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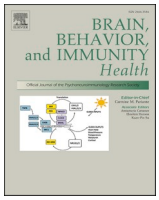

Acute natural killer cells response to a continuous moderate intensity and a work-matched high intensity interval exercise session in metastatic cancer patients treated with chemotherapy

Hugo Parent-Roberge [a,b], Adeline Fontvieille [a,b], Laurence Poirier [a,b], Lee-Hwa Tai [d,f], Michel Pavic [c,d,e], Tamàs Fülöp [b,c,d], Eléonor Riesco [a,b,*]

[a] University of Sherbrooke, Faculty of Physical Activity Sciences, 2500, boul. de l'Université, Sherbrooke, Qc, J1K 2R1, Canada
[b] Research Centre on Aging, affiliated with CIUSSS de l'Estrie - CHUS, 1036, rue Belvédère sud, Sherbrooke, Qc, J1H 4C4, Canada
[c] University of Sherbrooke, Faculty of Medicine and Health Sciences, 3001, 12e avenue Nord, Sherbrooke, QC, J1H 5N4, Canada
[d] Centre de recherche du Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Qc, J1H 5N4, Canada
[e] Institut de recherche sur le cancer de l'Université de Sherbrooke, Sherbrooke, Qc, Canada, J1H5N4
[f] University of Sherbrooke, Department of Immunology and Cell Biology, 3201 rue Jean-Mignault, J1E 4K8, Canada

A B S T R A C T

Background: It has been suggested that the acute natural killer (NK) cell response to aerobic exercise might contribute to the tumor suppressor effect of regular exercise observed in preclinical studies. Moreover, because this response is modulated by exercise intensity, high-intensity intervals exercise (HIIE) might represent an interesting therapeutic approach in cancer patients. However, this immune response remains unstudied in cancer patients currently undergoing chemotherapy.
Objective: To characterize the acute NK cell response following a moderate-intensity continuous aerobic exercise session (MOD), and a HIIE session in metastatic cancer patients treated with chemotherapy.
Methods: Twelve cancer patients (45–65 years old) underwent a MOD and a duration and work-matched HIIE trial, in a block-randomized order. Peripheral blood mononuclear cells (PBMC) were isolated before, after and 1h after each trial. NK cell subsets were enumerated using flow cytometry and complete blood counts. The surface expression of the cytotoxic NK cell (cNK; $CD56^{dim}CD16^{+}$) subset was evaluated for its expression of the differentiation markers CD57 and CD158a, the activating receptor NKG2D, the immune checkpoints TIM-3 and PD-1, and the chemokine receptors CXCR3, CXCR4 and CCR2.
Results: cNK cell blood counts increased immediately following MOD ($p < 0.001$) and decreased back to pre-exercise values 1 h after exercise cessation ($p < 0.001$). The most responsive cNK cell subsets were expressing CD57, CD158a, NKG2D, TIM-3 and CXCR3. The HIIE trial elicited a similar biphasic response, without any difference between trials (all $p \geq 0.38$). However, significant changes in the MFI values of CXCR4 and NKG2D were observed in the cNK cell subset following HIIE (all $p \leq 0.038$), but not MOD.
Conclusion: In metastatic cancer patients undergoing chemotherapy, both MOD and HIIE can elicit an acute mobilisation and egress of NK cells exhibiting phenotypic characteristics associated with high cytotoxicity and tumor homing. Future longitudinal trials are needed to determine if combining aerobic exercise training and chemotherapy will translate towards favorable immune and clinical outcomes.

1. Introduction

There is increasing epidemiological evidence highlighting the inverse relationship between post-diagnosis physical activity and cancer-specific mortality and recurrence (Cormie et al., 2017; Mctiernan et al., 2019). Likewise, a growing amount of tumor-bearing animal models have shown that aerobic exercise can supress tumor growth (Eschke et al., 2019). Many of these studies provided evidence of an increased tumoral infiltration and activity of cytotoxic lymphocytes, major effectors of the anti-cancer cell-mediated immunity (Spiliopoulou et al., 2021).

It has been proposed that the acute immune response to aerobic exercise might contribute to this tumor suppressive effect. Each exercise bout acutely provokes a rapid blood mobilization of effector cytotoxic lymphocytes (Bigley et al., 2014; Campbell et al., 2009). Within minutes after exercise cessation, their blood levels start to decrease, reaching a nadir within one to 2 h (Rooney et al., 2018). This decrease is believed to mainly reflect their margination and egress in peripheral tissues (Krüger et al., 2008; Peake et al., 2017), as the preferentially exercise-responding lymphocytes express high levels of endothelial adhesion and transmigration molecules (Simpson et al., 2006) and inflammatory chemokine receptors (Arroyo et al., 2022; Dimitrov et al., 2010), involved in

* Corresponding author. University of Sherbrooke, Faculty of Physical Activity Sciences, 2500, boul. de l'Université, Sherbrooke (Qc). J1K 2R1, Canada.
E-mail address: E.Riesco@USherbrooke.ca (E. Riesco).

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the infiltration of inflamed tissues, as well as tumor homing (Sackstein et al., 2017; Susek et al., 2018).

In this context, NK cells have recently attracted particular attention. NK cells can be broadly divided into two main subsets based on the surface expression of CD56 and the FC receptor CD16. The $CD56^{bright}CD16^{-}$ subset, sometimes referred to as immunoregulatory (iNK), express more inflammatory cytokines but less cytolytic molecules upon activation (Poli et al., 2009) and is less sensitive to exercise. The $CD56^{dim}CD16^{+}$ subset, often termed cytotoxic (cNK), is more differentiated, expresses more cytolytic molecules (Poli et al., 2009), and is the most responsive white blood cell subset to exercise (Natale et al., 2003). cNK cells can also be further stratified by their differentiation status. Terminally differentiated cNK cells, expressing CD57 and receptors of the KIR family, are particularly exercise-responsive and exhibit a strong cytotoxic capacity (Bigley et al., 2014), and their tumoral infiltration has been associated with a good prognosis in various epithelial and hematological cancers (Nielsen et al., 2013). Moreover, aerobic exercise has been shown to acutely increase the transcription level of the activating receptor NKG2D in cNK cells (Zimmer et al., 2015) and increase their cytotoxic activity (NKCA) per cell against various cancer cell lines *ex vivo* (Bigley et al., 2014; Schauer et al., 2022).

Based on these results, it has been proposed that each aerobic exercise session could mobilize highly differentiated cNK cells in the bloodstream, with some of these migrating within the tumor microenvironment in the hours following exercise. The cumulative effect of successive exercise bouts might thus "fuel" the increased tumoricidal activity that is observed in animal models of regular exercise (Hojman et al., 2018). Aerobic exercise could thus be envisaged as an adjuvant approach in cancer patients, in conjunction with conventional therapies.

