EXERCISE AND NATURAL KILLER CELL CYTOTOXICITY IN YOUTH

EVALUATING THE EFFECTS OF EXERCISE ON NATURAL KILLER CELL CYTOTOXICITY IN PRE- AND POST-PUBERTAL BOYS AND GIRLS

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Descriptive Note

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LAY ABSTRACT

The purpose of this study was to determine if exercise can increase the function of Natural Killer cells (NK cells) in children. These cells are a very important part of our immune system that work to protect us from harmful infections and cancers. We also wanted to see if puberty or sex influence how NK cells respond to exercise. We asked boys and girls who were 8-11 years old or 15-18 years old to visit our lab 5 times. During their first visit, we measured their fitness level. In the next 4 visits, they completed one of four different types of exercise including: 1) 30 minutes of hard cycling, 2) 5 minutes of stop-and-start hard cycling, 3) 30 minutes of less hard cycling, and 4) 5 minutes of stop-and-start less hard cycling. We collected blood at four times during each session, including: 1) before the exercise, 2) immediately after the exercise, 3) 30-minutes after exercise, and 4) 60-minutes after exercise. We took NK cells from this blood and added them to cancer cells. We measured how many cancer cells were killed by the NK cells. We were interested in learning if exercise changed the percent of cancer cells killed by NK cells. We also wanted to know if exercise helped make each NK cell a better killer. We found that immediately after exercise, NK cells were able to kill a greater percent of cancer cells than they could before exercise. We also found that at 60-minutes after exercise, each NK cell killed a greater number of cancer cells than they were able to before exercise. There were no differences in NK cell cancer killing capacity between boys and girls, or between pre-pubertal or post-pubertal

children. Our study helps us understand how we can increase important immune functions with exercise in children.

ABSTRACT

Natural killer (NK) cells are a type of lymphocyte involved in innate and adaptive immunity. In adults, exercise can increase NK cell cytotoxicity both as a total percent of target cell lysis and an increase in the function of each NK cell. Much less is known about this response in children. Thus, the objectives of this study were to examine: 1) the effects of exercise intensity and structure on NK specific lysis and NKCA per cell in youth, and 2) differences in baseline and post-exercise specific lysis and NKCA per cell by pubertal status and sex. We also examined the NK cell and NK subset response to these exercise protocols by pubertal status and sex to help contextualize the NK functional response.

We recruited healthy, recreationally active pre-pubertal (8-11 years, N=5) and late/post-pubertal (15-18 years, N=6) children from the Hamilton community. Participants completed 4 experimental cycling sessions in a randomized order including: high intensity intermittent (HI-INT) or continuous (HI-CONT), and moderate intensity intermittent (MOD-INT) or continuous (MOD-CONT) cycling. Blood was collected at baseline (PRE), immediately post-exercise (POST), and at 30- and 60-minutes recovery (REC1 and REC2, respectively). Peripheral Blood Mononuclear Cells (PBMC) were isolated from each timepoint and used in a cytotoxic assay with K562 targets. Flow cytometry was used to quantify target cell death (reported as specific lysis and NKCA per cell), and to determine NK cells and subsets as a proportion of lymphocytes.

Exercise significantly increased specific lysis from PRE (25.1± 6.7%) to POST (30.6±11.0%), while NKCA per cell increased from PRE (0.69±0.76) to REC2 (1.3±1.3); $p<0.05$ for both. These responses were seen irrespective of exercise intensity and structure, pubertal status, and sex. The % total NK cells increased from PRE to POST (6.8±2.8% vs. 13.6±7.2%), and was greater in post-pubertal children vs. pre-pubertal children (20.9±6.2% vs. 9.1±4.3%), and after HI-INT (15.5±8.1%) and MOD-INT (16.2±9.5%) vs. HI-CONT (12.0 ± 5.0%) and MOD-CONT exercises (10.6 ± 4.5%; $p<0.05$ for all).

Acute exercise can enhance NK cell cytotoxicity in youth regardless of exercise intensity, structure, pubertal status, or sex. NK function and mobilization responses to exercise did not always align, which suggests that unique post-exercise processes contribute to changes in NK cell proportions, specific lysis, and NKCA per cell. Our study has enhanced the understanding of exercise-induced changes NK cell cytotoxicity in the growing years. Future research will focus on explaining these effects, and exploring the clinical application of exercise programs in youth.

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CONTRIBUTIONS

Authors of funding grants for this project: JO

Conceived and designed the research: JO, IU, EH

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LIST OF ABBREVIATIONS AND SYMBOLS

β-adrenergic	Beta adrenergic
BMI	Body Mass Index
EPI	Epinephrine
FMO	Flow Minus One
FVD	Fixable Viability Dye eFlour 780
GH	Growth Hormone
HI-CONT	High Intensity Continuous Cycling
HI-INT	High Intensity Interval Cycling
HIIT	High Intensity Interval Training
HR	Hear Rate
ICAM	Intracellular Adhesion Molecule
IFNγ	Interferon gamma
IL	Interleukin
LT	Lactate Threshold
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MOD-CONT	Moderate Intensity Continuous Cycling
MOD-INT	Moderate Intensity Interval Cycling
PBMC	Peripheral Blood Mononuclear Cell
RPE	Rating of Perceived Exertion
SMP	Sample

STD	Spontaneous Target Cell Death
T:E	Target:Effector
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor alpha
TNFβ	Tumor Necrosis Factor beta
VO_2	Volume of oxygen uptake
VO_2max	Maximal volume of oxygen uptake
VT	Ventilatory Threshold
YPHV	Years from Peak Height Velocity

Chapter 1: Introduction

1.1 Natural Killer Cells

1.1.1 Overview of Natural Killer Cells

Natural killer (NK) cells are a type of large, granular lymphocyte involved in the immune system[1]. Although NK cells represent only 5-15% of total circulating lymphocytes, they are crucial for anti-viral and anti-cancer host defenses[1–5]. Unlike other immune cells such as B cells and T cells, NK cells can initiate the destruction of targets without the need for prior sensitization[1]. Their role in the innate immune system makes NK cells an important first line of host defense, as they are among the first to detect tumors and virally infected cells[3,6]. Upon recognition of these targets, NK cells promote target cell lysis through the release of perforin and granzymes[2,7]. Activated NK cells also release cytokines to communicate with and stimulate other parts of the immune system, suggesting their role in adaptive immunity[1,2,5].

1.1.2 Natural Killer Cell Development

The two primary sites of NK cell development are the bone marrow and lymph nodes[1,2]. In the bone marrow, NK cells originate from $CD34^+$ hematopoietic progenitor cells (HPCs) and respond to early growth factors that aid in their differentiation into mature, functional immune cells[1,5,8,9]. Two important early hematopoietic growth factors for NK cell differentiation are c-kit ligand and flt-3 ligand, both of which have been shown to enhance NK cell expansion in vitro[1]. Cytokines and chemokines also aid in the differentiation and proliferation of NK

cells[9]. The most noteworthy of these is IL-15, a cytokine that can be found in bone marrow[1,5]. IL-15 induces the transformation of $CD34^+$ HPCs into mature NK cells and works synergistically with the aforementioned ligands to promote NK cell expansion[1]. Mature NK cells are distributed into circulation and populate many peripheral tissues including the liver, lungs, and spleen[8,9]. Upon their cytokine-signalled activation, NK cells are trafficked to sites of tissue infection or malignancy where their functions are required[9].

1.1.3 Natural Killer Cell Subsets

NK cells are phenotypically defined by their expression of various cell surface molecules. All NK cells are $CD3^-/CD56^{+}$[1,5,6]. CD56 is a neural cell adhesion molecule (NCAM) whose function on human cells is not well known, however may facilitate the interaction between NK cells and their targets[5,6]. Importantly, the relative expression of CD56 on NK cells allows for their separation into two functionally distinct subsets[1]. CD16 (Fcγ receptor III) is an additional molecule used to classify NK cell subsets[10,11].

The two main subsets of NK cells are $CD56^{dim}$ cells and $CD56^{bright}$ cells. $CD56^{dim}$ cells have a low surface density of CD56, a high surface density of CD16 and account for ~90% of circulating NK cells[1,5]. The other ~10% of NK cells are classified as $CD56^{bright}$ cells, which express high levels of CD56 and little to no CD16[1,5]. Importantly, these two subsets are differentially capable of lysing target cells and producing cytokines. $CD56^{dim}$ cells have high cytotoxic potential that places them under the innate branch of immunity, while the immunoregulatory

functions of CD56^{bright} cells associate them best with adaptive immunity[1,5,6]. Compared to CD56^{dim} cells, CD56^{bright} cells express fewer genes for cytotoxic molecules and are less effective at mediating target cell lysis[6,12–14]. CD56^{bright} cells do, however, highly express genes that are involved in cell migration, cell-to-cell communication, and cellular adhesion[6,14]. For example, the CD62L/L-selectin adhesion molecule is more densely expressed on CD56^{bright} cells[5,6,15]. This molecule mediates the interaction between NK cells and the vascular endothelium and encourages the transportation of CD56^{bright} cells to peripheral tissues such as the lymph nodes and sites of inflammation[1,5]. CD56^{bright} cells also densely express the high affinity interleukin-2 (IL-2) receptor IL2-Rαβγ[1]. This allows them to respond to low cellular concentrations of IL-2, a cytokine released by T cells, demonstrating their crucial role in facilitating communication across branches of the immune system[1,5]. Once activated, CD56^{bright} cells produce a variety of cytokines to initiate immune reactions, including interferon gamma (IFNγ), tumor necrosis factor (TNFα and TNFβ), granulocyte macrophage colony stimulating factor (GM-CSF), and multiple interleukins such as IL-10 and IL-13[1,5]. These molecules help support the Th1 immune response and regulate inflammation[6,16].

Contrastingly, CD56^{dim} cells are the more cytotoxic subset of NK cells. Unlike CD56^{bright} cells, they produce negligible amounts of immunoregulatory cytokines following monokine-induced stimulation[17]. Instead, their major function is to lyse tumor- and virally-infected target cells through the release of cytotoxic molecules that are highly expressed in CD56^{dim} cells[12,14].

Although the two subsets of NK cells have distinct functions within the immune system, their common origin from HPCs within the bone marrow suggests a similar maturation and development process[6]. Accordingly, a linear differentiation model has been proposed, whereby $CD56^{dim}$ cells are directly derived from $CD56^{bright}$ cells[1,6,18]. This model suggests that $CD56^{bright}$ cells are the younger or less mature form of NK cell, evidenced by their longer telomeres and greater proliferative capacity when compared to $CD56^{dim}$ cells[5,18].

1.1.4 Natural Killer Cell Receptors

NK cells express a variety of activating and inhibitory receptors. Three superfamilies of NK cell receptors have been identified, and the relative expression and engagement of these determine NK cell activity[1,5,19]. The first family is the killer immunoglobulin-like receptors (KIRs) which can be either activating or inhibitory depending on the length of their cytoplasmic tail[1,5]. KIRs recognize human leukocyte antigen (HLA) complexes, otherwise known as the Major Histocompatibility Complex Class I (MHC-I)[1,5]. The second family is C-type lectin-like receptors (CLRs), which can recognize non-classical MHC-I molecules (e.g. HLA-E) that either activate or inhibit the NK cell[1,5]. The final superfamily is the natural cytotoxicity receptors such as NKp30, NKp44, and NKp46 whose density is directly correlated with NK cell function against tumor cells[19]. NK cells express different amounts of activating (e.g. NKG2D) and inhibitory (e.g. NKG2A) receptors within these receptor families that affect their function[1,19]. The acquisition of NK cell receptors occurs throughout development and previous work suggests that the

expression of various activating and inhibitory receptors differ between youth/young adults and adults (20+ years of age)[20].

NK cells also express a variety of chemokine receptors and adhesion molecules. Notably, the expression of these differs between $CD56^{dim}$ and $CD56^{bright}$ cells. For example, $CD56^{bright}$ cells express more of the adhesion molecules CD62L and CD44, and high levels of chemokine receptor 7 (CCR7)[1,10]. $CD56^{dim}$ cells express more of the CD11a adhesion molecule and the chemokine receptors CXCR1 and CX_3CR1[10]. The result of these chemokine and adhesion receptor profiles is the differential trafficking of NK cell subsets[5]. $CD56^{bright}$ cells preferentially migrate to secondary lymphoid organs, while $CD56^{dim}$ cells home to sites of acute inflammation[10].

1.1.5 Role in Innate and Adaptive Immunity

NK cells are traditionally noted for their unique role in the innate immune system because they promote the rapid lysis of target cells without the need for prior sensitization[1,5,6]. This distinguishes them from other lymphocytes, such as B cells and T cells, that require antigen exposure prior to mounting an immune response. The natural cytotoxicity of NK cells is crucial to early host defense, as NK cells function to protect the body in the window before the adaptive immune system can become fully activated[1].

The mechanism by which NK cells can immediately distinguish targets from healthy cells has been termed the ‘missing-self hypothesis’[21]. NK cells recognize healthy cells as those that express the Major Histocompatibility Complex Class I

molecule (MHC-I)[1,2,9,22]. MHC molecules are present on the surface of nearly all healthy, nucleated cells in the body, and are used to present antigens that allow for specific T cell recognition and activation[2]. When NK cells encounter healthy cells expressing the MHC-I, the MHC-1 molecule interacts with an NK cell inhibitory receptors to prevent target cell lysis[2,5,9,22]. Inhibition occurs when the immune tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic tail of the NK cell are phosphorylated, providing a negative feedback signal that blocks the cytotoxic machinery and cytokine effector functions of NK cells[9,22]. Abnormal cells that are either virally infected or cancerous have downregulated MHC-I molecules[2]. While this modification renders them invisible to detection by most other immune cells, it promotes NK cytotoxicity. This is because the activity of an NK cell is determined by the relative engagement of its activating and inhibitory receptors. Target cells have fewer MHC-I molecules to interact with and inhibit the NK cell, and as a result there is a greater likelihood that NK cell activation will be favoured[9,21].

The role of NK cells in the adaptive immune system is growing. This response is specifically facilitated by $CD56^{bright}$ cells through their release of immunoregulatory cytokines such as IFNγ and TNFβ[1,5]. These cytokines facilitate the cross-talk between NK cells and other important branches of the adaptive immune system, for example by activating and causing the proliferation of T cells[6]. NK cells also contribute to adaptive immunity by lysing target cells into antigenic debris for presentation to T cells, which allows T cells to generate antigen-specific immune responses[9].

1.1.6 Natural Killer Cell Cytotoxicity

During acute and chronic infection, NK cells receive signals from cytokines and chemokines that direct them to sites of inflammation[9]. Upon migrating to these tissues, adhesion molecules mediate the interaction between NK cells and their targets, and signals from activating receptors initiate NK function[7,9]. The CD16 antigen is expressed on many NK cell targets and binds to the CD16 receptor on $CD56^{dim}$ cells to mediate antibody-dependent cellular cytoxicity (ADCC)[2,23]. NCRs, a previously described class of activating receptors, are also crucial to initiating cytotoxicity because they facilitate the interaction between NK cells and a variety of pathogen-encoded ligands[19,24]. Accordingly, Moretta et al. reported that the more NCRs an NK cell expresses, the greater its cytotoxic potential[19].

Costimulatory factors including IL-2, IL-12, and IL-15 also influence NK function[9]. Researchers have previously demonstrated the ability of these molecules to enhance the cytotoxic and secretory functions of NK cells. For example, the administration of IL-2 or IL-12 in vitro have been shown to augment IFNγ production and the anti-metastatic function of NK cells[9].

The activation of NK cells results in blastogenesis, cytotoxic activity, and the release of cytokines[19]. The primary pathway employed by $CD56^{dim}$ cells to promote target cell lysis is the perforin/granzyme B pathway[9,25]. Upon conjugation with transformed cells, specialized lytic granules containing perforin and granzymes are released by NK cells and fuse with the target cell membrane[9,20,25]. These proteins are highly cytotoxic and act synergistically to mediate target cell destruction[25].

Perforin causes the formation of pores in the target cell membrane, which then allows granzymes to infiltrate the cell and proteolyze its contents[25].

1.2 Natural Killer Cell Response to Acute Exercise in Adults

1.2.1 Overview of the Natural Killer Cell Response to Exercise

Given that NK cells are a critical first line of defense against tumors and viruses, the ability to augment their number and cytotoxicity is of great clinical importance. Exercise is a feasible, non-invasive method of modulating NK number and cytotoxicity. In general, exercise drives an increase in lymphocytosis[26–28]. It is well known that NK cells are the most responsive type of lymphocyte to acute exercise in adults (>18 years)[3,8]. While a similar response pattern has been suggested in children, few studies have investigated the specifics of exercise-induced NK cell recruitment and function in youth[6,29–31]. As such, we have reviewed the adult literature to provide a more comprehensive overview of the effects of exercise on NK cells.

1.2.2 Natural Killer Cell Recruitment and Exercise

During exercise the proportion of circulating NK cells increases as they are recruited from peripheral tissues such as the spleen, lymph nodes, and lungs[3,8,32]. The mobilization of NK cells peaks immediately post-exercise, and is often followed by a decline below pre-exercise values during the recovery period from work[3,33]. This transient decrease is thought to reflect the redistribution of NK cells to sites of infection, suggesting a role for exercise in immunosurveillance. Accordingly, murine models have shown that exercise enhances NK cell recruitment and

infiltration into solid tumors, contributing to a reduction in tumor growth by 50-60%[34]. Following activity at peripheral sites, circulating NK cell counts return to baseline values over the next 2 - 24 hours[3,33,35]. This response pattern to exercise has been described consistently for more than 30 years[3,36].

1.2.3 Natural Killer Cell Subsets and Exercise

There is differential mobilization of $CD56^{bright}$ and $CD56^{dim}$ cells during exercise[6,37]. Given that these two subsets have unique immune contributions, this suggests that exercise may favour certain NK cell functions at various post-exercise timepoints. Notably, immunoregulatory $CD56^{bright}$ cells are less responsive to exercise than cytotoxic $CD56^{dim}$ cells[6]. For example, Suzui et al. had 6 males complete a 30-minute cycling protocol at 120% of their VT. There was a significant increase in the proportion of $CD56^{dim}$ cells from rest, while $CD56^{bright}$ cell proportions were unchanged[38]. These findings were later confirmed by the same group using an incremental cycling protocol where intensity was increased every 5-minutes from 50, 90, 120, to 140% of ventilatory threshold (VT)[39]. Post-exercise, there was an overall leukocytosis with an increase in both $CD56^{dim}$ and $CD56^{bright}$ counts. However, only the proportion of $CD56^{dim}$ cells increased significantly.

