459–464. https://doi.org/10.5114/BIOLSPORT.2021.101117.

Papayannopoulos, V., 2018. Neutrophil extracellular traps in immunity and disease. Nat. Rev. Immunol. 18 (2), 134–147. https://doi.org/10.1038/NRI.2017.105.

Pérez-Figueroa, E., Álvarez-Carrasco, P., Ortega, E., Maldonado-Bernal, C., 2021. Neutrophils: many ways to die. Front. Immunol. 12 https://doi.org/10.3389/FIMMU.2021.631821.

Pillon Barcelos, R., Freire Royes, L.F., Gonzalez-Gallego, J., Bresciani, G., 2017. Oxidative stress and inflammation: liver responses and adaptations to acute and regular exercise. https://doi.org/101080/1071576220171291942, 51 (2), 222–236. https://doi.org/10.1080/10715762.2017.1291942.

Pokrywka, A., Zembron-Lacny, A., Baldy-Chudzik, K., Orysiak, J., Sitkowski, D., Banach, M., 2015. The influence of hypoxic physical activity on cfDNA as a new marker of vascular inflammation. Arch. Med. Sci. 11 (6), 1156–1163. https://doi.org/10.5114/AOMS.2015.56341.

Robinson, M.M., Dasari, S., Konopka, A.R., et al., 2017. Enhanced protein translation underlies improved metabolic and physical adaptations to different exercise training modes in young and old humans. Cell Metab. 25 (3), 581–592. https://doi.org/10.1016/J.CMET.2017.02.009.

Sabbatini, M., Bona, E., Novello, G., Migliario, M., Renò, F., 2022. Aging hampers neutrophil extracellular traps (NETs) efficacy. Aging Clin. Exp. Res. 34 (10), 2345–2353. https://doi.org/10.1007/S40520-022-02201-0/FIGURES/4.

Simon, A.K., Hollander, G.A., McMichael, A., 2015. Evolution of the immune system in humans from infancy to old age. Proc. Biol. Sci. 282 (1821) https://doi.org/10.1098/RSPB.2014.3085.

Soriano-Maldonado, A., Carrera-Ruiz, Á., Díez-Fernández, D.M., et al., 2019. Effects of a 12-week resistance and aerobic exercise program on muscular strength and quality of life in breast cancer survivors: study protocol for the EFICAN randomized controlled trial. Medicine 98 (44), e17625. https://doi.org/10.1097/MD.0000000000017625.

Stoimenou, M., Tzoros, G., Skendros, P., Chrysanthopoulou, A., 2022. Methods for the assessment of NET formation: from neutrophil biology to translational research. Int. J. Mol. Sci. 23 (24), 15823. https://doi.org/10.3390/IJMS232415823, 2022, Vol 23, Page 15823.

Tseng, C.W., Kyme, P.A., Arruda, A., Ramanujan, V.K., Tawackoli, W., Liu, G.Y., 2012. Innate immune dysfunctions in aged mice facilitate the systemic dissemination of methicillin-resistant *S. aureus*. PLoS One 7 (7). https://doi.org/10.1371/JOURNAL.PONE.0041454.

Valeria Oliveira de Sousa, B., de Freitas, D.F., Monteiro-Junior, R.S., et al., 2021. Physical exercise, obesity, inflammation and neutrophil extracellular traps (NETs): a review with bioinformatics analysis. Mol. Biol. Rep. 48 (5), 4625–4635. https://doi.org/10.1007/S11033-021-06400-2.

Van Avondt, K., Strecker, J.K., Tulotta, C., Minnerup, J., Schulz, C., Soehnlein, O., 2022. Neutrophils in aging and aging-related pathologies. Immunol. Rev. 00, 1–19. https://doi.org/10.1111/IMR.13153.

Vorobjeva, N.V., Chernyak, B.V., 2020. NETosis: molecular mechanisms, role in physiology and pathology. Biochemistry (Mosc) 85 (10), 1178–1190. https://doi.org/10.1134/S0006297920100065.

Wewege, M., van den Berg, R., Ward, R.E., Keech, A., 2017. The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: a systematic review and meta-analysis. Obes. Rev. 18 (6), 635–646. https://doi.org/10.1111/OBR.12532.

Zharkova, O., Tay, S.H., Lee, H.Y., et al., 2019. A flow cytometry-based assay for high-throughput detection and quantification of neutrophil extracellular traps in mixed cell populations. Cytometry A 95 (3), 268–278. https://doi.org/10.1002/CYTO.A.23672.

Zwetsloot, K.A., John, C.S., Lawrence, M.M., Battista, R.A., Shanely, R.A., 2014. High-intensity interval training induces a modest systemic inflammatory response in active, young men. J. Inflamm. Res. 7 (1), 9–17. https://doi.org/10.2147/JIR.S54721.