Moreover, because this immune response is mainly driven by the signaling of adrenergic and corticosteroid hormones (Besedovsky et al., 2014; Graff et al., 2018; Okutsu et al., 2005), intensity is the exercise parameter having the largest influence on its magnitude (Bigley et al., 2014; Campbell et al., 2009). As such, high-intensity intervals exercise (HIIE) and supramaximal intervals can both provoke similar or stronger acute $CD56^{dim}$ and total lymphocytes responses in comparison to continuous, moderate exercise (MOD) despite lower total oxygen uptake and mechanical work (Arroyo et al., 2022; Jamurtas et al., 2018). Considering that HIIE was shown to be safe in cancer patients (Mugele et al., 2019), it could be envisaged as a promising strategy to acutely stimulate anti-cancer immunity during cancer treatments.

However, these hypotheses remain largely based on animal models and studies in healthy humans, and few trials have been conducted in cancer patients currently undergoing systemic treatments (Hanson et al., 2020; Schauer et al., 2022). Noteworthy, although the NK cell response to aerobic exercise does not seem compromised in prostate cancer patients undergoing androgen deprivation therapy (Hanson et al., 2020), a decreased resting NK cell level and weaker exercise-induced mobilization and egress were reported in breast cancer survivors 3–6 months after chemotherapy completion (Evans et al., 2015). This suggests that myelosuppressive treatments such as chemotherapy not only reduce lymphocytes resting levels, but also their sensitivity to acute exercise. However, to our knowledge, the acute NK cell response to MOD or HIIE in patients currently undergoing chemotherapy has not been studied so far.

Hence, the main objective of this study was to characterize the acute NK cells response to a MOD and a HIIE session, matched for duration and amount of external mechanical work, in metastatic cancer patients undergoing chemotherapy. Based on the literature, we hypothesized that exercise would elicit the preferential mobilization and post-exercise egress of highly differentiated cNK cells expressing markers associated with tumor infiltration and cytotoxicity, and that HIIE would elicit a stronger response than MOD.

2. Methods

2.1. Study design

This study was a single-blind cross-over trial with a randomized two period, two interventions allocation sequence (HIIE/MOD and MOD/HIIE). The design of this study, as well as the reporting of its methodology and results, were performed in accordance with the CONSORT guidelines for randomised crossover trials (Dwan et al., 2019), and the Consensus on Exercise Reporting Template (CERT) guidelines (Slade et al., 2016). The study consisted in three separate visits in the laboratory: a baseline assessment including an incremental exercise test, followed by two experimental conditions (MOD and HIIE) in a randomized order. Each visit was performed the day prior to a chemotherapy session, including the baseline assessment. For any given participant, all the exercise sessions were performed at the same time of day, to avoid the effects of circadian variations on the immune parameters of interest. Written informed consent was obtained from every participant before collecting data. All the procedures were approved by the Ethics Committee of the Centre Intégré Universitaire de Santé et de Services Sociaux de l'Estrie (CIUSSS de l'Estrie – CHUS, Canada).

The participants' chemotherapy cycles duration varied from 7 to 21 days, depending on each participant's treatment protocol. Considering that each experimental condition was realized the day before a chemotherapy treatment, the wash-out period between each visit varied between 7 and 21 days. This period is sufficient to wash out the acute effects of a single non-exhaustive exercise session on the blood immune parameters of interest (Peake et al., 2017). However, consecutive chemotherapy cycles can cumulatively lower the resting lymphocyte blood counts from cycle to cycle (Mackall et al., 1994). To minimize the period effect that might result from cumulative chemotherapy cycles, the randomization sequence was counterbalanced with an allocation ratio of 1:1, ensuring an even number of participants allocated to each sequence (HIIE/MOD and MOD/HIIE) at the end of the study, as recommended (Dwan et al., 2019). The random allocations sequence was generated using the Sealed Envelope online tool (Sealed Envelope Ltd. 2022).

2.2. Participants

A convenience sampling was realized in collaboration with the oncology unit of the CIUSSS de l'Estrie – CHUS. Medically eligible patients were contacted by a research nurse, whereupon interested patients meeting the inclusion criteria were provided the informed consent form and referred to the research team. Eligible and interested patients were then screened by phone and invited for the baseline visit.

Nineteen participants were recruited and randomized, of which 12 completed the study. (Fig. 1).

Inclusion criteria were: 1) Age between 45 and 65 years old, 2) diagnosis of a metastatic cancer (all solid cancer types included); 3) Currently undergoing chemotherapy treatments; 4) ECOG (Eastern Cooperative Oncology Group) functional status: 0–1; 5) Being able to exercise for 30 min at moderate to vigorous intensity. Exclusion criteria were: 1) any orthopedic, cardiorespiratory, or metabolic limitation contraindicating the prescribed exercise regimen (medical clearance to exercise required upon study initiation); 2) Surgery planned before study completion; 3) the use of beta-blockers.

2.3. Experimental protocol

2.3.1. Baseline assessment and incremental sub-maximal exercise test

The baseline assessment visit comprised, in this order, 1) the measurement of resting blood pressure (BP) and heart rate (HR), 2) anthropometric measurements, 3) medical and socio-demographic questionnaires, and 4) a submaximal incremental exercise test. A submaximal incremental exercise test was chosen based on the research