The selective mobilization of $CD56^{dim}$ cells is likely driven by multiple factors. Most notably, $CD56^{dim}$ cells have a high surface density of β_2-adrenergic receptors. This makes them especially sensitive and responsive to catecholamines such as epinephrine (EPI) that increase during physiological stress like exercise[3,40,41].

Additionally, $CD56^{dim}$ cells are primarily stored in the spleen and vascular endothelium, both of which are engaged during exercise[8].

The preferential mobilization of $CD56^{dim}$ cells post-exercise is noteworthy because of their potent cytotoxicity, with high perforin and granzyme content[5,12,14]. There is often a dip in $CD56^{dim}$ cell numbers during exercise recovery, which likely reflects the egress of cytotoxic NK cells to peripheral tissues and sites of infection[3]. A study in mice tracked T lymphocytes to the lungs, bone marrow, and Peyer's patches post-exercise[42]. While similar research has not been performed for NK cells, homing receptors present on $CD56^{dim}$ cells would indicate that these cells are destined for sites of inflammation[10]. Thus, exercise may increase the trafficking of cytotoxic NK cells to sites where enhanced immunosurveillance could be particularly advantageous. This provides a potential link between $CD56^{dim}$ cells and the reported beneficial effects of exercise, including decreasing tumor burden in rodents, and increasing cancer survivorship in humans[8,43].

As $CD56^{dim}$ cells move out of circulation, an increased proportion of $CD56^{bright}$ cells is left behind. These NK cells are capable of releasing cytokines to aid in post-exercise tissue recovery and homeostatic adaption[6]. For example, $CD56^{bright}$ cells express angiogenic growth factors and can therefore contribute to post-exercise angiogenesis[44]. The shift in $CD56^{dim}$ and $CD56^{bright}$ cells are typically apparent during one hour of exercise recovery, after which the proportion of NK cell subsets return to baseline[6].

1.2.4 Specific Lysis and Exercise

In addition to increasing NK cell number and proportion, acute exercise enhances NK cell cytotoxic activity (NKCA) or specific lysis. Specific lysis represents the proportion of a given number of target cells that are lysed by a fixed number of mononuclear cells. Most often, specific lysis is evaluated by the incubation of PBMC with K562 target cells, where chromium (Cr) release or flow cytometry is used to quantify cell numbers and target cell death[3,45]. Since K562 cells are MHC-deficient, target cell lysis can be attributed primarily to NK cells.

Changes in specific lysis often parallel those seen in NK cell number, with a peak immediately post-exercise and a decline during exercise recovery[3,32,46]. For example, Nieman et al. found that when young male participants (~22 years) cycled for 45-minutes at 80% of their VO_2 max, there was a significant increase in specific lysis immediately after exercise as measured by a Cr-release K562 assay. Specific lysis then tended to decrease below baseline at 1-hour recovery, with a return to pre-exercise values by 3.5 hours of recovery[3]. These changes followed the same pattern as those seen for NK cells ($CD3^-/CD56^+/CD16^+$) as a proportion of lymphocytes. Similar results have been reported by Tvede et al[47]. While their sample was small (N=6), 1-hour of cycling at 75% VO_2max caused a robust post-exercise increase in NK specific lysis, followed by a decline below baseline at 2-hours recovery. The percent of NK cells (defined as either $CD56^+$ or $CD16^+$) were also significantly increased post-exercise, however did not decrease below baseline values during 2-hour recovery. Taken together, these results suggest that

while post-exercise mobilization of NK cells is important to consider, it is not the only factor contributing to specific lysis. This point is further illustrated by a unique study from Moyna et al. Participants completed a progressive cycling protocol where exercise intensity was increased from 55 to 70 to 85% of VO_2max in 6-minute increments[48]. A significant increase in specific lysis occurred during the initial 6-minute bout, as did an increase in NK cell number. Following this, there were no further changes to specific lysis, despite a significant jump in NK cell counts at 8- and 12-minutes exercise (**Figure 1**). Thus, it appears that NK cell mobilization may be one of many factors to influence NK specific lysis after exercise.

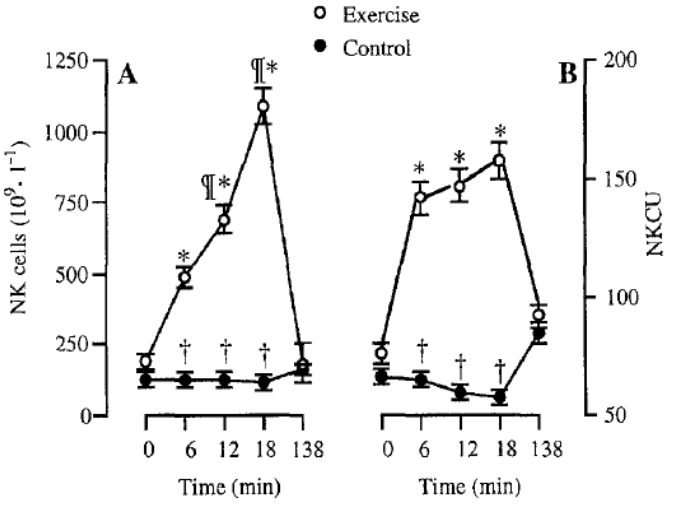

Figure 1. Exercise intensity was increased from 55 to 70 to 85% VO_2 max at 6, 12, and 18 minutes respectively. **A.** The absolute number of NK cells increased in a graded manner (18 min > 12 min > 6 min > 0 min). **B.** NKCU (an indicator of specific lysis) significantly increased from 0 to 6 minutes, followed by a smaller and non-significant upwards trend. Both count and lysis return to baseline 2-hours post-exercise. *Figure modified from Moyna et al (1996)*[48].

1.2.5 Effects of Exercise Intensity on Specific Lysis

Although the pattern of NK cell response described above is well-documented, the magnitude of change in specific lysis, and NK cell number, may depend on the level of physiological stress experienced by a participant[3,35,47–49]. For example, Pederson et al. investigated the NK cell response to 60-minutes of cycling at 80% VO_2 max vs. 60-minutes of back muscle training at 29% VO_2 max[35]. Immediately after high intensity exercise, there was a significant increase in specific lysis, followed by a decrease in 2-hour recovery and an eventual return to baseline within 24 hours. There were no significant differences in specific lysis at any point after low intensity exercise. While these results are limited by the inclusion of two different exercise modalities, intensity dependency was also found by Nieman et al.[3]. Young adult males (~22 years) cycled for 45 minutes at either a high or low intensity (50 vs. 80% VO_2 max). A similar pattern for changes in specific lysis were seen after both exercise protocols; however, the magnitude of increase in specific lysis was only significant following high intensity exercise (**Figure 2**). Intensity-dependent effects on specific lysis have also been reported in adult women[49].

Exercise intensity not only affects NK cell cytotoxicity immediately post-exercise but may also be an important determinant of specific lysis suppression during recovery. In a review of their own results Pederson et al. discussed how 1-hour of exercise at 25, 50, and 75% VO_2max all increased specific lysis, but that a decrease in specific lysis was only observed during recovery from the highest

intensity exercise[46,47]. Thus, exercise intensity may be manipulated to produce specific changes in NK cell specific lysis.

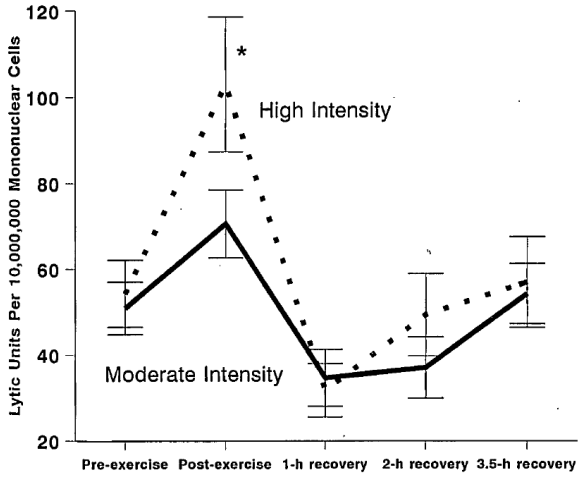

Figure 2. Lytic units per 10^6 mononuclear cells (an indicator of specific lysis) for high (dotted line; 80% VO_2max) and moderate (solid line; 70% VO_2max) intensity exercise. Relative to baseline, there is a significant increase in specific lysis immediately post-high intensity exercise, a decrease at 1-hour recovery, and a return to baseline by 3.5 hours recovery. Both exercises follow a similar trend. *Adopted from Nieman et al (1993)*[3].

1.2.6 Effects of Exercise Duration and Structure on Specific Lysis

Exercise duration is another important factor to consider when evaluating the NK cell response. An increase in either exercise intensity or duration places greater physiological stress on the body, which affects the exercise-induced release of catecholamines such as epinephrine (EPI), and myokines, including IL-6, that drive the demargination of NK cells[3,8,35,50]. Interestingly, NK cell are mobilized into circulation almost immediately after the initiation of exercise[51,52]. For example, Millard et al. observed a 6-fold increase in circulating NK cells ($CD3^-$/$CD56^+$) after only 70-seconds of stair climbing in 25- to 45-year-old males and females[51]. This is most likely because the exercise was intense enough to generate

a physiological response, as indicated by participant breathlessness, increased heart rate and catecholamine release. Interestingly, there was no change in specific lysis after the exercise. This suggests that a single, short bout of exercise may not be sufficient to enhance NK cell cytoxicity. However, Brenner et al. reported a significant increase in cytotoxicity after only 5-minutes of all-out cycling at 90% VO_2max in 20- to 40-year-old males[53]. Notably, this increase was very similar to that seen after 2-hours of cycling at 60-65% VO_2max in the same participants. Many other studies have similarly shown that short duration exercise can enhance NK cell cytotoxicity, for example two studies that each observed a significant increase in cytotoxicity after only 13 minutes of exhaustive exercise in younger (~18-27 years) and older adults (~58-77 years)[54,55]. These increases in specific lysis, which have also been found after 30- and 45-minute exercise protocols, are transient and typically return to baseline within 2-hours recovery or less[3,56]. However, longer duration exercise (2 – 3 hours) may cause prolonged immunosuppression that is evident up to 6-hours later[57,58].

Specific lysis appears sensitive to exercise duration, and may also be affected by exercise structure (i.e., continuous vs. intermittent protocols). A number of the above studies have found heightened NK cell cytotoxicity following continuous exercise protocols[3,49,54]. Intermittent exercise, which is reportedly more enjoyable for participants and produces similar cardiovascular and metabolic benefits, may also affect specific lysis[59,60]. For example, Brenner et al. had participants complete 2 x 30-minute bouts of cycling at 50% VO_2max, separated

by a 45-minute interval[56]. Total NK cytotoxicity increased after the first bout of exercise, but this response was attenuated and no longer significant after the second bout. In a different study, Nieman et al. compared the effects of continuous and intermittent exercise on specific lysis[61]. Total cycling duration was the same for both protocols (2-hours at 64% max Watts); however, in the intermittent protocol participants rested for 3-minutes every 10-minutes. There were no differences in specific lysis between the exercise protocols, and interestingly neither caused a significant increase in specific lysis post-exercise. Given that this was only one study with a small number of male participants (N=12), further research is needed to compare the effects of intermittent and continuous exercise. It would be of particular interest to examine intermittent exercise in children, given that their natural activity patterns occur in short, intermittent bursts[62].

1.2.7 NKCA per Cell and Exercise

A distinction must be made between post-exercise increases in specific lysis and changes in NKCA per cell. More specifically, post-exercise specific lysis is likely driven by changes in NK cell mobilization, while changes in NKCA per cell are indicative of the ability of exercise to promote changes in the cytotoxic function of each NK cell. Much like specific lysis, NKCA per cell can be measured through a Cr-release assay or via staining flow cytometry-based target and effector cell staining[3,45,63,64]. NKCA per cell is calculated by normalizing target cell death to the number of NK cells present in the assay. For example, one could directly provide the number of K562 lysed per NK cell. Alternatively, lytic units could be reported,

which is defined as the amount of effector cells required to lyse a set proportion of targets (here, a decrease indicates that the population of effector cells are more cytotoxic). Using these methods, a number of studies have shown that exercise can enhance NKCA per cell[3,54,65]. However, there is little consensus on the timeline for this change.

For example, Field et. al found that NKCA per cell was significantly increased immediately following a 13-minute bout of exhaustive cycling (100% VO_2max) in young males (~26 years)[54]. This was indicated by a significant decrease in lytic units immediately post-exercise, followed by a return to baseline at 1-hour recovery. A similar post-exercise increase in NKCA per cell was also reported by Targan et al[65]. However, a handful of additional studies have reported that NKCA per cell increases during 1-2 hour exercise recovery[3,66–68].

It is important to point out that NKCA per cell does not ubiquitously increase after exercise. This is an emerging field with little consensus on the timing and magnitude of changes. For example, Brenner et al. had participants complete two different cycling protocols on separate occasions, including 5-minutes at 90% VO_2max and 2-hours at 60% VO_2 max[53]. No significant differences were found in NKCA per cell at any measured timepoint (post-exercise or 3-, 24-, and 72-hour recovery). These results were confirmed by the same group after a moderate intensity, intermittent paradigm, as well as by other researchers[55,56,58]. Evidently, the literature to date is conflicting. While exercise may have the ability to enhance

NKCA per cell, the heterogeneity of study protocols limits any definitive conclusions. As such, additional research is needed to confirm these effects.

1.2.8 Effects of Exercise Intensity, Duration, and Structure on NKCA per cell

Few papers have directly compared the effects of exercise intensity, duration, or structure on NKCA per cell while holding all other factors constant. This was, however, done by Nieman et al. who evaluated NKCA per cell in young males (17-31 years) after separate 45-minute bouts of moderate (50% VO_2max) and high (80% VO_2max) intensity cycling[3]. NKCA per cell was significantly elevated only after 2-hours of high intensity exercise (**Figure 3**). This provides evidence that exercise intensity may affect the magnitude of increase in NKCA per cell after exercise. While possible, these results should be interpreted cautiously given that Targan et al. found that moderate exercise can, in fact, significantly increase NKCA per cell[65].

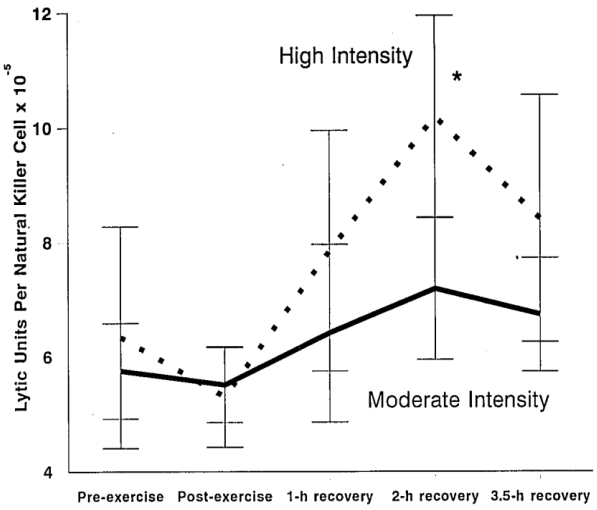

Figure 3. Participants cycled for 45 minutes at 80% of their VO_2 max (dotted line). NKCA per cell before, during and after exercise. Total NK cell activity shows a significant increase immediately post-exercise vs. NKCA per cell shows a significant increase 2-hours post-exercise. *Modified from Nieman et al (1993)*[3].

Regarding exercise duration, it appears that both short (e.g. 5-minutes, 13-minutes) and long (30-, 45-, 120-minutes) protocols can significantly affect NKCA per cell[3,54,65,66]. In an interesting comparison of these results, it appears that longer duration exercises (i.e. 30+ minutes) are associated with an increase in NKCA per cell during exercise recovery, while shorter duration exercises (<15 minutes) enhance NKCA per cell immediately post-exercise[3,54,65,66,68]. While an intriguing observation, more research is needed to compare potential effects of exercise duration on NKCA per cell.

A final modulating factor to consider is exercise structure. It is important to recognize that all of the aforementioned studies used continuous protocols. One study by Brenner et al. found that 2 x 30-minute bouts of cycling did not change NKCA per cell at any time post-exercise or in recovery. However, K562 lysis was expressed relative to either $CD56^+$ or $CD16^+$ cells alone rather than using these

markers in conjunction, which may have overestimated the number of NK cells in each calculation. A recent study found that after being challenged with cancer cells, obese mice who completed 4 weeks of HIIT had decreased lung tumor burden compared to sedentary mice[69]. Notably, mice who completed HIIT also demonstrated a significant increase in NK cell number and activation[69]. Intermittent exercise may be clinically beneficial, and as such, its effect on NKCA per cell in humans warrants further investigation.

1.2.9 Mechanisms of Exercise Induced Effects

Exercise increases the number and cytotoxicity of NK cells in circulation through a variety of mechanisms. The first of these is via catecholamines, namely EPI[3,70,71]. NK cells, specifically the $CD56^{dim}$ subset, have a high density of β-adrenergic receptors[40]. They are therefore sensitive to increased EPI concentrations that occur during exercise[3,8,34]. Catecholamines can induce the down-regulation of adhesion molecules such as CD62L and CD44[72–74]. Previous in vitro experiments have shown that in the presence of EPI, NK cells detach from cultured human endothelial cells, whereas a similar effect is not seen for T cells[75]. During exercise there is also an increase in heart rate and cardiac output, which increases shear stress in the blood vessels[76]. This, coupled with decreased adhesion properties, facilitates NK cell detachment from the vascular endothelium. Given their high affinity for EPI, $CD56^{dim}$ cell preferentially move into circulation during this time. As such, these factors contribute not only to NK cell mobilization, but likely confound post-exercise increases in specific lysis.