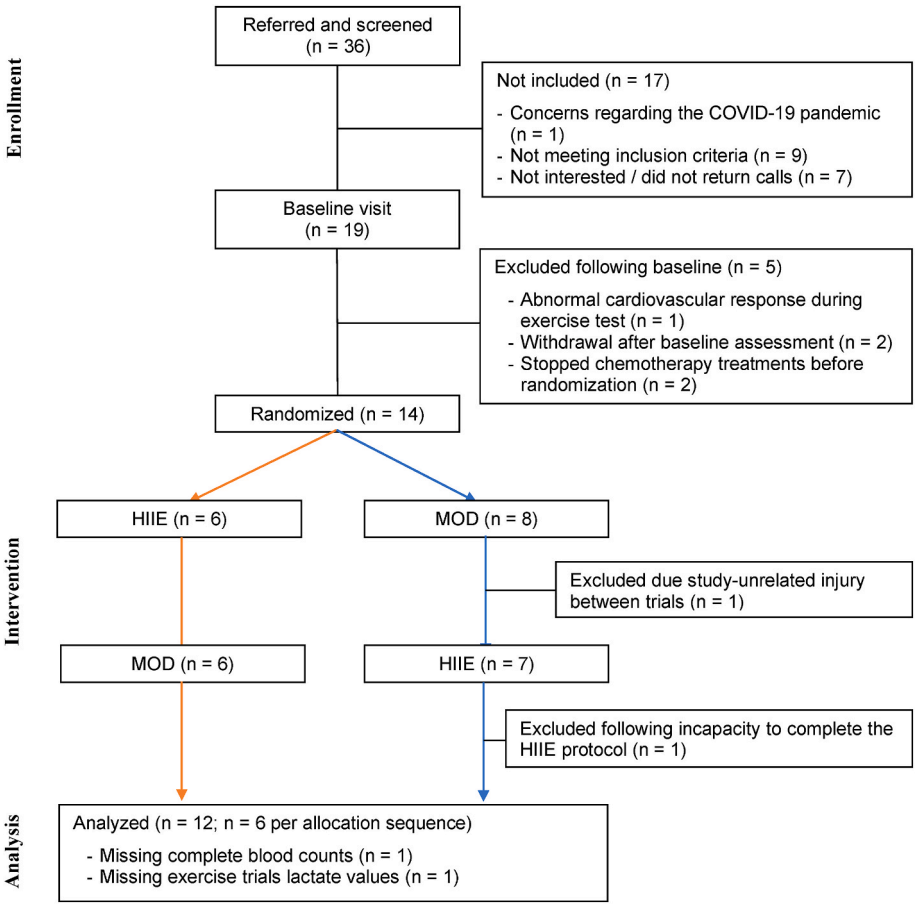

Fig. 1. Participants flow chart.

team's previous experience, to minimize the barriers and experimental mortality that could have been associated with a maximal testing procedure. Briefly, the test was initiated at a work rate of 25 Watts, and the work rate incrementation was of 20 Watts per stage in men and 15 Watts per stage in women. The participants could freely choose the pedalling cadence during the first stage but were then asked to maintain the same cadence during the whole test. Each stage lasted a minimum of 3 min but could be prolonged until a steady-state HR was reached (<5 bpm variation in 1 min). HR was monitored continuously (Polar H7 heart rate sensors, Polar Electro, Kempele, Finland), and BP was measured 2 min after each increase in work rate. The rate of perceived exertion (RPE) was obtained once per minute, using the Borg CR10 scale. Capillary blood lactate was measured before exercise, and during the last 10 s of each stage at the fingertip, using a Lactate Plus blood lactate analyzer (Nova Biomedical, Waltham, MA, USA) (Hart et al., 2013).

The test was terminated at the end of the stage corresponding to both a steady-state HR within 10 bpm of 80% of the estimated heart rate reserve (HHR) and a capillary lactate >4 mmol/L. The age-predicted maximal heart rate was computed using the Tanaka et al. equation (208-0.7 x age) (Tanaka et al., 2001). The target heart rate was then computed using the following equation: $((Predicted\ maximal\ HR - Resting\ HR) \times 0.8) + Resting\ HR$. This value is thereafter designated as 80% of the participant's age-predicted HRR. These criteria were selected to allow the determination of the work rate corresponding to a capillary lactate level above 4 mmol/L and close to 80% of the participant's predicted HRR, which would correspond to the work rate used during the high intensity bouts of the HIIE session. However, since there is a significant variability in aged-predicted maximal heart rate (Tanaka et al., 2001), the test could also be terminated if the participant reached a capillary lactate level >4 mmol/L combined with a RPE ≥8/10 even if the target HR was not attained, to preserve the submaximal nature of the test. The test could also be terminated if the participant was not able to maintain the chosen pedalling cadence (within 5 RPM), if an abnormal HR or BP response to exercise was registered, or upon request.

2.3.2. Experimental trials

The HIIE trial was composed of ten 1-min high intensity bouts, realized at the work rate (W) corresponding to the last completed stage of the submaximal test. Each bout was interspaced with a 2-min recovery bout at a work rate corresponding to 25% of the high-intensity bout, for a total duration of 30 min. The MOD session was realized at a steady work rate corresponding to 50% of the high intensity bouts for 30 min. Therefore, both exercise trials were matched in terms of duration and external work realized ($HIIE\ (10 \times 100\,\% + 20 \times 25\,\%) = MOD\ (30 \times 50\,\%) = 1500\ arbitrary\ units$). Both trials were preceded by an identical 5-min warm-up at a work rate corresponding to the HIIE recovery bouts. The MOD trial was also designed to correspond to the current exercise guidelines in cancer patients and survivors (K. L. Campbell et al., 2019).

During each trial, HR was monitored continuously, and capillary lactate was measured at the 9th, 18th and 27th minutes, the timepoints corresponding to the end of the 3rd, 6th, and 9th high-intensity bouts of HIIE. In some participants, adjustments to the prescribed work rate had to be realized when they estimated that they would not be able to complete trial. When this happened during the first trial, the prescribed work rate of the second trial was adjusted accordingly to maintain the work-matched design for this individual. However, this was not possible when adjustments had to be realized during the second trial. Hence, the amount of external work completed in each trial was computed as a control variable.

Both experimental visits were conducted following the same structure: 1) HR sensor installation and resting HR, BP, and lactate measurement after a 5-min seated period, 2) installation of an intravenous catheter in an antecubital vein, 3) pre-exercise blood draw, 4) exercise session, 5) post-exercise blood draw, 5) 1-h seated resting period, 6) 1h post-exercise blood draw. Each blood draw consisted of a 14-ml sampling in heparin-coated tubes for peripheral blood mononucleated cells (PBMC) isolation, and a 4-ml EDTA-coated tube that was immediately sent to the clinical biochemistry unit of the *CIUSSS de l'Estrie-CHUS* to obtain a complete blood count with white blood cell differential. The post-exercise (second) blood draw was realized immediately at the end of the 30th minute of exercise for the MOD trial, and immediately at the end of the last high-intensity bout for the HIIE trial (during the first minute of the cool-down), considering that post-exercise PBMC counts start to fall within the first minute after exercise cessation (Rooney et al., 2018).

2.3.3. Isolation and cryopreservation of PBMCs

Each heparinized blood sample was kept at room temperature and processed within 2 h for isolation of PBMC by density gradient centrifugation. Briefly, the samples were diluted 1:1 with phosphate buffered saline (PBS), layered on Ficoll–Paque™ Plus (GE Healthcare, Piscataway, NJ) and centrifuged at room temperature for 20 min at 400 g with no brake. The buffy coat was collected, washed twice with PBS, and resuspended on ice in Gibco™ Recovery™ Cell Culture Freezing Medium (Thermo Fisher Scientific, Waltham, MA, USA). Cell counting and viability determination was performed using the Trypan Blue exclusion technique. The cryovials were transferred in a Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA) at −80 °C for 24 h, and then cryopreserved in liquid nitrogen upon analysis.