Given this relationship, it follows that exercise intensity can affect the extent of NK cell trafficking and changes in specific lysis. EPI concentrations increase with exercise intensity[77]. Thus, lower intensity protocols with smaller effects on plasma EPI are less likely to affect NK cell trafficking. This has been demonstrated by multiple research teams[3,35]. For example, **Figure 4** shows EPI concentrations before, during, and following 45-minutes of high (80% VO_2max) and moderate (50% VO_2max) intensity cycling[3]. EPI was significantly greater following high vs. moderate intensity exercise, which paralleled with a greater increase in NK cell counts and cytotoxicity after this protocol.

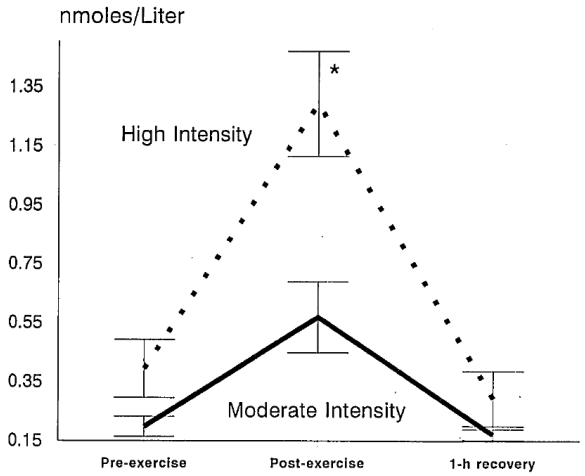

Figure 4. EPI levels (nmoles/Liter) before, during and 1-hour after 45-minutes of cycling at 50% (solid line) and 80% (dotted line) VO_2max. *Significant difference between conditions at one timepoint. The increase in EPI after high intensity exercise coincides with an increase in NK cytotoxicity (illustrated in Figure 2 above). *Taken from Nieman et al (1993)*[3].

Cytokines and myokines released during exercise may also affect NK cell mobilization and cytotoxicity. For example, muscle engagement during exercise can increase plasma IL-6 levels[8,50,78]. Previous studies have shown that NK cells mobilized in response to exercise are highly sensitive to IL-6, and thus have linked

the presence of IL-6 with NK cell redistribution[34]. One study found that IL-6 was particularly beneficial for the homing of NK cells to tumors, since IL-6 blockade diminished the infiltration of NK cells into solid tumors after wheel running in mice[34].

While the above factors help explain exercise-induced NK cell mobilization and specific lysis, they are unlikely to explain how exercise can augment NKCA per cell. One potential mechanism is through cytokines such as IL-2 and TNFα. While not unanimous, a number of studies have shown that TNFα increases with exercise and can affect NK cell cytotoxicity[79,80]. Additionally, exercise may recruit NK cells into circulation that are particularly responsive to IL-2[70]. Another factor to consider is growth hormone (GH), whose levels may increase during exercise. Animal studies have shown that GH-treated NK cells have greater function against glioma cell targets, and in humans exogenous administration of GH augments total NKCA against K562 cells[81–84].

Exercise may also preferentially recruit highly differentiated NK cells. Bigley et al. found that 30-minutes of cycling at 15% LT evoked a stepwise redeployment of NK cells, where highly differentiated NK cells ($KIR^+/NKG2A^-$) cells were mobilized more so than low differentiated NK cells ($KIR^-/NKG2A^+$)[68]. Post-exercise NK cells also had higher expression of activating receptors (e.g. NKG2C) and decreased expression of inhibitory receptors (CD158b) relative to baseline NK cell phenotype[68]. However, while interesting, findings do not explain why NKCA per cell can be enhanced against MHC-deficient targets.

To this point, all of the findings regarding the NK cell response to exercise have been derived through work in adults. There are important age-related differences in children's lymphocyte populations that limit the generalizability of these findings to the pediatric population. While limited, the available evidence in children is summarized below.

1.3 Natural Killer Cells in Children

1.3.1 Resting Natural Killer Cells

During childhood, the immune system undergoes many changes. Absolute leukocyte and lymphocyte counts decrease from infancy to 18 years[85,86]. However, age does not affect all lymphocytes subsets equally. As a proportion of total lymphocytes, B-cells decrease while NK cell proportions may actually increase over time, though this effect is not consistently reported[86–88]. Similar debate exists regarding the effect of age on absolute NK cell counts. One study showed a trend for NK ($CD3^-/CD56^+/CD16^+$) cell counts decreasing with age (birth – 18 years), while another study found significantly higher absolute number of NK ($CD3^-/CD56^+$) cells in youth (≤18 years) vs. adults (18-59 years)[89,90].

Age might also affect NK cell function. Mahapatra et al. found that after stimulation with PMA and ionomycin, CD69 was increasingly upregulated with age (20+ years > 16-20 > 11-15 > 5-10)[20]. While this would suggest greater NK cell activation in response to stimuli in adults, lysis appears similar between children and adults. A study evaluated NK cytotoxicity against K562s from the neonatal period to adulthood[91]. Cytotoxicity was the lowest at 1-3 days old and reached

adult-like levels by 5 months. Bartlett et al. similarly found no relationship between age and specific lysis in a large group of children aged 8-12 years[92]. However, specific lysis in these studies was measured using only resting NK cells. There is a non-significant trend for the frequency of $CD56^{bright}$ cells to decrease, and $CD56^{dim}$ cells to increase with age (5-60 years)[20]. Given that these two NK cell respond differentially to exercise, it is possible that children have a unique post-exercise NK cell response pattern from adults.

1.3.2 Response to Exercise

To date, only a handful of studies have examined the effects of exercise on NK cell mobilization and specific lysis in children. These studies have found that while NK cells remain the most responsive type of lymphocyte to exercise in children, this response is dampened by ~40% when compared to adults[93,94]. For example, Timmons et al. asked boys (~10 years) and men (~22 years) to cycle for 60-minutes at 70% of their VO_2 max. They found that boys experienced a significantly smaller post-exercise increase in NK cell mobilization than men (78 vs. 236% increase), and had a significantly lower concentration (cells/L) and proportion of NK cells post-exercise[93]. This may be partially explained by the fact that only men had a significant increase in their plasma IL-6 concentration post-exercise, which is important for NK cell trafficking[34,93].

Age can also influence exercise-induced immunosuppression[95]. Timmons et al. found that the while the total number of lymphocytes was significantly below baseline at 1-hour recovery in men, lymphocyte counts in boys had returned to pre-

exercise values[93]. The faster recovery in children appears to be a consistent finding across multiple physiological outcomes. For example, youth have demonstrated a faster heart rate recovery than adults[96,97]. Other physiological functions also recover faster after exercise in children, including minute ventilation[98]. Children's attenuated stress response and faster recovery times may be a protective strategy to conserve growth and development.

Interestingly, the mobilization of children's NK cells appears to be complete by 30-minutes of exercise. This has been demonstrated repeatedly by Timmons et al[6,29–31]. Each time, youth cycled for 60-minutes at 70% VO_2max and blood was taken mid-exercise (30-minutes) and immediately post exercise (60-minutes). By 30-minutes there was a significant increase in NK cell counts, and exercise beyond this did not further augment the NK cell response[29–31].

Despite children experiencing an overall dampened NK cell response to exercise, they demonstrate a similar pattern of change in their $CD56^{dim}$ and $CD56^{bright}$ subsets as adults. Immediately after 60-minutes of cycling at 70% VO_2max, 12-year old boys had a significant increase in the proportion of $CD56^{dim}$ cells[31]. $CD56^{dim}$ cells were then recruited out of circulation, leaving behind a larger portion of $CD56^{bright}$ cells, evidenced by an increase in the $CD56^{bright}$:$CD56^{dim}$ ratio at 1-hour recovery[31]. Similar responses were also observed for 12-year old girls[29]. The effects of exercise on the $CD56^{bright}$:$CD56^{dim}$ ratio in youth is demonstrated in **Figure 5**.

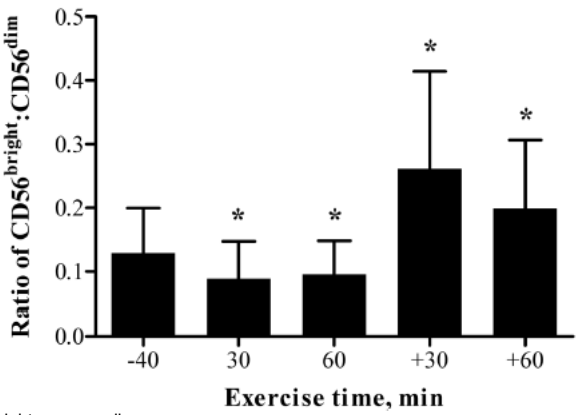

Figure 5. The ratio of $CD56^{bright}$:$CD56^{dim}$ cells before (-40), during (30) and after (60, +30, +60) 60-minutes of cycling at 70% VO_2max. Higher values indicate a shift towards a higher proportion of $CD56^{bright}$ in circulation. There was a significant decrease in this ratio at 30- and 60-minutes exercise, followed by a significant increase in this ratio at +30- and +60-minutes recovery. *Adopted from Timmons et al.*[6]

The effects of exercise on specific lysis in youth have received little attention to date. However, Boas et al. did investigate NK cell mobilization and cytoxicity in boys (8-17 years) following the Wingate Anaerobic Test (WAnT)[99]. The concentration of NK cells ($CD3^-/CD56^+/CD16^+$) was significantly increased 3-minutes after the test and returned to baseline by 1-hour recovery. As a measure of cytotoxicity, they used a whole blood Cr-release assay with K562s and reported NK cytolytic units (specific lysis corrected for the number of targets present in the whole blood assay). Much like adults, cytotoxicity was significantly increased post-exercise, followed by a decline below baseline at 1-hour recovery. This same research group reported similar results following a maximal aerobic exercise test (~6-7 minutes) in children (8-18 years)[100]. Once again, NK cell ($CD3^-/CD56^+/CD16^+$) concentrations increased significantly post-exercise and returned to baseline by 1-hour recovery. NK cytotoxicity, reported this time as specific lysis,

was also significantly increased post-exercise; however, it did not decrease in recovery. With little research and somewhat contradictory results, further investigation is warranted to determine the effects of exercise on specific lysis and NKCA per cell in youth.

1.3.3 Effects of Pubertal Status and Sex

Limited evidence suggests that NK cell mobilization is affected by chronological and biological age throughout childhood. Timmons et al. compared NK cell recruitment ($CD3^-/CD56^+/CD16^+$) in young girls (~12 years) vs. older girls (~14 years) after 60-minutes of cycling (70% VO_2max). When grouped according to age, older girls had a 90% greater increase in their NK cell counts post-exercise compared with young girls[101]. However, girls within age groups reported varying levels of pubertal development, ranging from pre- to post-pubertal. There were no significant differences when examined by pubertal status; however, researchers observed that the least developed girls consistently showed the smallest post-exercise increase in NK cells[101]. In another study, Timmons et al. had 12-year old boys grouped by pubertal status (pre-pubertal, early pubertal, mid/post-pubertal) complete the same cycling protocol and measured their $CD56^{dim}$ and $CD56^{bright}$ cells[31]. All groups had a significant increase in the proportion of $CD56^{dim}$ cells post-exercise; however, the proportion of $CD56^{dim}$ cells at post-exercise were significantly greater for mid/post-pubertal boys compared with pre- and early-pubertal boys. Interestingly, mid/post-pubertal boys also experienced a significant increase in the proportion $CD56^{bright}$ cells post-exercise, while values were

unchanged in the pre- and early-pubertal groups. At all timepoints, mid/post-pubertal boys had a significantly greater proportion of $CD56^{bright}$ cells than pre- or early pubertal boys. Taken together, these studies suggest that pubertal development enhances the response of both cytotoxic and immunoregulatory NK cells to exercise. Pubertal status may also effect NK cell cytotoxicity, however evidence in this area is severely limited[99].

Sex may be another important factor to consider when studying the NK cell response to exercise. Timmons et al. found that 14-year-old girls had a significantly greater amount of $CD56^{dim}$ and $CD56^{bright}$ cells post-exercise than age-matched boys despite no difference at baseline[30]. Interestingly, the effects of sex in youth may appear with age and/or pubertal development. For example, Timmons et al. found that after cycling for 60-minutes at 70% VO_2max, 12-year-old girls and boys had a similar NK cell response, while NK cell counts post-exercise were 82% greater in 14-year old girls vs. boys[101]. In a separate study, these researchers also showed that only 14-year old girls had a significant increase in the density of CD69 on their NK cells during 1-hour recovery from exercise[30]. CD69 is a marker of NK cell activation that correlates with cytotoxicity, but does not provide a direct indication of target cell lysis[102]. Thus, it remains unclear if post-exercise NK cell cytotoxicity differs between boys and girls.

Taken together, the evidence in children indicate an effect of pubertal status and sex on NK cell mobilization post-exercise. It is unclear how these findings

translate to changes in specific lysis or NKCA per cell after exercise. Thus, future research should aim evaluate these effects.

1.4 Summary

NK cells are a crucial component of the immune system that provide the body with a first line of defense against tumor and virally infected cells. Exercise represents a non-invasive, feasible approach to increasing NK cell counts, overall cytotoxicity, and may also enhance cytotoxicity on a per NK cell basis.[3,35] Research in adults suggest that exercise intensity, duration, and structure are important to consider when evaluating this response[3,49,51]. In children, the effects of exercise on NK cells remain poorly understood. There is evidence that exercise can increase NK cell proportions and counts in youth, and that pubertal status and sex influence this response[101]. However, the effects of exercise on specific lysis, and even more so NKCA per cell, in youth remain unclear. Given the many health benefits that have been linked to NK cell cytotoxicity, it is incredibly important to evaluate the potential for exercise to augment specific lysis and NKCA per cell in boys and girls.

Chapter 2: Research Questions, Objectives, and Hypotheses

Questions:

To fulfill the existing gaps in our knowledge of the NK cell response to exercise in children, this thesis will address the following research questions:

1. What are the effects of exercise intensity (high vs. moderate) and structure (continuous vs. intermittent) on specific lysis and NKCA per cell in children?
2. Does the NK cell cytotoxic response to exercise differ in pre-pubertal vs. late/post-pubertal and male vs. female youth?

Objectives:

The **primary objectives** of this study are to:

1. Evaluate the effects of exercise intensity and structure on a) specific lysis and b) NKCA per cell in children.
2. Compare a) specific lysis and b) NKCA per cell at rest and post-exercise in pre- vs. late/post-pubertal children.
3. Compare a) specific lysis and b) NKCA per cell at rest and post-exercise in males vs. females.

To help explain changes in specific lysis and NKCA per cell, the **secondary objective** of this study is to evaluate the effects of exercise, pubertal status and sex on NK mobilization, and specifically total NK, $CD56^{bright}$, and $CD56^{dim}/CD16^{+}$ cells as a proportion of lymphocytes.

Hypotheses:

Primary Objectives: We hypothesize that high-intensity exercise will have the greatest effect on specific lysis and NKCA per cell in children. We predict the greatest increase in specific lysis will occur immediately post-exercise and the greatest increase in NKCA per cell will occur during recovery from exercise. Moreover, we hypothesize that specific lysis and NKCA per cell will be higher in late/post-pubertal children vs. pre-pubertal children at all exercise timepoints, and will be greater in females vs. males.

Secondary Objective: Mobilization of $CD56^{dim}$ cells post-exercise will drive increases in specific lysis at this time.

Chapter 3: Methods

3.1 Participant Recruitment

3.1.1 Eligibility and Exclusion Criteria

Healthy boys and girls were recruited from the local Hamilton community to participate in a total of five study visits. To be eligible for our study, children had to be 8-11 years old or 15-18 years old (approximation for pre-pubertal late/post-pubertal status, respectively; confirmed at visit #1). All participants were required to be recreationally active, defined as participation in physical activity 2-3 times/week[103], and fall within the 5th – 85th BMI percentile. Children were excluded based on the following criteria:

1) *Pubertal development:* Tanner's scale of breast development for girls and pubic hair development for boys was used to assess puberty. This method is commonly used to self-report puberty and has been well validated against physician assessments[104]. Five stages of increasing pubertal development are illustrated by diagrams and a brief text description, and the child circles the stage that best represents themselves[105,106]. Tanner 1 indicates that a child is pre-pubertal (no breast development for girls or pubic hair for boys), while Tanner 4-5 indicate late/post-pubertal status (protruding nipple for females and pubic hair spread near the thigh for boys). Peri-pubertal children were excluded (Tanner 2-3) because of the large fluctuations in sex hormones at this time, and the difficulty controlling for a child's progression through puberty, both of which may impact our NK cell assessments[107].

2) *Activity level:* Children were excluded from our study if they were considered elite athletes. Using similar criteria to the International Olympic Committee, we defined elite athletes as those participating in organized training/sport more than 5 times/week for 3+ hours each session[108]. Previous work has demonstrated that training can affect the cytotoxicity of NK cells[37,109–111]. Thus, we wanted to ensure that exercise training did not affect our results.
3) *Weight status:* In children, normal/healthy weight status is indicated by a BMI between the 5th – 85th percentile. A BMI >85th percentile indicates overweight/obesity. Obesity is associated with systemic inflammation and an increase in circulating immune cells[112,113]. Thus, we excluded children with a BMI >85th percentile to minimize the possibility that these effects would confound our results.
4) *Health Status:* Children were excluded from our study if they had any medical condition such as asthma or allergies, or indicated a family history of this. This is because certain conditions, for example asthma and allergic rhinitis, are associated with dysregulation of the immune system and more specifically, NK cell cytotoxicity[114,115]. Thus, only healthy children were included to reduce the effect of these potential confounding variables.
5) *Medication Use:* Children regularly taking any medications were excluded from our study. This is because medications (e.g. corticosteroids) have known effects on the immune system which could have interfered with our results[116]. Females were excluded if they had taken any hormonal contraceptives in the past 6

moths or if they were using an IUD. These agents may have affected female hormones, for example estradiol, which are known to influence NK cells[117,118].

3.1.2 Recruitment Strategy

Participants were recruited via posters, online advertisements, and word of mouth. Interested participants and/or parent/guardians contacted our research team to schedule a phone call where an eligibility questionnaire was administered. During this screening, participants (if 16+ years) and/or parent/guardian (if <16 years) provided their age, approximate height and weight, participation in physical activity, and health status. Where possible, participants indicated their pubertal status. Participants who passed the phone screen were invited to our lab for Visit #1. During this visit their eligibility was confirmed using physical activity and medical questionnaires, anthropometric measurements, and Tanner stages, as described below.

3.2 Study Overview

Participants took part in a total of five study visits which all occurred in the CHEMP laboratory located at McMaster Children's Hospital. Before any questionnaires or measurements were administered, informed consent/assent was obtained. Youth who were 16+ years old provided their own consent, and in participants <16 years old, parental consent and child assent was provided. This study was approved by the Hamilton Integrated Research Ethics Board (#5618).

3.2.1 Visit #1: Anthropometric and Fitness Assessment

Upon arrival to the lab, participant eligibility was confirmed using the CHEMP lab physical activity and medical questionnaires. We then measured participant standing and sitting height using a wall-mounted stadiometer (Harpenden Stadiometer 2109, CMS Weighing Equipment Ltd, London, UK). These measurements were taken to the nearest 0.1 cm, and were repeated twice or until within 0.4 cm of each other. Sitting height was used to calculate predicted years from peak height velocity (YPHV) which represents the difference between a child's current chronological age and the age at which they are expected to achieve their maximal upwards growth[119]. Given that linear growth occurs with pubertal development, YPHV was used as a secondary indicator of puberty. Using a digital scale (Tanita BWB-800, Tokyo, Japan), weight in minimal clothing (kg) was recorded to the nearest 0.1 kg at least twice, or until values were within 0.1 kg of each other. BMI was calculated as weight (kg) ÷ height^2 (cm). BMI, height and weight percentiles were calculated based on CDC growth charts[120]. Body composition was determined via the InBody machine (InBody 570 Cerritos, California, USA), which employs bioelectrical impedance analysis to determine percentage body fat. Upon completion of anthropometric assessments, children were asked to complete a self-report Tanner stage assessment for pubertal status, as described above. Children were provided Tanner charts in a private room and asked to circle the stage that best represents their own body.

Once eligibility was confirmed, participants completed the *McMaster All-Out Progressive Continuous Cycling Test*. This aerobic fitness test was performed on a calibrated cycle ergometer (Corival and Corival Pediatric, Lode, Groningen, The Netherlands). Throughout the test participants wore a Polar heart rate monitor (Polar Electro OY, Kempele, Finland) to enable continuous measurement of their heart rate (HR). Participants also donned a mouthpiece connected to a calibrated metabolic cart (Vmax29, Sensor Medics) to allow the direct collection of their expired gases (O_2 and CO_2) throughout the test. The test began with a two-minute rest phase to determine baseline HR and gas collection parameters, followed by a warm-up cycling phase at a workload of 10 watts (W). After the warm-up, workload was then increased at a constant increment every 2 minutes, and participants received steady verbal encouragement to cycle until exhaustion (typically seen around 8-12 minutes)[76,121]. Participants reported their exertion level every 2-minutes using Borg's Rating of Perceived Exertion Scale (6-20), which has been validated in children[122]. The test was terminated when participants reached maximal effort, defined as a HR of ≥180bpm and/or a respiratory exchange quotient ≥1.0, and an inability to maintain a pedalling cadence of 60-80rpm, despite strong verbal encouragement[123]. Aerobic fitness was defined as the highest volume of oxygen uptake (VO_2max) over a 20-sec period and measured in mL/kg/min. Ventilatory threshold (VT) was determined using V-slope methodology[124]. VO_2 uptake was graphed against VCO_2 output, and the line of identity (y = x) was plotted. VT was defined as the first point of deviation above

this line, indicating excess CO_2 that is expected as the body buffers increases in lactate[125]. VT was determined independently for each participant by two CHEMP team members.

3.2.2 Visits #2-5: Experimental Sessions

Participants were invited to complete 4 experimental sessions. At each session they completed one of four exercise protocols in a randomized, counterbalanced fashion (**Figure 6**). These included 1) high intensity all-out intermittent cycling (HI-INT), which is most characteristic of children's natural activity patterns[62]; 2) high intensity continuous cycling (HI-CONT), which is the most widely used exercise stimulus in existing pediatric studies[30,31,101]; 3) moderate intensity all-out intermittent cycling (MOD-INT); and 4) moderate intensity continuous cycling (MOD-CONT). Exercise intensity was set above (high) and below (moderate) ventilatory threshold. The desired VO_2 for a participant's high intensity protocol was calculated using **Equation 1** and the desired VO_2 for moderate intensity exercise was calculated using **Equation 2**. The workload corresponding to each of these values was interpolated by plotting the VO_2 and watts from the participant's fitness test.

Equation 1: VO_2 at VT + 0.25(VO_2 at VO_2 max - VO_2 at VT)

Equation 2: VO_2 at baseline + 0.75(VO_2 at VT - VO_2 at baseline).

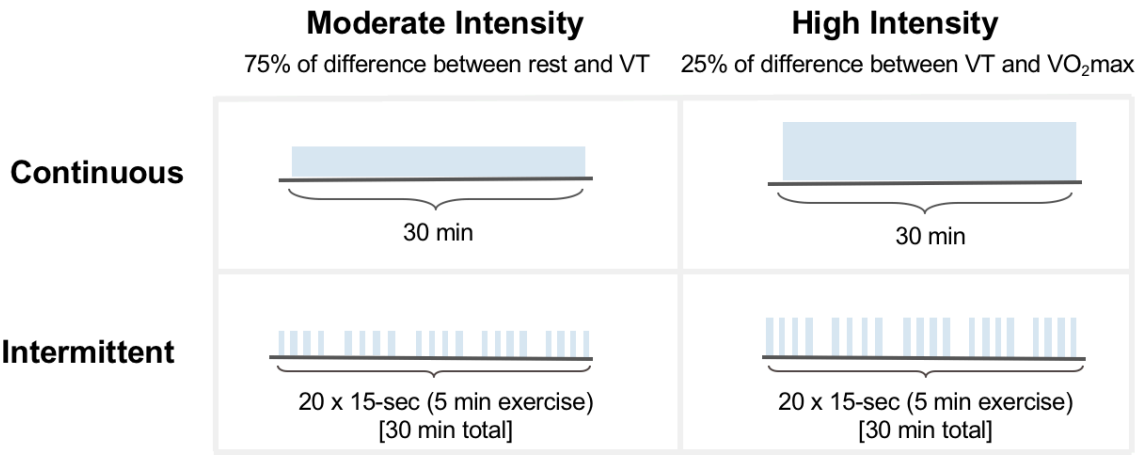

Figure 6: The four experimental protocols were performed by each child in a randomized, counterbalanced order.

Throughout each exercise protocol, HR was continuously monitored. During continuous exercise HR was recorded every 2-minutes, and during intermittent exercise we recorded peak HR roughly 10-15 seconds after each exercise bout. This was done to confirm that participants met the target heart rate of about 64-76% of max during moderate intensity protocols, and 77-93% of max during high intensity protocols[126]. During continuous and intermittent exercise, RPE was assessed every 5 minutes. Ratings between 6-11 represented minimal effort; 12-16 represented hard, but manageable effort; 17-19 indicated very hard work; and 20 was an indicator of maximal effort[122].

3.2.3 Blood Draws

Upon arrival to the laboratory, participants rested in a supine position for 10 minutes and an indwelling catheter was placed in the antecubital region of their arm for ease of blood draw. Whole blood samples were taken pre-exercise (PRE), immediately post-exercise (POST), and at 30- and 60-minutes into exercise

recovery (REC1 and REC2, respectively). At each blood draw a serum and plasma (EDTA) tube were collected and placed on ice, and the catheter was flushed with saline to prevent clotting and sample contamination from previous exercise time-points. Only plasma samples were used for the purposes of this thesis.

3.2.4 Experimental Controls

We implemented several experimental controls to minimize differences between experimental visits. All visits were scheduled in the afternoon to avoid diurnal fluctuations in hormones that could affect the exercise response[127,128]. Each participant's visits were scheduled within 2-3 hours of each other and a minimum of 4 days apart to allow for full NK cell recovery between exercises. Post-menarcheal females all scheduled their first experimental session to take place at the end of their menstrual cycle. This ensured that all post-menarcheal females progressed through similar phases of their cycle throughout the study. Prior to each visit participants were contacted and their current health status was assessed. If a participant had any sign of illness/infection in the past 2 weeks, their visit was rescheduled to 2-weeks post-illness to minimize these effects on NK cells[129,130]. Participants were asked to refrain from participating in any strenuous physical activity the day before and day of each visit to control for confounding exercise effects. Participants were additionally asked to avoid high-fat foods and caffeine in the 24-hours prior to their visit to mitigate the possibility that consumption of these would suppress NK function[131]. Pre-exercise diet was further controlled by asking participants complete a 24-hour diet log prior to each session, and to recreate their

original diet-log as closely as possible for each subsequent visit. On the day of the visit, participants were asked not to eat or drink anything other than water in the 4 hours leading up to their visit. These restrictions allowed us to control for the known effect of pre-exercise diet on the immune system[93,132,133].

3.3 Post-Visit Blood Work

3.3.1 PBMC Isolation

Our protocol for PBMC isolation was modified from the Bowdish lab at McMaster University[134]. PBMC isolation was performed in biosafety cabinet using blood samples collected from the four timepoints (PRE, POST, REC1, and REC2). 15mL of density gradient cell separation medium (Ficoll Histopaque, Sigma-Aldrich, Germany) was added to Leucosep tubes (Grenier, BioOne) labelled for each of the four timepoints and these were centrifuged for 30 seconds at 1000rcf. Blood from PRE, POST, REC1, and REC2 were added to the respective Leucosep tube, and an equal volume of MACS Buffer (phosphate-buffered saline, 0.5% bovine serum albumin, and 2mM EDTA prepared by mixing 1-part MACS BSA Stock Solution: 20-parts autoMACS Rinsing Solution, Miltenyi Biotech Inc.) was then added. Tubes were gently mixed and then centrifuged for 10 minutes at 390rcf. Upon completion of centrifugation, PBMC were extracted using a serological pipette and transferred into a pre-labelled 15mL Falcon Tube. PBMC were washed twice, first using 10mL of MACS Buffer and then using 5mL. Each time, samples were centrifuged for 10 minutes at 390rcf and the supernatant was discarded using aspirator. After the second wash, PBMC were suspended in 40mL

of PBMC-cRPMI (RPMI-1640 Medium, SigmaAldrich supplemented with 10% FBS [Fetal Bovine Serum, Canada, ThermoFisher Scientific] and 1% Abx [Antibiotic Antimycotic Solution 100x, Sigma Aldrich]) and vortexed for 5-seconds. PBMC number and viability were recorded using a Cell Countess (Fisher Scientific) with a mixture of 10uL of cell suspension and 10uL of Trypan blue (EVE Cell Counting Slides, NanoEnTek). Cells were centrifuged for 7 minutes at 250rcf and resuspended at a concentration of 1 x 10^7 PBMC/mL using 12.5% FBS solution which had been previously prepared by combining 2mL of FBS (Fetal Bovine Serum, Canada, ThermoFisher Scientific) with 14mL of 1640-RPMI (RPMI-1640 Medium, SigmaAldrich). An equal volume of 20% DMSO freezing medium was then added dropwise. This 20% DMSO freezing medium was created by combining 12.8mL of 12.5% FBS solution with 3.2mL DMSO (Dimethyl sulfoxide, SigmaAldrich). Tubes were immediately placed on ice to avoid agitation. Finally, 1mL of cell suspension was added to pre-labelled 1.5mL cryovials. These were placed in a Mr. Frosty and transferred to a -80°C freezer. Cryovials were transferred to liquid nitrogen within a week.

3.4 Analysis of Natural Killer Cell Cytotoxicity

3.4.1 K562 Culture and Preparation

K562 cells (HLA-null immortalized myelogenous leukemia cell line; ATCC, Manassas, USA) line were used as targets in our experiments. K562s are MHC-deficient, meaning that upon incubation with PBMC target cell lysis can be

promoted only by NK cells. Our protocols for K562 culture were developed based on previously published K562 culture methods[45,135–137].

In preparation for cytotoxic experiments, K562 were thawed in a 37°C water bath. K562 were then transferred into 15mL Falcon tube containing 10mL of pre-warmed K562-cRPMI (1640-RPMI supplemented with 10% FBS, 1% Abx, and 1% L-glutamine [L-glutamine solution, 200mM, Sigma Aldrich]). Cells were centrifuged for 5 minutes at 300rcf and the supernatant was aspirated and discarded. K562 were resuspended in 10mL warm K562-cRPMI and transferred to a T25 flask (Falcon Tissue Culture Flask, Sterile, Corning). This was stored in a 37°C incubator (5% CO_2) for optimal target cell growth conditions. Cells were counted every 2-3 days via the Cell Countess described above. When cells reached confluence at 0.8-1x10^6 cells/mL they were spun down (300rcf for 5 minutes) and resuspended in 10mL K562-cRPMI at a concentration of 2.5x10^5 cells/mL. Passage number was recorded each time, and log phase growth was achieved when doubling time was 24 hours.

3.4.2 PBMC Thawing and Preparation

PBMC were thawed the night before a scheduled cytotoxic experiment. Two cryovials of PBMC from each time point were thawed to ensure enough viable cells were available to perform our experiments. Each cryovial was thawed in a 37°C water bath and contents were transferred to a 50mL Falcon tube containing 16mL of pre-warmed PBMC-cRPMI. Where two cryovials were not available, one was thawed and transferred into a 15mL Falcon tube with 8mL of pre-warmed PBMC-

cRPMI. This was done in a dropwise fashion to avoid cell agitation. PBMC were then centrifuged at 250rcf for 7 minutes. The supernatant was discarded and cells were resuspend in 10mL of warm PBMC-cRPMI. This volume was transferred to a labelled T25 and stored in a 37°C incubator (5% CO_2) for 12-18 hours.

3.4.3 Cytotoxic Assay

The cytotoxic assay involved the incubation of PBMC with K562 target cells. K562 for all experiments came from the same parent line, were in log phase growth, and had a viability of >85% on the day of the experiment. Target cell death was compared across two conditions: 1) K562 cells incubated in media alone to assess for spontaneous target cell death (STD) and 2) K562 cells incubated with sample PBMC (SMP). For SMP tubes, PBMC from each exercise timepoint were incubated with K562 according to 3 different Target:Effector (T:E) ratios, including: 1:12.5, 1:25, and 1:50. All ratios were run in duplicate. A detailed description of control and sample tubes is provided in **Table 1.** All incubations took place in 5mL Falcon flow tubes (Round-Bottom Polystyrene Tubes, Sterile, VWR), which were pre-labelled before each experiment.

At the start of the experiment, the T25 containing K562 were removed from the incubator and counted. 20uL of trypan blue was mixed with 20uL of cell suspension, and 10uL of this solution was counted in duplicate using the Cell Countess. The volume needed for $6.0x10^5$ cells was aliquoted from the T25 into a labelled 15mL falcon tube. This tube was centrifuged for 5 minutes at 300rcf, the supernatant was discarded, and cells were resuspended at a concentration of

$1x10^5$ cells/mL. 100uL of this suspension was added into the unstained control tube and the fluorescence minus one (FMO) for DiO. DiO (Vybrant DiO Cell-Labelling Solution, ThermoFisher Scientific) was added to the remaining solution of K562 to label live target cells. This was vortexed and incubated for 10 minutes at 37°C as per product protocol. This and all subsequent steps were done in dim lighting to protect the light sensitive stains. After incubation, K562 were washed to ensure that no unbound DiO was present in the rest of the assay. Three washes were carried out using K562-cRPMI and centrifugation for 5 minutes at 300rcf. The final pellet was resuspend in K562-cRPMI to achieve a concentration of $1x10^5$ cells/mL. 100uL of this solution was added into all remaining flow tubes.

PBMC from each timepoint were counted in duplicate using the Cell Countess. Where possible $2.0x10^6$ PBMC from each timepoint were kept and spun down at 250rcf for 7 minutes. This pellet was then resuspended in 400uL of PBMC-cRPMI to achieve a concentration of $5x10^6$ cells/mL. 2 x 100uL aliquots of this solution were added into the respective flow tubes for the 1:50 ratio. The stock suspension was then reconstituted to a concentration of $2.5x10^6$ cells/mL and 2 x 100uL aliquots of this were added into the respective flow tubes for the 1:25 ratio. Finally, the stock suspension was diluted to a concentration of $1.25x10^6$ cells/mL. 2 x 100uL aliquots of this were added into the respective flow tubes for the 1:12.5 ratio. 100uL of leftover stock suspension were added to the unstained tube, and the FMOs for DiO, FVD, and CD16. The remaining stock suspension was discarded.

All tubes were supplemented with an extra 200uL of media. Tubes with only K562 received 200uL of K562-cRPMI, and tubes with both K562 and PBMC received 100uL each of K562-cRPMI and PBMC-cRPMI media. Finally, all flow tubes were centrifuged for 3 minutes at 300rcf and incubated as a pellet for 4 hours at 37°C.

3.4.4 Cell Staining

After incubation, flow tubes were put on ice and stained to quantify NK cell number and K562 cell death. This process was done in dim lighting and the centrifuge was set to 4°C. All flow tubes were centrifuged for 5 minutes at 300rcf. The supernatant was removed and stored in a -80°C freezer for future analysis. Cells were resuspended in 500uL of MACS Buffer. Fluorophore-conjugated antibodies were used to identify NK cells (Militenyi Biotec). Ebioscience Fixable Viability Dye eFlour 780 (FVD) was used to label dead cells (ThermoFisher Scientific). 1uL of each antibody and 0.5uL of FVD were used to stain flow tubes according to the panel in **Table 2**. Tubes were vortexed and incubated in the dark for 30 minutes at 4°C. Cells were then washed by adding 2mL of MACS Buffer to each flow tube and centrifuging for 10 minutes at 300rcf. The supernatant was discarded and cells were fixed. This was done by adding 100uL of fixation buffer (Ic Fixation Buffer, ThermoFisher Scientific) to each flow tube and vortexing to mix. Tubes were incubated for 30 minutes at room temperature, and fixation buffer was washed off. 2mL of MACS Buffer was added to each flow tube and then centrifuged for 5 minutes at 300rcf. The supernatant was discarded, and cells were

resuspended in a final volume of 200uL of MACS Buffer. Cells were stored overnight at 4°C and analyzed the next day.