2.3.4. Immunostaining and flow cytometry

After thawing and washing, the cells from each sample were resuspended at a concentration of 1×10^6 in 100 µl Stain Buffer, and FC receptor blockade was performed using Human BD Fc Block™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Immunophenotyping was performed by multicolor surface immunostaining, using a 12-color panel (Sup. Table 1). Same concentration isotype control tubes were performed for each analyzed sample. All antibodies and isotypes were purchased in a single batch from BD Biosciences (Franklin Lakes, NJ, USA). Optimal concentrations of each antibody were previously determined in titration assays. The samples were incubated on ice in the dark for 30 min. Data acquisition was performed within 2 h of labelling, using a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA). Dead cell exclusion was performed using DAPI (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (Davis et al., 2013). The DAPI solution was added to the samples at a final concentration of 0.2 mg/ml five to 10 min before data acquisition, as recommended by the manufacturer.

2.3.5. Flow cytometry gating strategy and mean fluorescence intensity

Analysis was performed using the CytExpert 2.4.0 software (Beckman Coulter, Brea, CA, USA). For each sample, lymphocytes were gated on the Forward against Scatter Side plot, then dead cells ($DAPI^+$) and doublets were excluded. NK cells were defined as $CD3^-CD56^+$ lymphocytes. Within the NK cell population, the $CD56^{bright}CD16^-$ subset was gated and defined as immunoregulatory NK cells (iNK), and the $CD56^{dim}CD16^+$ subset was gated and defined as cytotoxic NK cells (cNK). The cNK subset was then evaluated for its expression of the differentiation markers CD57, CD158a and NKG2D, the immune checkpoint PD-1 and TIM-3 and the chemokine receptors CXCR3, CXCR4 and CCR2. Isotype controls were used as negative controls to set the gates when continuous expression levels rather than discrete populations were visible, which was the case for chemokine receptors, TIM-3, and CD158a. In addition to the number of positive gated events for each of these markers, their median fluorescence intensity (MFI) was also analyzed in the cNK cell subset, as an estimate of each marker's surface density at the single cell level (Maher and Fletcher, 2005).

Table 1
Baseline characteristics.

	Mean ± SD
Demographic and treatment characteristics	
Age	57.9 ± 7.1
Sex (%)	
Women	7 (58%)
Men	5 (42%)
Cancer type (%)	
Breast	4 (33%)
Colorectal	5 (42%)
Oesophagus	1 (8%)
Prostate	1 (8%)
Lung	1 (8%)
Chemotherapy regimen (%)	
5-Fluorouracile-based (FOLFIRI, FOLFOX, Capecitabine)	7 (58 %)
Taxane (docetaxel, paclitaxel)	3 (25%)
Miscellaneous (Carboplatin + Gemcitabine, Vinorelbine)	2 (18%)
Treatment frequency (%)	
Every 14 days	6 (55%)
Every 21 days	1 (9%)
2 weekly doses every 21 days	2 (18%)
3 Weekly doses every 28 days	2 (18%)
Time since initial cancer diagnosis (months)	50 ± 49
Use of G-CSF (%)	5 (42%)
Use of glucocorticoids (%)	12 (100%)
Time since initial cancer diagnosis (months)	50 ± 49
Use of G-CSF (%)	5 (42%)
Use of glucocorticoids (%)	12 (100%)
Body composition and anthropometrics	
Weight (kg)	79.8 ± 16.2
Height (m)	165.8 ± 9.2
BMI (kg/m^2)	29.0 ± 5.4
Waist circumference (cm)	103.2 ± 15.7
Baseline resting hematological parameters	
RBC ($10^{12}/L$)	3.7 ± 0.5
Hemoglobin (g/L)	116.3 ± 12.5
Hematocrit (%)	0.35 ± 0.04
Platelets ($10^9/L$)	191.1 ± 79.5
WBC ($10^9/L$)	4.79 ± 1.97
Granulocytes ($10^9/L$)	3.13 ± 1.89
Lymphocytes ($10^9/L$)	1.18 ± 0.66
Monocytes ($10^9/L$)	0.49 ± 0.3
Submaximal exercise test end-parameters	
Final stage power output (Watts)	84.6 ± 27.7
Capillary blood lactate (mmol/L)	6.15 ± 2.09
Percent estimated maximal heart rate (%)	83 ± 7
Percent estimated reserve heart rate (%)	70 ± 11
Perceived exertion (CR10 scale) [a]	7.75 [5.25–8.00]

[a] Perceived exertion values are presented as median [IQR].

2.3.6. Determination of NK cell subsets blood counts

Each blood draw included a 4-ml EDTA coated tube, to obtain a complete blood count with differential (performed by the clinical biochemistry unit of the *CIUSSS de l'Estrie-CHUS*). The blood count of each gated subset was obtained by multiplying the percent events of total live singlet lymphocytes by the total lymphocyte count of the corresponding complete blood count. The post-exercise and 1h post-exercise total lymphocytes blood counts were corrected to account for the exercise-induced hemoconcentration. The corrected values were computed using the whole-blood biomarkers correction method proposed by Matomäki et al. (2018). The corrected lymphocyte counts were then used for each gated subset computation, as explained above.

2.3.7. Medical and disease-related variables

Medical and disease-related control variables were collected during the baseline assessment visit or retrieved in the participants' medical file, including: 1) cancer type, 2) prescribed chemotherapy protocol 3) time since diagnosis at inclusion, and 4) the use of corticosteroids and/or hematopoietic factors (G-CSF).

2.4. Statistical analysis

Data were analyzed using SPSS version 26.0. The Shapiro-Wilk test and visual inspection of the frequency histograms were used to verify the normality of the distributions. Sample size computation was based on the acute post-exercise increase in total NK cells blood count following a moderate-intensity intermittent exercise session in recent breast cancer survivors (Evans et al., 2015). It was determined that 12 participants would allow to detect an acute total NK cells blood mobilization (Post-exercise – Pre-exercise) corresponding to an effect size of 0.8 (Cohen's d) with a power of 80% and an alpha value of 0.05.

NK cell counts were normally distributed, as well as baseline and control variables, apart from perceived exertion. Thereby, Wilcoxon matched-pairs signed-ranks tests were used to compare perceived exertion values between trials (Table 2), and all perceived exertion values are reported as Median [interquartile range]. All other results are from parametric analyses and are presented as mean ± standard deviation. For each analysis, the alpha level was set at 0.05. To answer the main objective of this study, repeated measures two-way ANOVAs were used to assess the main effects of time, exercise trial, and time × trial interaction for each cell subset blood counts, and for the changes in MFI in the cNK cell subset. The Greenhouse-Geiser correction was used when the assumption of sphericity was violated. When significant effects were found, post-hoc comparisons were made using paired T-tests, and the Benjamini-Hotchberg False Discovery Rate (FDR) was used to account for multiple testing. Q-values (FDR-adjusted p-values) are reported for post-hoc analyses. For each subset blood counts, effect sizes for significant post-hoc comparisons were computed as standardised mean differences (SMD), using the paired samples Hedges' G with averaged standard deviations, as follow: $G_{av} = \left(\frac{\overline{X}_2 - \overline{X}_1}{(SD_2 - SD_1)/2}\right) \times \left(1 - \frac{3}{4n-9}\right)$, where $\overline{X}_{Post-Pre}$ is the mean of differences between paired values (Goulet-Pelletier and Cousineau, 2018).