3.5 Flow Cytometric Analysis

NK cell number and target cell death were quantified on a 7-channel Militenyi MACSQuant Analyzer. On each day of analysis, the flow cytometer was cleaned with bleach, flushed with running buffer, and calibrated using MACSQuant calibration beads (Militenyi Biotec Inc.). Separate compensations were created for all of the fluorochrome combinations present in our sample. This was done using MACS Compensation Bead Kits (Militenyi Biotec Inc.). All flow tubes were run using the appropriate compensation, which allowed us to control for the spectral overlap between channels and fluorochromes.

3.5.1 Flow Minus One Controls

Fluorescence Minus One (FMO) controls were used for DiO, FVD, and CD16. By definition, each FMO tube contained all stains except one (e.g., the FMO for DiO had all stains except DiO). These tubes were subject to the exact same incubation and staining procedures as all other samples on the same day. FMOs were used to control for interference between stains and delineate between positive and negative populations in later analysis.

3.5.2 Gating for Natural Killer Cells

All gating was accomplished via FlowJo software. The first step in the analysis of NK cells was to exclude CD14+ cells, a common marker of monocytes. The next step was to gate lymphocytes within the CD14- population based on size

(forward scatter) and granularity (side scatter). Within this population, NK cells were identified as $CD3^-/CD56^+$. Within the NK cell population, $CD56^{dim}$ and $CD56^{bright}$ NK cells classified based on the intensity of CD56 expression. Finally, $CD56^{dim}$ cells were further classified as $CD16^+$ using the threshold for positive events identified with the FMO. Our complete gating strategy for NK cells is outlined in **Figure 7**.

3.5.3 Gating for K562 Cells

The first step in gating for K562 cells was to identify those that were originally live. This was done using the FMO for DiO, which indicated the threshold for positive events. Next, we characterized lysed K562 cells within this population. The FMO for FVD was used to delineate positive and negative events. The FMOs were consistently set so that <3% of events were positive, and these gates were routinely applied to each sample and then confirmed visually. This gating strategy is outlined in **Figure 8**.

3.6 Calculations for NK Specific Lysis and NKCA per Cell

Specific lysis was calculated according to **Equation 3**. Specific lysis represents the percent of target cell death relative to total target cells in each tube that can be attributed to NK cells. NKCA per cell was calculated according to **Equation 4**. NKCA per cell is reported as the number of K562 cells lysed per $CD56^{dim}/CD16^+$ NK cell. Spontaneous target cell death was accounted for in our equations. Calculations were made individually for each sample tube and the average of duplicates is reported.

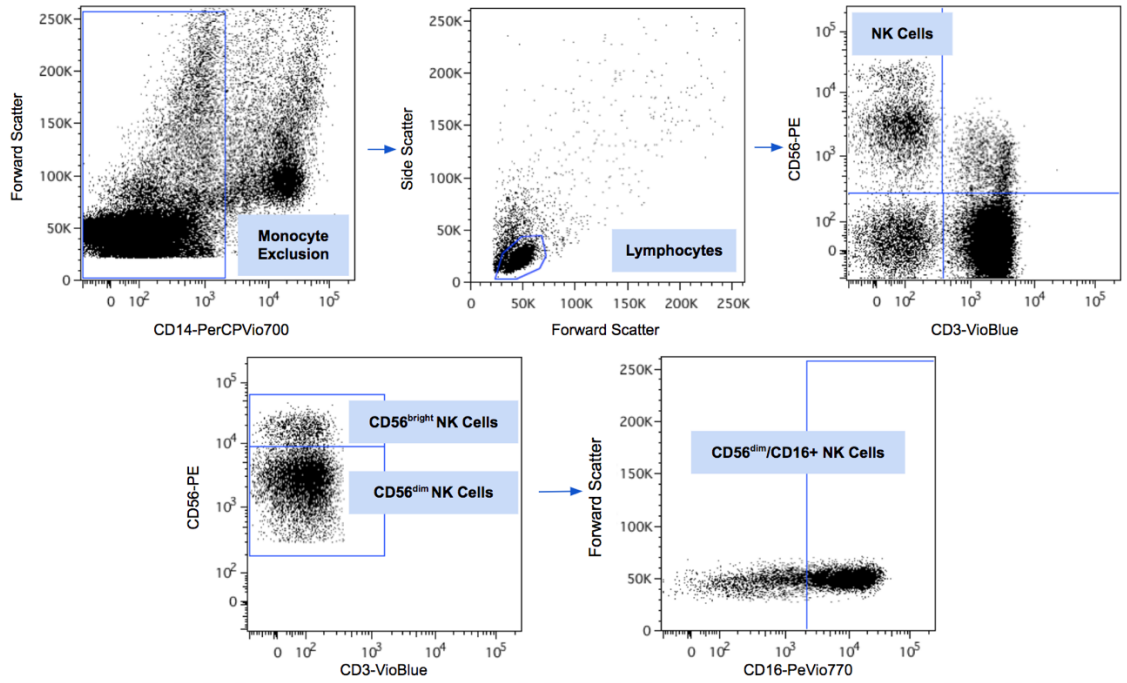

Figure 7: NK Cell Gating Strategy

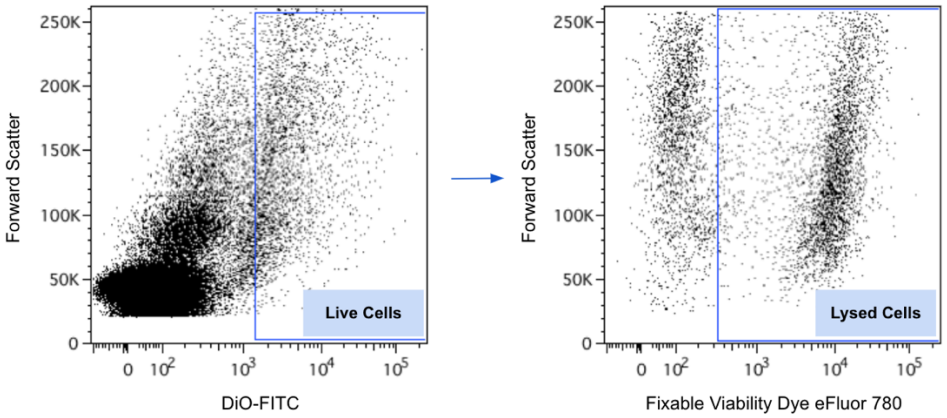

Figure 8: K562 Cell Gating Strategy

Equation 3:

Specific Lysis = % Lysis in SMP tube - % Death in STD_{avg} tube

Equation 4:

K562 Lysed per $CD56^{dim}/CD16^{+}$ cell = (# K562 lysed in SMP tube – *# K562 lysed spontaneously*) ÷ # of $CD56^{dim}/CD16^{+}$ cells in SMP tube

where *# K562 lysed spontaneously* = (# K562 in SMP tube) x (% Death in STD_{avg} tube)

Table 1: Cytotoxic Assay: Tube Breakdown

TYPE OF SAMPLE	TUBE	# of PBMC	# of K562
Staining Controls	Unstained	1.25 x 10^5	1 x 10^4
	FMO for DiO	1.25 x 10^5	1 x 10^4
	FMO for FVD	1.25 x 10^5	1 x 10^4
	FMO for CD16	1.25 x 10^5	1 x 10^4
Spontaneous Death	STDa	N/A	1 x 10^4
	STDb	N/A	1 x 10^4
Sample Tubes	1:50a **PRE**	5 x 10^5	1 x 10^4
	1:50b **PRE**	5 x 10^5	1 x 10^4
	1:25a **PRE**	2.5 x 10^5	1 x 10^4
	1:25b **PRE**	2.5 x 10^5	1 x 10^4
	1:12.5a **PRE**	1.25 x 10^5	1 x 10^4
	1:12.5b **PRE**	1.25 x 10^5	1 x 10^4
***Note:** Sample tubes for POST, REC1, and REC2 were the exact same as for PRE			

Table 2: Staining Panel

TIME ADDED	UNSTAINED	K562 PANEL	NK PANEL
Before Incubation	None	DiO-FITC	CD14-PerCPVio700
After Incubation		Fixable Viability Dye e-Fluor 780	CD56-PE
			CD3-VB
			CD16-PEVio770

3.7 Statistical Analyses

General participant and fitness characteristics were compared between pre- and post-pubertal children using independent samples t-tests. Comparisons of measures of exercise intensity by exercise protocol were examined using one-way repeated measures ANOVA, with pairwise comparisons when appropriate. The first objective was to characterize the effects of exercise intensity and structure on a) specific lysis and b) NKCA per cell in children. For each of these measures of cytotoxicity, a separate two-way repeated measures ANOVA was performed (factors [levels]: exercise [HI-INT, HI-CONT, MOD-INT, MOD-CONT] × timepoint [PRE, POST, REC1, REC2]). The second and third objectives were to compare a) specific lysis and b) NKCA per cell at rest and post-exercise in pre- vs. late/post-pubertal children and in males vs. females. To evaluate the effects of puberty a two-way ANOVA was performed using the data from the high-intensity intermittent exercise visit (factors [levels]: timepoint [PRE, POST, REC1, REC2] × pubertal group [pre-pubertal; post-pubertal]). To evaluate the effects of sex a two-way ANOVA was performed using the data from the high-intensity intermittent exercise visit (factors [levels]: timepoint [PRE, POST, REC1, REC2] × pubertal group [pre-pubertal; post-pubertal]). These were done separately for specific lysis and NKCA per cell. The decision to examine puberty and sex separately was based on the small sample size in this study; including both puberty and sex in a 3-way ANOVA was not feasible or statistically sound. The secondary objective of this thesis was to evaluate the effects of exercise intensity and structure, pubertal status, and sex

on the total NK cells, $CD56^{bright}$ cells, and $CD56^{dim}/CD16^{+}$ cells as a proportion of lymphocytes. The statistical analyses for each of these were identical to those described above (two-way ANOVAs), and were done separately for total NK cells, $CD56^{bright}$ cells, and $CD56^{dim}/CD16^{+}$ cells. Where appropriate a Tukey's Honestly Significant Differences post-hoc was used to identify differences between levels. Effect sizes for main and interaction effects are reported as partial eta squared (η^2_p) and Cohen's values are used as reference for small (0.01), medium (0.06), and large (0.14) effect sizes. Where possible, graphs include individual participant data to illustrate the range of responses seen in our sample. STATISTICA for Windows 7 software was used for all analyses related to the primary and secondary objective. Independent samples t-tests and one-way repeated measures ANOVA were performed in SPSS version 27. All data are presented as mean ± standard deviation unless otherwise stated.

Chapter 4: Results

4.1 Participant Characteristics

A total of 59 individuals contacted our research team seeking additional information about our study. Of these, 44 were screened for eligibility, and 26 were invited to our lab for Visit #1. After screening, 21 participants met all of our eligibility criteria and enrolled in the study. To date, 11 participants (55% male) have completed the study; 8 participants were placed on hold due to the COVID-19 research restrictions. Among the 11 participants who completed the study, 5 identified as pre-pubertal and 6 were late- or post-pubertal according to Tanner stages. All 11 participants completed all 5 study visits. Participant flow is presented in **Figure 9**.

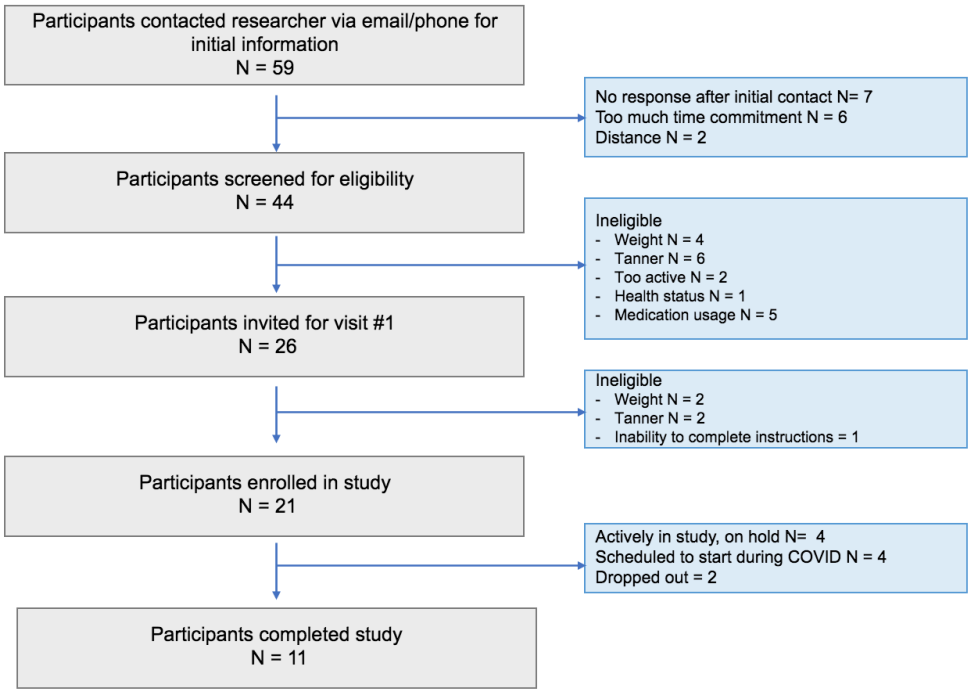

Figure 9: Participant eligibility flow chart, adopted from Inna Ushcatz

4.1.1 Descriptive Participant Characteristics

Our pre-pubertal participants were on average seven years younger than our post-pubertal participants. All participants were within normal weight, height, and BMI percentiles as well as body fatness for their age and sex[138]. Independent samples t-tests revealed that pre-pubertal children had a significantly lower BMI percentile compared with post-pubertal children ($p = 0.034$). All pre-pubertal children reported being Tanner stage 1, which was confirmed by the negative YPHV values. Two post-pubertal children self-reported Tanner 4 and six post-pubertal children self-reported Tanner 5, which was also confirmed positive YPHV values. Descriptive participants characteristics are provided in **Table 3**.

Table 3: Descriptive Participant Characteristics

	ALL (N=11; 6M, 5F)	PREPUBERTAL (N=5; 3M, 2F)	POSTPUBERTAL (N=6; 3M, 3F)	t-test statistic
Age (years)	13.9 ± 3.9 (8.6 – 18.3)	10.0 ± 0.9 (8.6 - 11)	17.2 ± 1.0 (15.6 – 18.3)	12.2*
Height Percentile	45.4 ± 22.6 (1.9 – 77.0)	53.5 ± 13.8 (39.0 – 69.2)	38.7 ± 27.4 (1.9 – 77.0)	1.1
Weight Percentile	54.6 ± 20.2 (23.0 – 89.4)	47.4 ± 22.5 (23.0 – 68.4)	60.6 ± 17.8 (39.4 – 89.4)	1.1
BMI Percentile	57.4 ± 23.0 (17.0 – 85.0)	42.0 ± 25.0 (17.0 – 72.0)	70.2 ± 11.3 (52.0 – 85.0)	2.5*
Percent Body Fat	18.9 ± 7.1 (9.2 – 32.6)	15.6 ± 3.4 (11.7 – 19.3)	21.7 ± 8.5 (9.2 – 32.6)	1.5
YPHV	0.3 ± 3.2 (-3.2 – 3.9)	-2.9 ± 0.2 [(-3.2) – (-2.8)]	3.0 ± 0.9 (1.7 – 3.9)	14.9*

Note: All data are presented as mean ± SD and (range). *indicates a significant difference between pre- and post-pubertal groups, $p < 0.05$. Height, weight, and BMI percentile calculated using the CDC percentile calculators for children.

4.1.2 Participant Fitness Characteristics

The fitness characteristics of our participants are provided in **Table 4**. Independent samples t-tests indicated that post-pubertal children had a significantly higher absolute VO_2max, absolute peak power, and VT as expected ($p = 0.006$; $p = 0.020$; $p = 0.008$). However, pre-pubertal children had a significantly greater relative VO_2max ($p = 0.008$). There was no difference between pubertal groups for VT as a % of VO_2max.

Table 4: Participant Fitness Characteristics

	ALL (N=11; 6M, 5F)	PREPUBERTAL (N=5; 3M, 2F)	POSTPUBERTAL (N=6; 3M, 3F)	t-test statistic
VO_2 max absolute (L/min)	2.1 ± 0.7 (1.3 – 3.6)	1.5 ± 0.2 (1.3 – 1.8)	2.5 ± 0.6 (2.0 – 3.6)	3.6*
VO_2 max relative (mL/kg*min)	44.1 ± 7.3 (35.3 – 55.0)	49.8 ± 6.2 (39.5 – 55.0)	39.4 ± 4.1 (35.3 – 45.8)	3.4*
VO_2 at VT (L/min)	1.2 ± 0.3 (0.8 – 1.8)	1.0 ± 0.2 (0.8 – 1.3)	1.5 ± 0.2 (1.2 – 1.8)	3.4*
VT as a % of VO_2 max	61.0 ± 7.1 (50.7 – 71.8)	63.9 ± 7.0 (57.9 – 71.8)	58.6 ± 6.7 (50.7 – 70.1)	1.3
Absolute peak power (watts/kg)	146 ± 73 (75 – 285)	94 ± 16 (75 – 113)	189 ± 75 (121 – 285)	2.8*
Relative peak power (watts/kg)	2.9 ± 0.6 (2.1 – 3.6)	3.0 ± 0.3 (2.6 – 3.2)	2.9 ± 0.7 (2.1 – 3.6)	0.7

Note: All data are presented as mean ± SD and (range). All data are presented as mean ± SD and (range). *indicates a significant difference between pre- and post-pubertal groups, $p < 0.05$

4.1.3 Exercise Intensity During Experimental Sessions

Measures of exercise intensity, including power output, % max HR and RPE, were averaged and provided in **Table 5**. Most physiological and self-reported measures suggest that pre- and post-pubertal children worked at similar intensities. Absolute power output was greater for post-pubertal children during high intensity protocols ($p = 0.015$), which is to be expected given their larger body size. We also found that during HI-INT exercise, average RPE values reported by post-pubertal children were significantly greater than those reported by pre-pubertal children ($p = 0.010$). Relative power output was lower in post-pubertal children during moderate intensity protocols compared with pre-pubertal children ($p = 0.048$). We also found that during HI-INT exercise, average RPE values reported by post-pubertal children were significantly greater than those reported by pre-pubertal children ($p = 0.010$). One-way ANOVAs indicated that % Max HR was significantly lower during MOD-CONT than all other exercises, and that RPE was significantly lower during MOD-CONT than HI-CONT and MOD-INT ($p<0.05$ for all).

Table 5: Measurements of Exercise Intensity

	ALL (N=11, 6M, 5F)	PREPUBERTAL (N=5, 3M, 2F)	POSTPUBERTAL (N=6, 3M, 3F)	t-test statistic
HI-INT				
% Max HR	88.6 ± 5.2 (79.2 – 96.4)	86.8 ± 4.5 (79.2 – 91.0)	90.0 ± 5.6 (79.9 – 96.4)	1.0
RPE	14.1 ± 2.8 (9.0 – 17.6)	12.1 ± 2.4 (9.0 – 14.5)	16.1 ± 1.2 (14.7 – 17.6)	3.4*
HI-CONT				
% Max HR	83.5 ± 7.3 (70.4 – 95.1)	82.4 ± 6.2 (74.6 – 89.2)	84.4 ± 8.6 (70.4 ± 95.1)	0.4
RPE	14.2 ± 2.4 (9.5 – 17.2)	14.2 ± 1.7 (12.6 – 17.0)	14.1 ± 3.2 (9.5 – 17.2)	0.06
MOD-INT				
% Max HR	87.5 ± 5.9 (75.2 – 96.8)	84.6 ± 6.0 (75.2 – 91.0)	90.0 ± 5.1 (81.9 – 96.8)	1.6
RPE	13.8 ± 2.8 (9.8 – 20.0)	14.3 ± 3.5 (10.7 – 20.0)	13.3 ± 2.4 (9.8 – 16.0)	0.5
MOD-CONT				
% Max HR	68.8 ± 8.4[#] (55.5 – 86.0)	70.8 ± 4.5 (64.1 – 76.4)	67.1 ± 10.9 (55.5 – 86.0)	0.7
RPE	10.5 ± 2.3^ (6.0 – 13.4)	11.3 ± 2.0 (8.4 – 13.4)	9.6 ± 2.4 (6.0 – 12.0)	1.2
HI power output				
Absolute power (W)	91.7 ± 35.4 (51.0 – 155.0)	65.6 ± 13.3 (51.0 – 81.0)	113.5 ± 33.4 (80.0 – 155.0)	3.2*
Relative power (W/kg)	1.9 ± 0.3 (1.4 – 2.3)	2.1 ± 0.2 (1.8 – 2.3)	1.8 ± 0.3 (1.4 – 2.3)	2.0
MOD power output				
Absolute power (W)	52.8 ± 22.6 (28.0 – 111.0)	39.0 ± 8.3 (28.0 – 48.0)	64.3 ± 24.9 (44.0 – 111.0)	2.2
Relative power (W/kg)	1.1 ± 0.2 (0.8 – 1.4)	1.2 ± 0.1 (1.1 – 1.4)	1.0 ± 0.2 (0.8 – 1.3)	2.3*

Note: All data are presented as mean ± SD and (range). *significant difference between pre- and post-pubertal groups, $p<0.05$. [#]HI-INT, HI-CONT, and MOD-INT > MOD-CONT, $p<0.001$. ^HI-CONT and MOD-INT > MOD-CONT, $p<0.05$.

4.2 Descriptive Note on Analysis of Primary and Secondary Outcomes

1) T:E ratios: While 3 T:E ratios were used as a quality control measure for the cytotoxic assays, results below are presented using data obtained at the 1:50 ratio. Specific lysis for each timepoint at the 1:50 ratio was consistently higher than the corresponding timepoint at the 1:25 and 1:12.5 ratio ($F(2, 86) = 223.97$, $p < 0.001$; $\eta^2_p = 0.839$). Therefore the 1:50 ratio provided the most robust response for detecting effects of exercise type, time, pubertal status, and sex on our outcomes of interest. A detailed description of the T:E comparisons, as well as values for specific lysis and NKCA per cell at each T:E ratio are included in Appendix B.

2) Pubertal status and sex comparisons: Data from HI-INT was used for all analyses on the effect of pubertal status and sex. This decision was made a priori. We expected that high-intensity exercise would elicit the greatest physiological response since NK cell mobilization is highest with this stimulus[3,49]. As such, we expected that high-intensity exercise would provide the most robust response for the detection of puberty- and sex-related effects. The intermittent protocol was selected because it is most representative of children's natural activity patterns[62].

3) ANOVA tables: ANOVA results are summarized in the text below. Complete ANOVA tables are provided in Appendix C.

4.3 NK Specific Lysis

4.3.1 Effects of Exercise on NK Specific Lysis

There was no statistically significant interaction between exercise type and timepoint on specific lysis ($F(9, 90) = 1.45$, $p = 0.180$; $\eta^2_p = 0.126$; Figure 10A). There was no main effect of exercise type on specific lysis ($F(3, 30) = 1.52$, $p = 0.229$; $\eta^2_p = 0.132$). There was a main effect of timepoint on specific lysis ($F(3, 30) = 12.80$, $p<0.001$; $\eta^2_p = 0.561$; Figure 10B). Tukey's post-hoc analysis revealed that specific lysis at POST (30.6 ± 11.0%) was significantly higher than PRE (25.1 ± 6.7%; $p<0.05$), REC1 (22.8 ± 8.2%; $p<0.001$), and REC2 (20.1 ± 6.5%; $p< 0.001$). Specific lysis at REC2 (20.1 ± 6.5%) was significantly lower than at PRE (25.1 ± 6.7%; $p<0.05$). For clarity, participant data are averaged and presented in the figures below. Individual participant specific lysis data by exercise type are presented in Appendix A1 and A3.

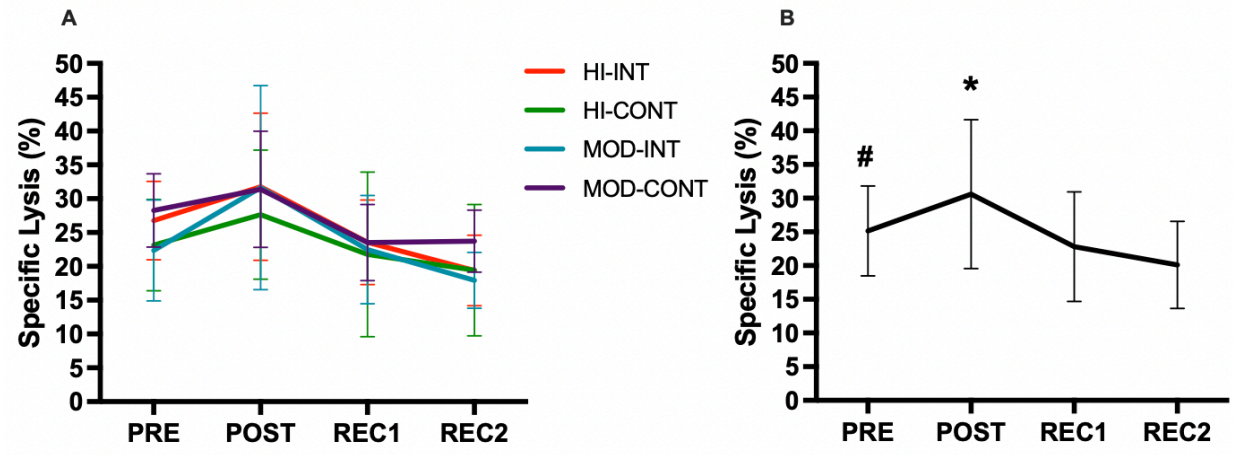

Figure 10. Effects of exercise on specific lysis. A: There were no differences in specific lysis between HI-INT, HI-CONT, MOD-INT, MOD-CONT exercises. **B**: Main effect of timepoint on specific lysis, # = PRE > REC2, * = POST > PRE, REC1 and REC2; $p<0.05$. All data are presented as mean ± SD.

4.3.2 Effects of Exercise on NK Specific Lysis by Pubertal Status

There was a main effect of timepoint during HI-INT on specific lysis ($F(3, 27) = 11.51$, $p<0.001$; $\eta^2_p = 0.561$; Figure 11A). Tukey's post-hoc analysis revealed that specific lysis at PRE (26.8 ± 5.8%) was significantly greater than at REC2 (19.4 ± 5.2%; $p<0.05$). Moreover, specific lysis at POST (31.8 ± 10.9%) was significantly greater than at REC1 (23.6 ± 6.3%; $p<0.005$) and REC2 (19.4 ± 5.2%; $p< 0.001$). There was no statistically significant interaction between pubertal status and timepoint on specific lysis ($F(3, 27) = 1.85$, $p = 0.162$; $\eta^2_p = 0.171$; Figure 11B). Overall, average specific lysis did not differ between pre-pubertal (23.5 ± 8.5%) and post-pubertal children (27.0 ± 8.3%; $F(1, 9) =1.00$, $p = 0.344$; $\eta^2_p = 0.100$).

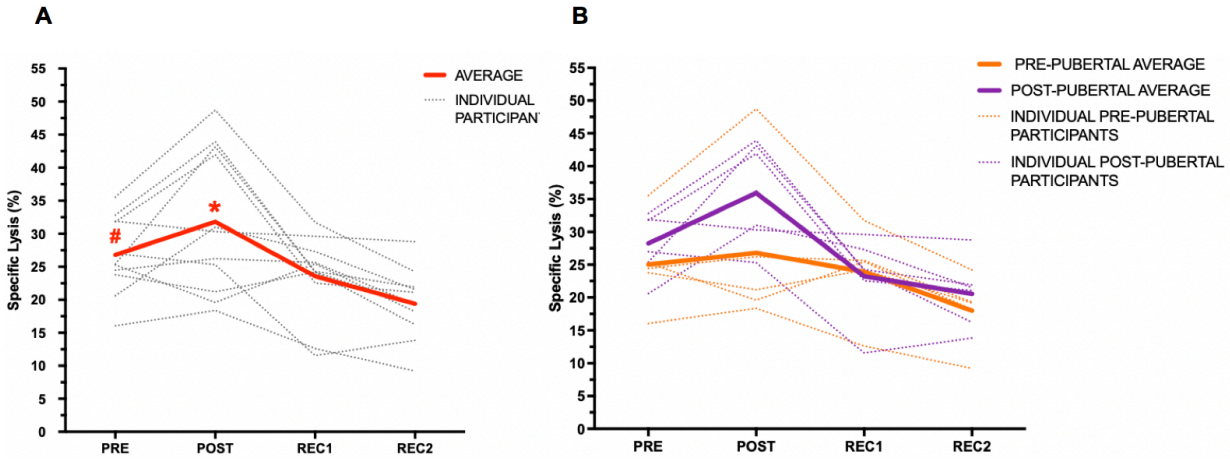

Figure 11. Effects of exercise on specific lysis by pubertal status. A: Main effect of timepoint on specific lysis, # = PRE > REC2, * = POST > REC1 and REC2, $p<0.05$. The solid red line represents mean specific lysis averaged by time point across all participants during HI-INT, and the grey dotted lines represent individual participant data during HI-INT. **B.** There were no differences in specific lysis between pre- and post-pubertal children during HI-INT. The solid lines represent mean values for pre-pubertal (orange) and post-pubertal (purple) children. Individual data are presented in dotted lines for pre-pubertal (orange dotted line) and post-pubertal (purple dotted line) children.

4.3.3 Effects of Exercise on NK Specific Lysis by Sex

There was no statistically significant interaction between sex and timepoint on specific lysis ($F(3, 27) = 0.01$, $p = 0.998$; $\eta^2_p = 0.001$; Figure 12). There were no differences in average specific lysis between male (25.8 ± 8.2%) and female participants (24.9 ± 9.0%; $F(1, 9) = 0.07$, $p = 0.801$; $\eta^2_p = 0.007$).

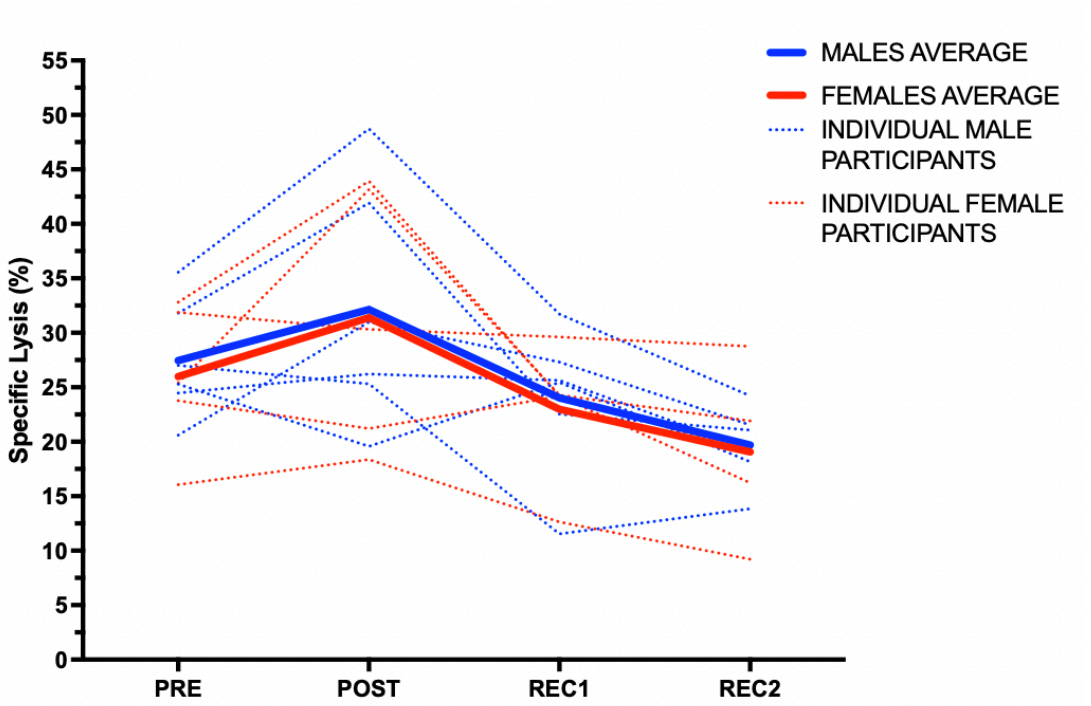

Figure 12. Effects of exercise on specific lysis by sex. There were no differences in specific lysis during HI-INT in male vs. female children. The solid lines represent mean values for males (blue) and females (red). Individual data are presented in dotted lines for males (blue dotted line) and females (red dotted line).

4.4 NKCA per Cell

4.4.1 Effects of Exercise on NKCA per Cell

There was no statistically significant interaction between exercise type and timepoint on NKCA per cell ($F(9, 90) = 1.95$; $p = 0.055$; $\eta^2_p = 0.163$; Figure 13A). There was no main effect of exercise type on NKCA per cell ($F(3, 30) = 0.20$; $p = 0.895$; $\eta^2_p = 0.020$). There was a main effect of timepoint on NKCA per cell ($F(3, 30) = 8.33$; $p<0.001$; $\eta^2_p = 0.454$; Figure 13B). Tukey's post-hoc analysis revealed that NKCA per cell at REC1 (1.3 ± 1.6) was significantly greater than at POST (0.36 ± 0.39; $p<0.01$). Additionally, NKCA per cell at REC2 (1.3 ± 1.3) was significantly greater than at PRE (0.69 ± 0.76; $p <0.05$) and POST (0.4 ± 0.4; $p<0.01$). For clarity, participant data are averaged and presented in the figures below. Individual participant NKCA per cell data by exercise type are presented in Appendix A2 and A4.

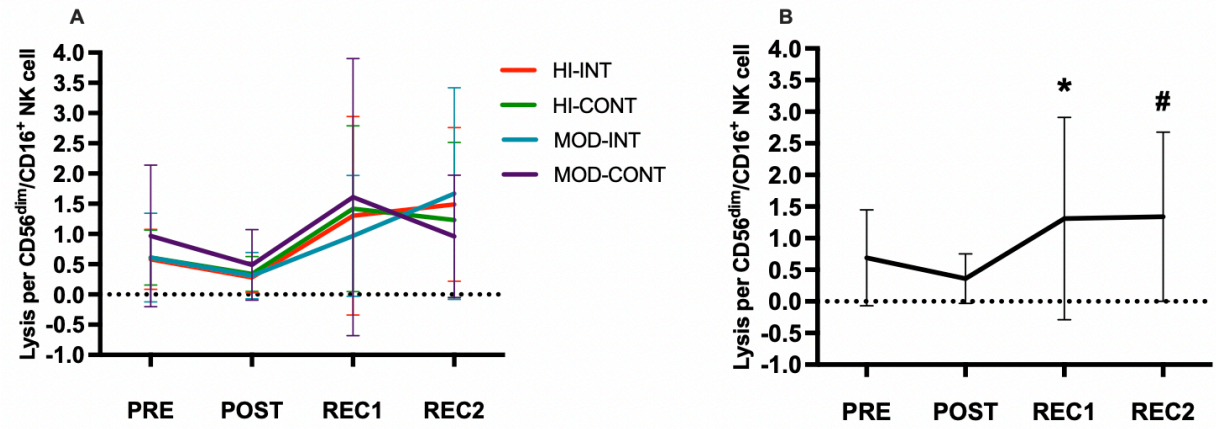

Figure 13. Effects of exercise on NKCA per cell. A: There were no differences in NKCA per cell, defined as the number of K562 lysed per $CD56^{dim}/CD16^{+}$ NK cell, between HI-INT, HI-CONT, MOD-INT, MOD-CONT exercises. **B**: Main effect of timepoint on NKCA per cell, * = REC1 > POST, # = REC2 > PRE and POST, $p<0.05$. All data are presented as mean ± SD.

4.4.2 Effects of Exercise on NKCA per Cell by Pubertal Status

There was a main effect of timepoint on NKCA per cell ($F(3, 27) = 6.57$, $p < 0.01$; $\eta^2_p = 0.422$; Figure 14A). Tukey's post-hoc analysis revealed that NKCA per cell increased significantly from PRE (0.58 ± 0.50) to REC2 (1.5 ± 1.3; $p<0.05$) as well as from POST (0.28 ± 0.25) to REC1 (1.3 ± 1.6; $p<0.05$) and REC2 (1.5 ± 1.3; $p<0.005$). There was no statistically significant interaction between pubertal status and timepoint on NKCA per cell ($F(3, 27) = 0.50$, $p = 0.688$; $\eta^2_p = 0.052$; Figure 14B). There were no differences in average NKCA per cell among pre-pubertal (1.1 ± 1.5) vs. post-pubertal children (0.78 ± 0.78; $F(1, 9) = 0.25$, $p = 0.627$; $\eta^2_p = 0.027$).