3. Results

3.1. Recruitment and baseline sample characteristics

Recruitment took place from December 2020 to May 2022. The study was ended when 12 participants completed both exercise trials (MOD and HIIE). Complete blood counts were missing for one participant, which was therefore excluded from analyses performed on blood counts but included in analyses performed on MFI values. Details regarding participants flow in the study are provided in Fig. 1.

Baseline characteristics, including parameters measured at the end of the submaximal exercise test, are reported in Table 1. Noteworthy, half of the participants (6/12) did not reach the test termination target HR (steady-state HR within 10 bpm of 80% of the estimated HHR), in which cases the test was stopped when a combination of RPE ≥8/10 and blood lactate >4 mmol/L were achieved. There was no difference regarding end-test capillary lactate between the participants who did not reach and the ones who reached the target HR (6.2 ± 2.7 and 6.1 ± 1.3 mmol/L respectively, p = 0.38).

Table 2
Exercise trials work-related and physiological parameters.

	MOD	HIIE
Mean power output (W)	41.3 ± 15.8	*80.4 ± 28.4*[b] [a]
External work (kJ)	74.1 ± 27.8	71.7 ± 21.9
Capillary blood lactate (mmol/L)		
Pre-exercise	1.59 ± 0,47	1.88 ± 0.58
9 min	3.26 ± 0.97	*4.02 ± 1.06*[a]
18 min	3.30 ± 0.95	*4.52 ± 1.34*[a]
27 min	3.28 ± 1.05	*4.95 ± 1.20*[a]
Percent of predicted maximal HR (%)		
9 min	66 ± 7	68 ± 8
18 min	68 ± 8	71 ± 9
27 min	68 ± 8	73 ± 10
Perceived exertion [c]		
9 min	3.00 [2.13–4.00]	*3.50 [3.00-4.88]*[a]
18 min	3.50 [3.00–5.00]	*4.50 [3.00-4.88]*[a]
27 min	4.25 [3.00–5.00]	*6.00 [3.88-7.00]*[a]

[a] Significantly different from MOD trial, p < 0.05.
[b] Mean power output of the 10 high-intensity bouts.
[c] Perceived exertion values are presented as median [IQR].

3.2. Exercise trials parameters

The physiological and work-related parameters for each exercise session are presented in Table 2 The mean power output of HIIE represents the mean power (watts) of the ten 1-min high intensity bouts. Regarding capillary lactate values during MOD, a steady level was reached and maintained between the 9th and the 27th minutes of MOD (mean increase: 0.03 ± 0.82 mmol/L, p = 0.94). During HIIE, a significant increase was observed between the 3rd and 9th high intensity bouts (mean increase: 0.93 ± 0.29 mmol/L, p < 0.0001)

3.3. Acute immune response to MOD and HIIE trials

3.3.1. Lymphocytes, inflammatory and cytotoxic NK cells subsets

The blood concentrations of total circulating lymphocytes, total NK cells, iNK and cNK cells are reported in Table 3. Significant effects of time were found for every subset, but no trial effect or time by trial interaction effect was found for any cell population. Post-hoc analyses revealed significantly increased blood counts after exercise (Post – Pre-exercise) for every cell population and lower blood counts 1 h after exercise relatively to post-exercise, but not relatively to pre-exercise levels. Effect sizes computation revealed very large effects for total and cK cells, and moderate effects for iNK cells. Similarly, the frequency of cNK cells among the whole lymphocyte population significantly increased during both exercise trials, followed by decreases 1 h after exercise cessation. Following MOD, the cNK frequency among lymphocytes increased from 9.03 ± 5.45% to 15.81 ± 9.76% (q = 0.003) and decreased back to 8.05 ± 6.98% 1 h post-exercise (q = 0.003). Following HIIE, the cNK frequency among lymphocytes increased from 8.60 ± 6.62% to 16.20 ± 8.57% (q = 0.003) and decreased back to 6.59 ± 5.38% 1 h post-exercise (q = 0.003). The 1 h post-exercise cNK cell frequency among lymphocytes was lower than the pre-exercise level following HIIE (q = 0.021), and a similar tendency was found following MOD (q = 0.061). No significant trial effect or time by trial interaction was found.

3.3.2. Cytotoxic NK cells response to exercise trials by differentiation markers expression

The blood concentrations of cNK cells expressing CD57, NKG2D and CD158a are reported in Table 4. Significant effects of time were found for every subset, but no trial or time x trial effect was found. Post-hoc analyses revealed significantly increased blood counts after exercise and lower counts 1 h after exercise relatively to post-exercise, but not relatively to pre-exercise levels. Effect sizes computation revealed, for both trials, the largest effects on $CD57^+$ cells, followed by $NKG2D^+$ cells and $CD158a^+$ cells. Of all the subsets included in this study analysis, $CD57^+$ cNK cells exhibited the largest responses.

3.3.3. Cytotoxic NK cells response to exercise trials by chemokine receptor expression

The blood concentrations of cNK cells expressing CXCR3, CXCR4 and CCR2 are reported in Table 5. Significant effects of time were found for both $CXCR3^+$ and $CXCR4^+$ cNK cell, but no trial or time x trial effect was found. Although borderline, the time effect for $CCR2^+$ cells did not reach significance, and thus no post-hoc analysis was conducted. For both $CXCR3^+$ and $CXCR4^+$ subsets, post-hoc analyses revealed significantly increased blood counts after exercise, and significantly lower counts 1 h

Table 3
Total lymphocytes, inflammatory and cytotoxic NK cells blood concentrations.

	Blood counts (cells/μL)			Main effects F (p-value)		Interaction F (p-value)	Post-hoc tests for time – Hedge's G (q-value)		
	Pre	Post	1h Post	Time	Trial		Post - Pre	1h Post - Post	1h Post - Pre
Lymphocytes				**F = 25.75 (p < 0.001)**	F = 0.28 (p = 0.87)	F = 1.81 (p = 0.20)			
MOD	1009 ± 401	1461 ± 544	1104 ± 516				**G = 0.92 (q = 0.001)**	**G = -0.65 (q = 0.002)**	G = 0.20 (q = 0.146)
HIIE	1191 ± 863	1444 ± 609	974 ± 463				**G = 0.63 (q = < 0.001)**	**G = -0.84 (q = 0.001)**	G = 0.02 (q = 0.808)
NK cells ($CD3^{-}CD56^{+}$)				**F = 63.95 (p < 0.001)**	F = 0.061 (p = 0.81)	F = 1.65 (p = 0.23)			
MOD	107 ± 48	238 ± 78	97 ± 49				**G = 2.00 (q = < 0.001)**	**G = -2.15 (q = < 0.001)**	G = −0.21 (q = 0.449)
HIIE	111 ± 70	262 ± 91	87 ± 51				**G = 1.84 (q = < 0.001)**	**G = -2.34 (q = < 0.001)**	G = −0.32 (q = 0.175)
Regulatory NK cells ($CD56^{bright}$ $CD16^{-}$)				**F = 13.52 (p = 0.003)**	F = 0.41 (p = 0.54)	F = 0.039 (p = 0.94)			
MOD	12 ± 8	17 ± 11	12 ± 8				**G = 0.49 (q = 0.009)**	**G = -0.52 (q = 0.013)**	G = −0.02 (q = 0.79)
HIIE	13 ± 9	19 ± 11	13 ± 8				**G = 0.50 (q = 0.013)**	**G = -0.53 (q = 0.013)**	G = −0.02 (q = 0.79)
Cytotoxic NK cells ($CD56^{dim}$ $CD16^{+}$)				**F = 70.57 (p < 0.001)**	F = 0.011 (p = 0.92)	F = 1.69 (p = 0.22)			
MOD	69 ± 35	176 ± 58	61 ± 31				**G = 2.21 (q = < 0.001)**	**G = -2.49 (q = < 0.001)**	G = −0.23 (q = 0.51)
HIIE	70 ± 50	196 ± 64	50 ± 30				**G = 2.15 (q = < 0.001)**	**G = -2.95 (q = < 0.001)**	G = −0.41 (q = 0.16)

Table 4
Cytotoxic NK cells blood concentrations by NK cytotoxicity marker expression.

	Blood counts (cells/μL)			Main effects F (p-value)		Interaction F (p-value)	Post-hoc tests for time – Hedge's G (q-value)		
	Pre	Post	1h Post	Time	Trial		Post - Pre	1h Post - Post	1h Post - Pre
$CD56^{dim}$ $CD16^{+}$ $CD57^{+}$				**F = 68.88 (p < 0.001)**	F = 0.048 (p = 0.83)	F = 1.55 (p = 0.25)			
MOD	43 ± 23	116 ± 37	36 ± 15				**G = 2.38 (q = < 0.001)**	**G = -2.98 (q = < 0.001)**	q = 0.34
HIIE	44 ± 31	128 ± 43	30 ± 17				**G = 2.21 (q = < 0.001)**	**G = -3.14 (q = < 0.001)**	q = 0.14
$CD56^{dim}$ $CD16^{+}$ $NKG2D^{+}$				**F = 44.07 (p < 0.001)**	F = 0.012 (p = 0.91)	F = 0.89 (p = 0.40)			
MOD	50 ± 28	129 ± 50	44 ± 18				**G = 1.94 (q = < 0.001)**	**G = -2.39 (q = < 0.001)**	q = 0.43
HIIE	53 ± 44	139 ± 59	37 ± 24				**G = 1.64 (q = < 0.001)**	**G = -2.35 (q = < 0.001)**	q = 0.20
$CD56^{dim}$ $CD16^{+}$ $CD158a^{+}$				**F = 9.70 (p = 0.01)**	F = 0.042 (p = 0.84)	F = 0.92 (p = 0.38)			
MOD	3 ± 3	10 ± 9	3 ± 2				**G = 1.06 (q = 0.022)**	**G = -1.14 (q = 0.022)**	q = 0.88
HIIE	3 ± 2	11 ± 9	2 ± 1				**G = 1.32 (q = 0.022)**	**G = -1.55 (q = 0.022)**	q = 0.44

after exercise relatively to post-exercise, but not relatively to pre-exercise levels. Larger effect sizes were found regarding the $CXCR3^{+}$ cNK cell subset.

3.3.4. Cytotoxic NK cells response to exercise trials by immune checkpoints expression

It was not possible to detect a PD-1^{+} NK cell subset in any participant, and this marker was thus excluded from further analyses. The blood levels of TIM-3^{+} cNK cells are reported in Table 6. A significant effect of time, but no trial or time x trial effect, was found. Post-hoc analyses revealed significantly increased blood counts after exercise relatively to pre-exercise levels, and significantly lower blood count 1h post-exercise relatively to post-exercise. Effect sizes computation revealed very large effects of both exercise trials on TIM-3+ cells.

3.3.5. Effects of exercise trials on cytotoxic NK cells expressing multiple markers

Considering that the most exercise-responsive cNK cells were expressing NKG2D, CD57, CXCR3 and TIM-3, we sought to determine the relative proportion and the exercise-responsiveness of the cNK cell subset expressing all these markers. The $CD56^{dim}CD16^{+}NKG2D^{+}CD57^{+}CXCR3^{+}TIM\text{-}3^{+}$ NK subset was detected in every participant, representing 16.3 ± 8.3% of the total NK cells and 2.0 ± 1.5 % of the whole lymphocyte count (in combined pre-exercise samples). The blood counts of this subset are reported in Table 7. A significant effect of time was found, but no trial or time × trial interaction. For both trials, significantly increased blood counts following exercise and significantly lower counts 1h post-exercise were revealed by post-hoc analyses. Effect sizes were very large for both trials.

Table 5
Cytotoxic NK cells blood concentrations by chemokine receptor expression.

	Blood counts (cells/μL)			Main effects F (p-value)		Interaction F (p-value)	Post-hoc tests for time – Hedge's G (q-value)		
	Pre	Post	1h Post	Time	Trial		Post - Pre	1h Post - Post	1h Post - Pre
$CD56^{dim}$ $CD16^{+}$ $CXCR3^{+}$				**F = 50.37 (p < 0.001)**	F = 0.035 (p = 0.86)	F = 0.54 (p = 0.55)			
MOD	27 ± 19	64 ± 27	23 ± 12				**G = 1.49 (q = 0.001)**	**G = -2.01 (q < 0.001)**	q = 0.38
HIIE	26 ± 18	68 ± 28	19 ± 14				**G = 1.75 (q < 0.001)**	**G = -2.24 (q < 0.001)**	q = 0.16
$CD56^{dim}$ $CD16^{+}$ $CXCR4^{+}$				**F = 13.97 (p = 0.002)**	F = 0.05 (p = 0.83)	F = 0.24 (p = 0.73)			
MOD	34 ± 32	78 ± 59	27 ± 20				**G = 0.93 (q = 0.010)**	**G = -1.24 (q = 0.012)**	q = 0.38
HIIE	39 ± 49	79 ± 70	24 ± 29				**G = 0.66 (q = 0.010)**	**G = -1.06 (q = 0.010)**	q = 0.15
$CD56^{dim}$ $CD16^{+}$ $CCR2^{+}$				F = 4.80 (p = 0.052)	F = 0.85 (p = 0.38)	F = 0.88 (p = 0.43)			
MOD	32 ± 33	70 ± 84	30 ± 30						
HIIE	46 ± 59	85 ± 103	33 ± 40						

Table 6
Cytotoxic NK cells blood concentrations by TIM-3 expression.