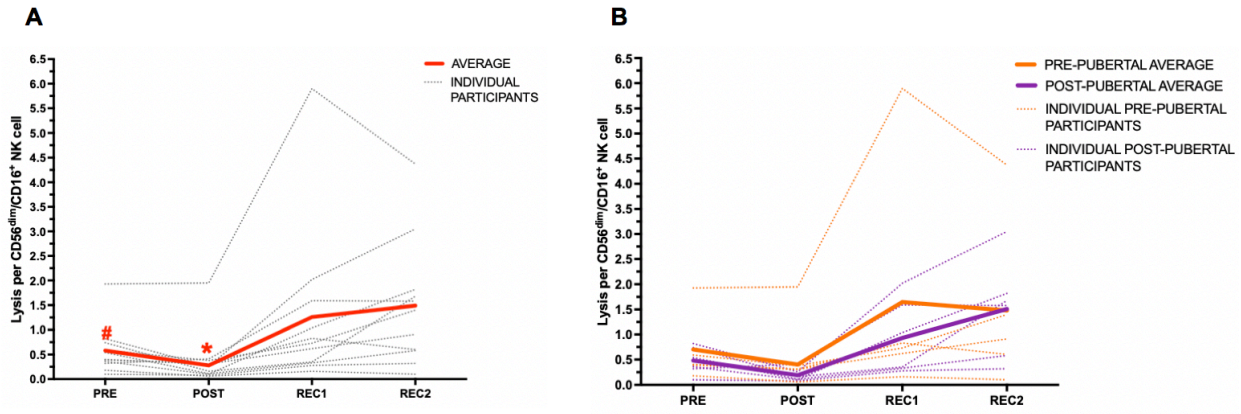

Figure 14. Effects of Exercise on NKCA per Cell by Pubertal Status. A: Main effect of timepoint on NKCA per cell, # = PRE < REC2, * = POST < REC1 and REC2, $p<0.05$. The solid red line represents mean specific lysis averaged by time point across all participants during HI-INT, and the grey dotted line represents individual participant data during HI-INT. **B**: There were no differences in NKCA per cell in pre- and post-pubertal children during HI-INT. The solid lines represent mean values for pre-pubertal (orange) and post-pubertal (purple) children. Individual data are presented in dotted lines for pre-pubertal (orange dotted line) and post-pubertal (purple dotted line) children.

4.4.3 Effects of Exercise on NKCA per Cell by Sex

There was no statistically significant interaction between sex and timepoint on NKCA per cell ($F(3, 27) = 0.70$, $p = 0.562$; $\eta^2_p = 0.072$; Figure 15). There were no differences in average NKCA per cell between male (0.73 ± 0.52) and female youth (1.1 ± 1.6; $F(1, 9) = 0.46$, $p = 0.514$; $\eta^2_p = 0.049$).

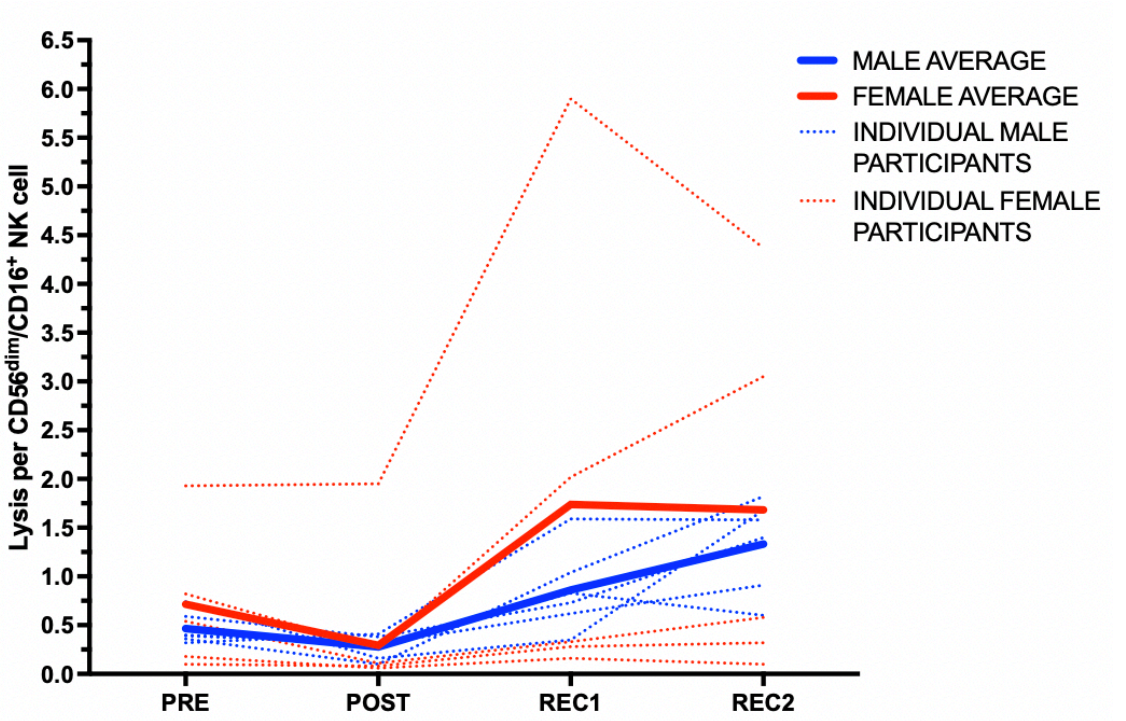

Figure 15. Effects of Exercise on NKCA per Cell by Sex. There were no differences in NKCA per cell during HI-INT in male vs. female children. The solid lines represent mean values for males (blue) and females (red). Individual data are presented in dotted lines for males (blue dotted line) and females (red dotted line).

4.5 Total NK Cells as a Proportion of Lymphocytes

4.5.1 Effects of Exercise on Total NK Cells as a % of Lymphocytes

There was a statistically significant interaction between exercise type and timepoint on %NK cells ($F(9, 90) = 5.19$; $p < 0.001$; $\eta^2_p = 0.342$; Figure 16A). %NK cells at POST were greater during HI-INT (15.5 ± 8.1%) than HI-CONT (12.0 ± 5.0%; $p < 0.005$) and MOD-CONT (10.6 ± 4.5%; $p < 0.001$). Similarly, %NK cells at POST were greater during MOD-INT (16.2 ± 9.5%) than HI-CONT and MOD-CONT ($p < 0.001$). There was also a main effect of timepoint on %NK cells ($F(3, 30) = 25.76$, $p < 0.001$; $\eta^2_p = 0.720$). As expected, Tukey's post-hoc analysis revealed that %NK cells at POST (13.6 ± 7.2%) was significantly greater than at PRE (6.8 ± 2.8%), REC1 (5.6 ± 2.7%), and REC2 (4.9 ± 2.0%); $p<0.001$ for all (Figure 16B).

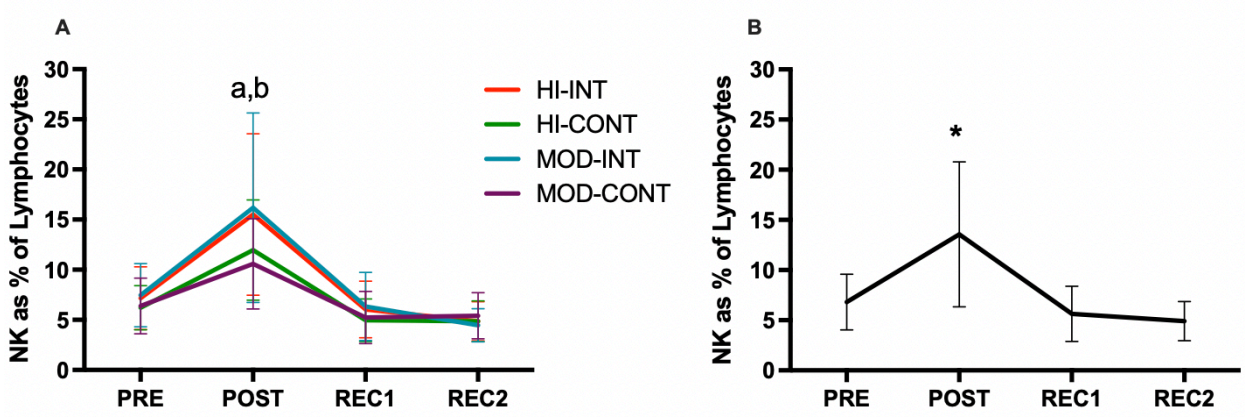

Figure 16. Effects of Exercise on Total NK Cells as a % of Lymphocytes. A: Interaction between exercise and timepoint, a = HI-INT > HI-CONT and MOD-CONT at POST, b = MOD-INT > HI-CONT and MOD-CONT at POST; $p < 0.005$. **B**: Main effect of timepoint on NK proportions, * = POST > PRE, REC1, and REC2; $p < 0.001$. All data are presented as mean ± SD.

4.5.2 Effects of Exercise on Total NK Cells as a % of Lymphocytes by Pubertal Status

There was a statistically significant interaction between pubertal status and timepoint on %NK cells ($F(3, 27) = 10.07$, $p < 0.001$; $\eta^2_p = 0.528$; Figure 17). At POST, pre-pubertal children had a significantly lower %NK cells than post-pubertal children (pre-pubertal vs. post-pubertal: 9.1 ± 4.3% vs. 20.9 ± 6.2%; $p<0.001$).

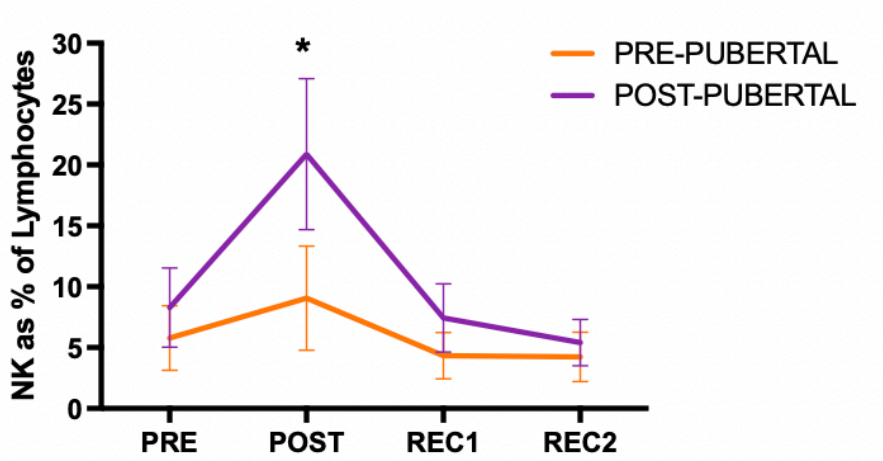

Figure 17. Effects of Exercise on Total NK Cells as a % of Lymphocytes by Pubertal Status. Interaction between pubertal status and timepoint on %NK cells, * = POST-PUBERTAL > PRE-PUBERTAL at POST; $p < 0.001$. All data are presented as mean ± SD.

4.5.3 Effects of Exercise on Total NK Cells as a % of Lymphocytes by Sex

There were no statistically significant differences in %NK cells between males and females at any timepoint during HI-INT ($F(3, 27) = 0.37$, $p = 0.773$; $\eta^2_p = 0.040$; Figure 18).

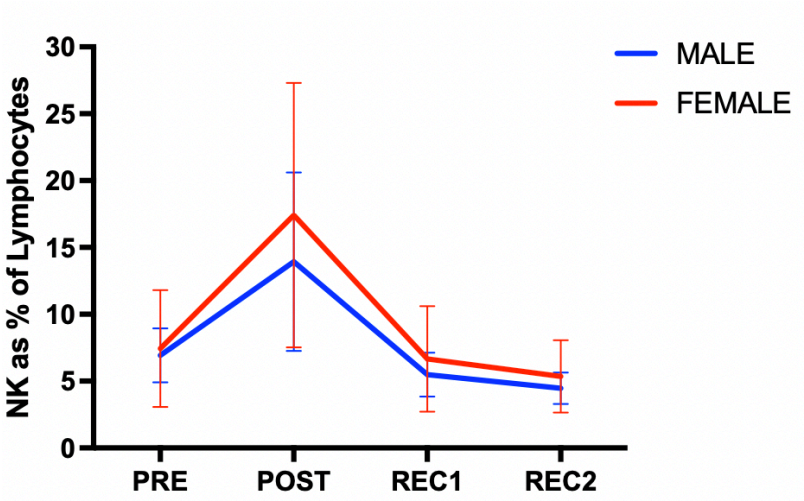

Figure 18. Effects of Exercise on Total NK Cells as a % of Lymphocytes by Sex. There were no differences in %NK cells during HI-INT in male vs. female children. All data are presented as mean ± SD.

4.6 CD56^{bright} NK Cells as a Proportion of Lymphocytes

4.6.1 Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes

There was no statistically significant interaction between exercise and timepoint on %CD56^{bright} cells ($F(9, 90) = 1.37$; $p = .215$; $\eta^2_p = 0.120$; Figure 19A). However, there was a main effect of timepoint on %CD56^{bright} cells ($F(3, 30) = 8.31$, $p<0.001$; $\eta^2_p = 0.454$; Figure 19B). Tukey's post-hoc revealed that %CD56^{bright} cells increased significantly from PRE (0.92 ± 0.39%) to POST (1.1 ± 0.55%; $p<0.005$), and this was maintained at REC1 (1.2 ± 0.63%), which was significantly greater than PRE (0.92 ± 0.39%; $p<0.001$) and REC2 (1.0 ± 0.43%; $p<0.05$).

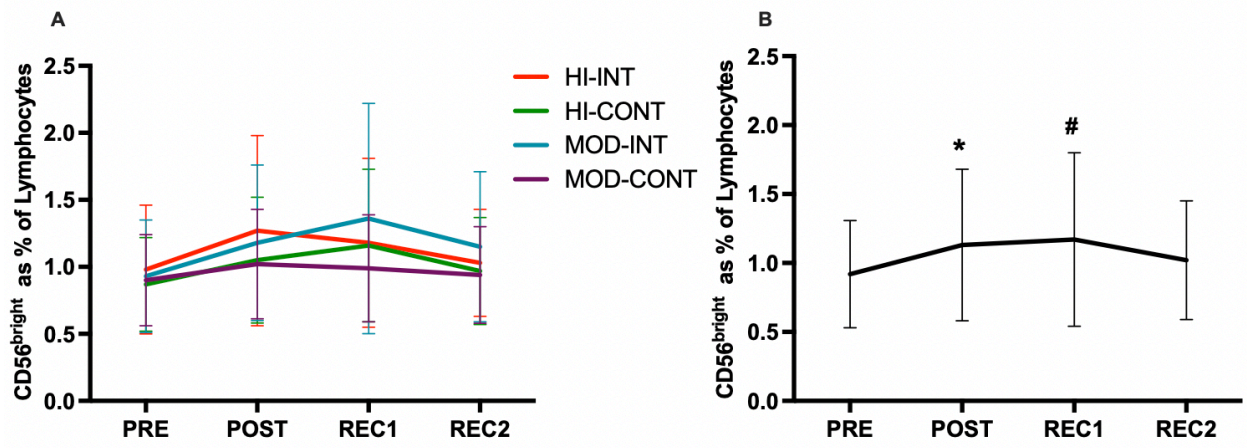

Figure 19. Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes. A: There were no differences in %CD56^{bright} cells between HI-INT, HI-CONT, MOD-INT, AND MOD-CONT exercises. **B**: Main effect of timepoint on CD56^{bright} proportions, * = POST > PRE, # = REC1 > PRE and REC2; $p<0.05$. All data are presented as mean ± SD.

4.6.2 Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes by Pubertal Status

There were no statistically significant differences in %CD56^{bright} cells between pre-pubertal and post-pubertal children at any timepoint ($F(3, 27) = 0.234$, $p = 0.872$; $\eta^2_p = 0.025$; Figure 20).

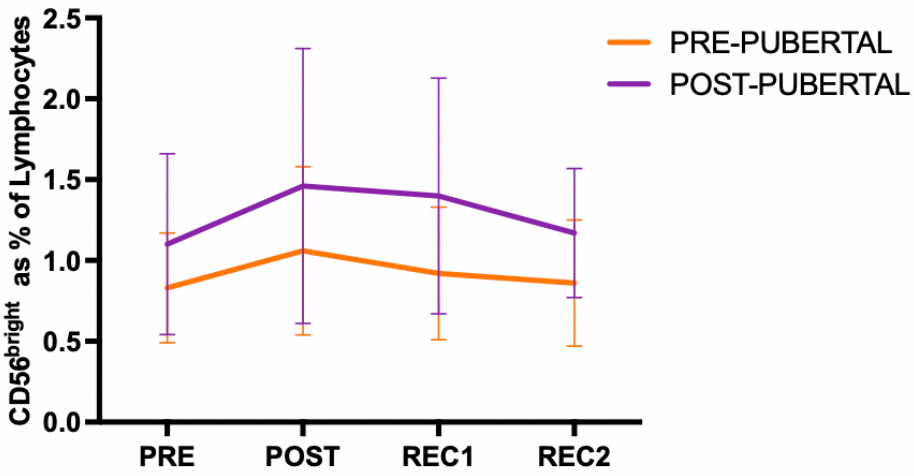

Figure 20. Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes by Pubertal Status. There were no differences in %CD56^{bright} cells between pre- and post-pubertal children during HI-INT. All data are presented as mean ± SD.

4.6.3 Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes by Sex

There were no statistically significant differences in %CD56^{bright} cells between males and females at any timepoint ($F(3, 27) = 0.416$, $p = 0.743$; $\eta^2_p = 0.044$; Figure 21).