	Blood counts (cells/μL)			Main effects F (p-value)		Interaction F (p-value)	Post-hoc tests for time – Hedge's G (q-value)		
	Pre	Post	1h Post	Time	Trial		Post - Pre	1h Post - Post	1h Post - Pre
$CD56^{dim}$ $CD16^{+}$ $TIM\text{-}3^{+}$				**F = 49.01 (p < 0.001)**	F = 0.003 (p = 0.96)	F = 0.54 (p = 0.54)			
MOD	35 ± 16	95 ± 43	33 ± 21				**G = 1.97 (q = < 0.001)**	**G = -1.87 (q = < 0.001)**	q = 0.74
HIIE	35 ± 26	106 ± 39	27 ± 16				**G = 2.08 (q = < 0.001)**	**G = -2.78 (q = < 0.001)**	q = 0.20

Table 7
$CD56^{dim}CD16^{+}NKG2D^{+}CD57^{+}CXCR3^{+}TIM\text{-}3^{+}$ NK cell subset blood concentrations.

	Blood counts (cells/μL)			Main effects F (p-value)		Interaction F (p-value)	Post-hoc tests for time – Hedge's G (q-value)		
	Pre	Post	1h Post	Time	Trial		Post - Pre	1h Post - Post	1h Post - Pre
				F = 51.40 (p < 0.001)	F = 0.081 (p = 0.78)	F = 0.14 (p = 0.87)			
MOD	17 ± 11	44 ± 21	14 ± 8				**G = 1.63 (q < 0.001)**	**G = -2,07 (q < 0.001)**	G = −0,37 (q = 0.17)
HIIE	16 ± 12	47 ± 18	12 ± 7				**G = 1,97 (q < 0.001)**	**G = -2,68 (q < 0.001)**	G = −0,47 (q = 0.26)

3.3.6. *Effect of exercise trials on the MFI values in the cNK cells subset*

For both trials, the CD16 MFI significantly increased immediately following exercise (q = 0.02) (Fig. 2A). Significant decreases in the MFI values of NKG2D (q = 0.01) and CXCR4 (q = 0.003) were found immediately following the HIIE trial, which were followed by subsequent increases in the hour following exercise cessation (NKG2D: q = 0.002; CXCR4: q = 0.038) (Fig. 2B–C). A similar tendency for a decrease following the HIIE trial was found regarding the MFI values of CD158a (q = 0.056) (Fig. 2D) followed by a non-significant increase 1h post-exercise. No other significant changes were observed, and no significant trial effect or trial × time interaction was found.

4. Discussion

4.1. *Main findings*

The main objective of this study was to characterize the acute NK cell response to a MOD and a HIIE trial matched for duration and external work done, in metastatic cancer patients treated with chemotherapy. The main finding is that both trials elicited the preferential response of cNK cells expressing the differentiation markers CD57 and CD158a, the immune checkpoint TIM-3 and the activating receptor NKG2D, which is indicative of a high differentiation status, an activated state and a strong tumoricidal potential (Abel et al., 2018; Bigley et al., 2014; Ndhlovu et al., 2012).

The expression of CXCR3 by preferentially responsive cNK cells is also suggestive of their tumor migratory potential. CXCR3 is an

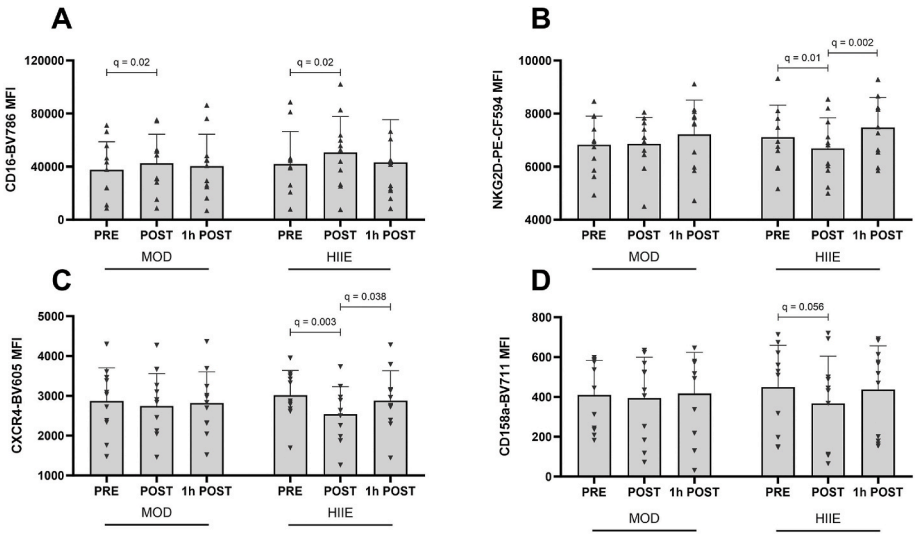

Fig. 2. Median Fluorescence Intensity changes in the cytotoxic NK cell subset Data are presented as mean ± standard deviation, with triangles representing individual data points. Q: Benjamini-Hochberg FDR adjusted p-value. MFI: Median fluorescence intensity. MOD: Moderate intensity continuous exercise trial. HIIE: High-intensity intervals exercise trial.

inflammatory chemokine receptor upregulated upon activation (Wennerberg et al., 2014) and involved in tumor homing in many carcinomas (Susek et al., 2018). In this regard, the fact that a highly exercise-responsive NKG2D^{+}CD57^{+}CXCR3^{+}TIM-3^{+} cNK subset was detected in every participant of this study demonstrates that both a MOD and a HIIE session can elicit an acute response of cNK cells exhibiting phenotypic characteristics associated with a strong tumoricidal capacity and tumor homing potential.

Interestingly, pre-clinical data from pre-operative exercise training in early-stage prostate cancer patients suggest that exercise may promote NK cell tumor infiltration in a dose-dependent manner. While a single aerobic exercise bout 12 h before prostatectomy failed to increase tumoral NK cell density (Schenk et al., 2022), more completed exercise sessions resulted in a significant effect (Djurhuus et al., 2023). In this regard, the present study showed that a potentially clinically relevant cNK response to aerobic exercise is conserved in metastatic patients undergoing long-term chemotherapy, both following MOD and HIIE. Taken together, these results further support the idea of investigating the longitudinal effects of training regimen incorporating multiple sessions of MOD or HIIE on the tumor-immune microenvironment, and its implications on cancer outcomes such as survival in this population.

Another therapeutic perspective naturally following these results is to study the combination of acute exercise and immunotherapy. Indeed, cancer immune evasion strategies suppressing NK cells infiltration and cytolysis are well documented (Hu et al., 2019). In this regard, it is noteworthy that TIM-3^{+} cNK cells showed to be highly responsive in both trials. TIM-3 is a well-known inhibitory checkpoint which contributes to the immune evasion of cancers against NK cells (Jiang et al., 2022; Yu et al., 2021). Preclinical trials evaluating the safety and clinical relevance of TIM-3 blockade are currently ongoing (He et al., 2018), and owing its efficacy, combining aerobic exercise and anti-TIM-3 antibodies might represent an interesting avenue.

4.2. Comparison between exercise trials

We hypothesized that work and duration matched HIIE would elicit a stronger response. However, we did not observe any significant interaction or between-trial difference in any of the studied subsets, which appear contradictory with what has been reported in healthy adults (Arroyo et al., 2022; Jamurtas et al., 2018).

A dampened adrenergic hormonal secretion in response to high-intensity exercise might have contributed to this lack of difference. In healthy individuals, adrenergic hormonal secretion is driven by sympathetic outputs and rises exponentially with exercise intensity (Urhausen et al., 1994). Yet, dampened exercise-induced increases in plasma epinephrine concentrations have been reported in prostate (Hanson et al., 2018) and breast (Evans et al., 2016) cancer survivors, in the latter case following adjuvant chemotherapy. There is accumulating evidence that anti-neoplastic treatments are associated with autonomic nervous system dysfunctions (Lakoski et al., 2015), including maintenance chemotherapy in metastatic cancers (Jones et al., 2012). As such, the adrenergic response to high intensity could be blunted in some cancer patients, which would presumably hinder the immune response following HIIE. Although speculative, this hypothesis could have important implications in terms of exercise prescription in this population and should be further studied.

On another hand, it would be too simplistic to conclude that both trials elicited the same effect.

Interestingly, HIIE induced a decrease in the MFI of NKG2D and CXCR4 during exercise, followed by increases 1 h latter, suggesting that the surface density of these receptors in post-exercise circulating cNK was lower in comparison to the baseline and 1h post-exercise levels. These changes may reflect either acute alterations of the surface expression of these receptors in circulating cNK cells, or the preferential blood mobilization and egress of these specific cell subsets during and after exercise (or a combination of both). Although functional assays were not performed in the present study, it cannot be excluded that these changes may have a functional significance in terms of homing or cytotoxic capacity. Noteworthy, previous findings also suggest that different exercise intensities might differentially influence the functional behavior of cNK cells. Indeed, a decreased NK cytotoxic activity (NKCA) per cell immediately following maximal intensity exercise, followed by an increase 1h later, has been reported in cancer survivors (Schauer et al., 2022). Similar changes have also been reported in healthy individuals (Bigley et al., 2014; Graff et al., 2018). Interestingly, Nieman et al. (1993) measured an increased NKCA per cell 2h after exercise cessation following high intensity (80% VO_2 max), but not moderate intensity exercise (50% VO_2 max). Finally, in cancer survivors, a training regimen combining sessions of MOD and HIIE increased the expression of NKG2D in NK cells, while it was decreased following a more classical endurance training regimen comprising only MOD sessions (Pal et al., 2021). Based on these findings and the results of the present study, it could be hypothesized that MOD and HIIE may exert differential, and maybe complementary, effects on NK cells, although

future longitudinal trials comparing both exercise regimen will be needed to confirm this.

Finally, it cannot be excluded that potentially different effects of HIIE and MOD may have been blunted by methodological considerations, such as the use of the OBLA as a threshold between moderate and high intensity. The OBLA has been retained as it is, on average, the theoretical value corresponding to the maximal lactate steady state (MLSS), often used as the physiological threshold between moderate (sustainable) and high (unsustainable) intensity. Reassuringly, the steady capillary lactate level observed during the last 20 min of the MOD trial, the continuous increase in capillary lactate from the 3rd to the 9th high intensity bouts of the HIIE trial, and the homogeneity of the capillary lactate results all suggest that each trial was performed in the targeted intensity zone. However, on an individual basis, the MLSS can range from under 2 mmol/L to over 7 mmol/L (Goodwin et al., 2007). As such, the submaximal exercise test used in this study did not allow the precise identification of a lactate or ventilatory threshold on an individual basis. This may have increased the heterogeneity of the observed immune responses, in comparison to trials that would have been conducted at fixed proportions of a more precisely identified physiological threshold.

Similarly, another important consideration that should be kept in mind is that this pilot study was primarily designed, and thus powered, to characterize the acute response of the studied subsets following both trials. In this regard, it may have lacked the sufficient statistical power to detect interactions effects between the trials. As such, future and larger studies are still needed to verify if a work-matched HIIE session may or may not provoke a larger immune response in cancer patients undergoing chemotherapy.

4.3. Limitations

This study carries certain limitations, beginning with its small sample size and heterogeneity. The plurality of different cancer diagnoses included, resulting in a plurality of chemotherapy regimen of varying cycle lengths, may have increased the heterogeneity of the obtained results. Considered in the light of the small sample size, these factors may explain why smaller or more heterogenous effects could not be statistically detected, although they might have a significant clinical implication. Another limitation is that no tumor tissue analysis or NK cell cytotoxic assay has been conducted, and thus, the tumor homing and cytotoxic potential of the exercise-responding cells was derived from their phenotypic analysis. Moreover, this phenotypic analysis included only one activatory (NKG2D) and two inhibitory (CD158a and TIM-3) receptors, representing only a small proportion of the balance between activatory and inhibitory receptors found in NK cells. Thus, whether the observed acute immune response did translate in an increased tumor migration or tumoricidal activity after exercise remains unknown.

5. Conclusion

In conclusion, to our knowledge, this is the first study to demonstrate that a significant acute NK cell response to both MOD and HIIE is conserved in metastatic cancer patients undergoing chemotherapy. Moreover, the phenotypic characteristics of the preferentially responding cNK subsets are suggestive of their tumor migratory and tumoricidal potential. These results will further support the design of future trials to investigate the combined effect of anti-cancer treatments with MOD and HIIE on tumor outcomes and immune infiltration in cancer patients.

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CRediT authorship contribution statement

Hugo Parent-Roberge: Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adeline Fontvieille:** Writing – review & editing, Project administration, Methodology, Investigation, Conceptualization. **Laurence Poirier:** Writing – review & editing, Investigation, Conceptualization. **Lee-Hwa Tai:** Writing – review & editing, Validation, Formal analysis. **Michel Pavic:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Tamàs Fülöp:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Eléonor Riesco:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

There are no conflict of interest among all authors.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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