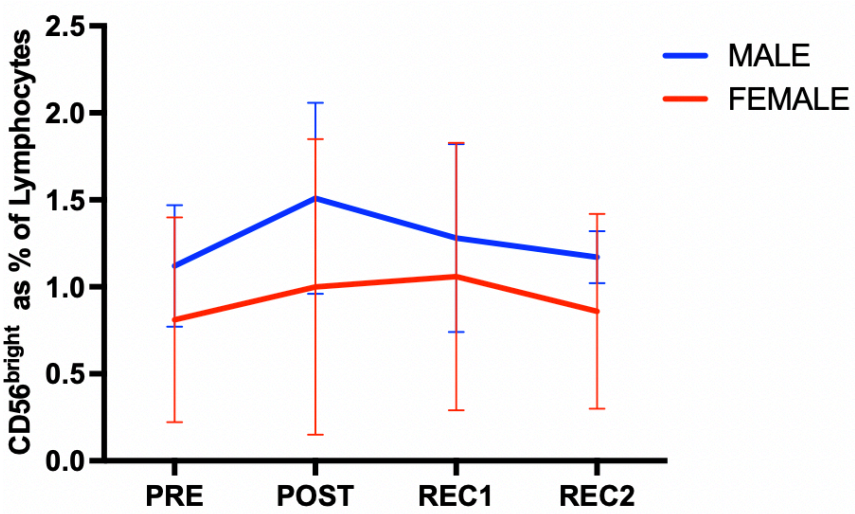

Figure 21. Effects of Exercise on CD56^{bright} Cells as a % of Lymphocytes by Sex. There were no differences in %CD56^{bright} cells during HI-INT in male vs. female youth. All data are presented as mean ± SD.

4.7 $CD56^{dim}/CD16^{+}$ NK Cells as a Proportion of Lymphocytes

4.7.1 Effects of Exercise on $CD56^{dim}/CD16^{+}$ Cells as a % of Lymphocytes

There was a statistically significant interaction between exercise protocol and timepoint for $\%CD56^{dim}/CD16^{+}$ cells ($F(9, 90) = 4.14$; $p < 0.001$; $\eta^2_p = 0.293$; Figure 22A). At POST, the $\%CD56^{dim}/CD16^{+}$ cells was higher for HI-INT (8.5 ± 6.0%) than HI-CONT (5.6 ± 3.6%) and MOD-CONT (5.6 ± 3.9%); $p<0.005$ for both. Similarly, the $\%CD56^{dim}/CD16^{+}$ cells at POST were greater for MOD-INT (9.2 ± 8.4%) than either HI-CONT or MOD-CONT ($p < 0.001$ for both). There was no main effect of exercise protocol on $\%CD56^{dim}/CD16^{+}$ cells ($F(3, 30) = 2.60$, $p = 0.070$; $\eta^2_p = 0.206$). However, there was a main effect of timepoint on $\%CD56^{dim}/CD16^{+}$ cells ($F(3, 30) = 16.86$, $p < 0.001$; $\eta^2_p = 0.628$; Figure 22B). The $\%CD56^{dim}/CD16^{+}$ cells at POST (7.2 ± 5.8%) was significantly greater than at PRE (3.2 ± 2.4%), REC1 (2.1 ± 1.8%), and REC2 (1.8 ± 1.3%); $p<0.001$ for all.

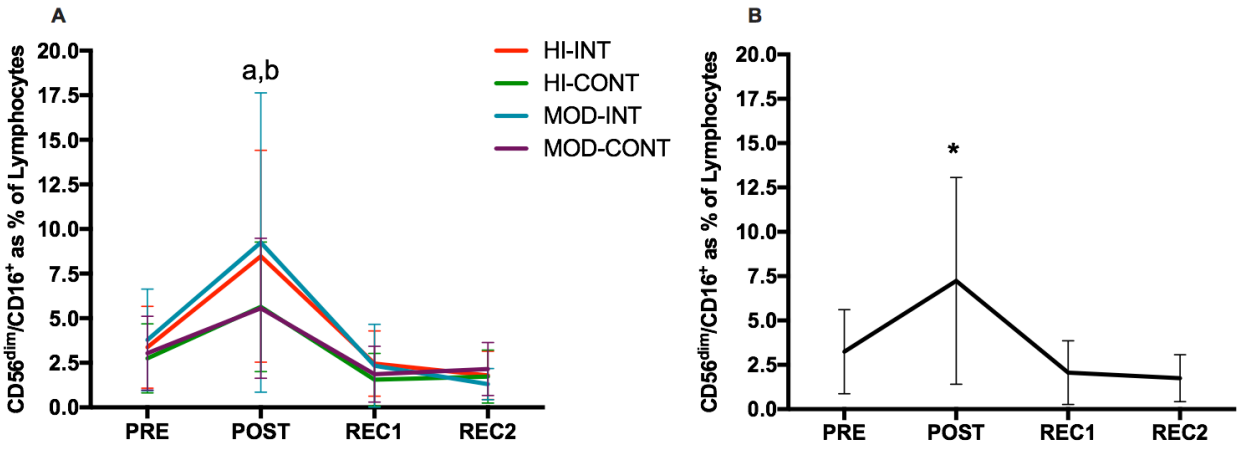

Figure 22. Effects of Exercise on $CD56^{dim}/CD16^{+}$ Cells as a % of Lymphocytes. A: Interaction between exercise and timepoint, a = HI-INT > HI-CONT and MOD-CONT at POST, b = MOD-INT > HI-CONT and MOD-CONT at POST; $p < 0.005$. **B**: Main effect of timepoint on $\%CD56^{dim}/CD16^{+}$ cells, * = POST > PRE, REC1, and REC2; $p < 0.001$. All data are presented as mean ± SD.

4.7.2 Effects of Exercise on CD56^{dim}/CD16^{+} Cells as a % of Lymphocytes by Pubertal Status

There was a statistically significant interaction between pubertal status and timepoint ($F(3, 27) = 5.18$, $p<0.01$; $\eta^2_p = 0.365$; Figure 23); however, none of the differences between pre- and post-pubertal children were seen at matched timepoints. For example, %CD56^{dim}/CD16^{+} cells in post-pubertal children at POST (11.3 ± 6.0%) were different from pre-pubertal children at REC2 (1.9 ± 1.4%; $p<0.001$).

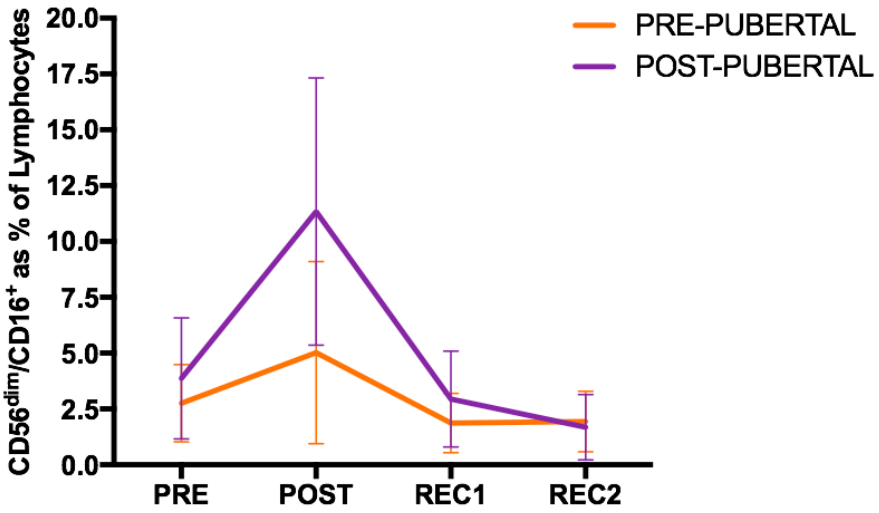

Figure 23. Effects of Exercise on CD56^{dim}/CD16^{+} Cells as a % of Lymphocytes. There were no meaningful significant differences in %CD56^{dim}/CD16^{+} cells between pre- and post-pubertal children during HI-INT. All data are presented as mean ± SD.

4.7.3 Effects of Exercise on $CD56^{dim}/CD16^{+}$ Cells as a % of Lymphocytes by Sex

There were no statistically significant differences in $\%CD56^{dim}/CD16^{+}$ cells between males and females at any timepoint ($F(3, 27) = 0.44$, $p = 0.730$; $\eta^2_p = 0.046$; Figure 24).

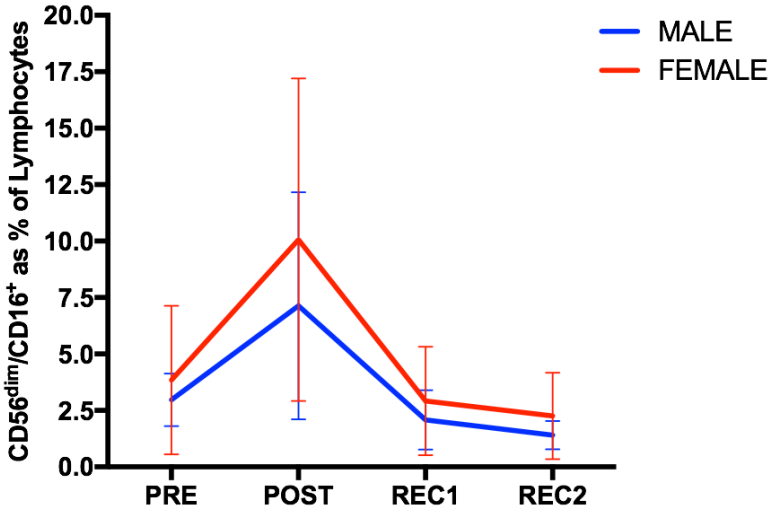

Figure 24. Effects of Exercise on $CD56^{dim}/CD16^{+}$ Cells as a % of Lymphocytes. There were no differences in $\%CD56^{dim}/CD16^{+}$ cells during HI-INT for male vs. female youth. All data are presented as mean ± SD.

Chapter 5: Discussion

To our knowledge, this is the first study to investigate NK specific lysis and NKCA per cell in response to exercise of different intensities and structures in children. We demonstrated that an acute bout of exercise can increase total NK cytotoxicity (specific lysis), as well as cytotoxicity per cell (NKCA per cell), in youth. Much like the adult literature, we confirmed that exercise-induced changes in total cytotoxicity and cytotoxicity per cell occur along different timelines. NKCA per cell was elevated during 60-minute recovery, while increases in specific lysis were noted immediately post-exercise. Immediately post-exercise, there was also an increase in the proportion of total NK cells ($CD3^-/CD56^+$), as well as the two main subsets, $CD56^{dim}/CD16^+$ and $CD56^{bright}$ cells. Interestingly, we found that the increase in total NK and $CD56^{dim}/CD16^+$ cell proportions were significantly greater after intermittent vs. continuous exercise, with no effect of exercise intensity. Additionally, post-pubertal children had a greater proportion of $CD3^-/CD56^+$ cells post-exercise than pre-pubertal children. While $CD56^{dim}/CD16^+$ cells returned to baseline by 30-minutes recovery, $CD56^{bright}$ cells remained elevated until 60-minutes post-exercise. Surprisingly, there were no effects of exercise intensity and structure on specific lysis or NKCA per cell in children. Importantly, we also found that the effects of exercise on NK cytotoxicity did not differ according to pubertal status or sex. Taken together, our results suggest that acute exercise affects NK cell recruitment, specific lysis, and NKCA per cell in children, and that unique processes likely contribute to each of these findings.

5.1 NK Cell Recruitment

In our cohort we observed a significant increase in total NK cells as a proportion of lymphocytes immediately after acute exercise. This post-exercise increase was also apparent for both the $CD56^{dim}/CD16^{+}$ or $CD56^{bright}$ subsets. While this is in contrast to previous studies in adults showing an increase in only $CD56^{dim}$ cell proportions post-exercise, our results align with studies in children[6,31,38]. Children may have a unique immune response to exercise, whereby $CD56^{bright}$ cells facilitate a quick recovery and return to homeostasis after exercise in an effort to protect growth processes[6,139].

Although the proportion of both NK cell subsets increase with acute exercise in youth, $CD56^{dim}$ cells consistently respond with a greater magnitude[6]. Accordingly, we found that exercise increased $CD56^{dim}/CD16^{+}$ cell proportions from approximately ~3 to 7%, while only increasing the proportion of $CD56^{bright}$ cells from ~0.9% to 1.1%. Preferential mobilization of cytotoxic $CD56^{dim}$ cells after exercise may be explained by their high density of β-adrenergic receptors and storage in sites that are highly engaged during exercise, such as the vascular bed[8,40,72–74].

We also observed that the proportion of $CD56^{dim}/CD16^{+}$ cells returned to baseline by 30-minutes of recovery, while the proportion of $CD56^{bright}$ cells remained elevated at this time. These findings are in line with previous pediatric exercise studies and likely reflect the preferential redistribution of cytotoxic NK cells to peripheral tissues and sites of infection such as the lungs and mucosa[31,101]. The

movement of $CD56^{dim}$ cells out of circulation would have resulted in a larger proportion of $CD56^{bright}$ cells during recovery, and all NK cell proportion returned to baseline by 60-minutes.

When evaluating the effects of exercise structure on NK cell recruitment, we observed a greater post-exercise increase in total NK and $CD56^{dim}/CD16^{+}$ cell proportions during our intermittent vs. continuous protocols. This may be driven by a greater increase in both EPI and IL-6 concentrations during intermittent vs. continuous exercise, which has been found in adults[140,141]. Recent work suggests that exercise induces selective mobilization of IL-6-sensitive NK cells, and that blocking IL-6 receptor signalling attenuates post-exercise NK cell recruitment[34,142]. However, the extent to which these signals can explain our observations is unclear given that we did not measure either EPI or IL-6.

We also observed that NK cells as a proportion of total lymphocytes at POST were significantly greater in post-pubertal vs. pre-pubertal children. Though not significant, $CD56^{dim}/CD16^{+}$ cell proportions also tended to be greater in post-pubertal children ($p = 0.0505$) at POST. These results align with previous studies that indicate an effect of biological maturity on post-exercise $CD56^{dim}$ cell recruitment[31,99]. There is an age-related increase in lymphocyte β-adrenergic receptor density, and our post-pubertal participants were on average 7 years older than our pre-pubertal group[143,144]. Thus, post-pubertal children may have been more responsive to catecholamines released during exercise. Another possibility is that post-exercise cortisol levels may have been higher in our pre-pubertal vs.

post-pubertal participants[145]. Cortisol promotes lymphocytopenia through the redistribution of circulating cells to peripheral sites, and thus may have trafficked NK cells out of circulation faster in our pre-pubertal children[146].

5.2 Timeline for Effects of Exercise on NK Cell Cytotoxicity

As hypothesized, we observed an effect of exercise on both measures of NK cell cytotoxicity. Relative to baseline, there was an increase in specific lysis at POST, and an increase in NKCA per cell at REC2. These unique timelines suggest the importance of considering different post-exercise processes that contributed to each change. For example, the increase in specific lysis may be driven by the increase in $CD56^{dim}/CD16^{+}$ cell proportions that also occurred after exercise. In our study, we evaluated specific lysis through the incubation of K562 targets with a given number of PBMC. While consistent T:E ratios were used for all timepoints, $CD56^{dim}/CD16^{+}$ cells accounted for a larger proportion of PBMC at POST than at PRE. Given their high cytotoxicity and perforin content, this likely drove time-matched increases in specific lysis[12,13]. Similar effects have been shown in adults, where a post-exercise peak in the % of $CD3^{-}/CD56^{+}/CD16^{+}$ NK cells is paralleled by an increase in total NK cell cytotoxicity[3]. Fewer studies have assessed these effects in children; however, one research group reported a significant increase in both $CD16^{+}$ NK cells and NK cytotoxicity after a Wingate anaerobic test in boys (age 7-18 years)[99]. Our study demonstrates that these parallel increases in $CD16^{+}$ NK cells and total NK cytotoxicity occur after both continuous and intermittent aerobic exercise in youth.

Another factor that can uniquely contribute to the increase in specific lysis at POST is EPI. As previously discussed, EPI drives NK cell mobilization during exercise and may therefore also be important for facilitating the increase in specific lysis at POST. To test this hypothesis, Kappel et al. had 8 young males (age 20 - 29 years) complete a 60-minute cycling protocol at 75% VO_2max and recorded their EPI concentrations at baseline and during the last minute of exercise[147]. On a later day, the same participants were given an intravenous infusion of EPI at a similar plasma concentration as was seen with exercise. The EPI infusion alone led to significant increases in both $CD16^+$ NK cell concentrations and cytotoxicity, which were similar to the magnitude of change observed after exercise. This supports the observation that cytotoxic NK cell mobilization during exercise facilitates specific lysis, as seen in our study, and that EPI is key for both of these effects.

While specific lysis in our study had returned to baseline at REC1, it was significantly lower than baseline by REC2. Similar findings have been reported in many adult studies[32,35,47]. To explain these effects we can again draw on alterations in NK cell mobilization. $CD56^{dim}/CD16^+$ cell proportions returned to baseline values by 30- and 60-minutes recovery. This is thought to reflect the preferential recruitment of cytotoxic NK cells to peripheral tissues where, for example, they can control infections and tumor growth[8,34,148,149]. However, biopsies to confirm the sites of NK cell infiltration after acute exercise are lacking.

Nonetheless, the normalization of $CD56^{dim}/CD16^{+}$ cell proportions by REC1 likely drove the return to baseline specific lysis values seen at this time.

Given that $CD56^{dim}/CD16^{+}$ proportions remained similar to baseline by REC2, they cannot explain the significant decrease in specific lysis observed at this time. In the only other study to examine these factors in children, Boas et al. also found that across all participants, the concentration of $CD3^{-}/CD56^{+}/CD16^{+}$ cells returned to baseline 1-hour post-exercise while NK cell cytotoxicity was significantly decreased[99]. This suppression may be explained in part by prostaglandins, which are well-known to inhibit NK cell function. This has important clinical implications as prostaglandin release by tumors facilitate their metastatic capacity[150,151]. Prostaglandin signalling can increase NK intracellular cAMP levels, which favours cell inhibition and the interferes with NK cell migration to targets by chemotaxis[150,152]. Importantly, monocytes, which increase during exercise recovery, produce prostaglandins when cultured[153]. The relationship between monocytes, prostaglandins, and post-exercise NK cytotoxicity has been explored by Pedersen et al. In two separate studies, this group found a 2-fold increase in monocyte proportions (defined as CD20+ or CD14+) following 2 hours of recovery from 60-minutes of cycling in young adults[35,147]. In both studies this was paralleled by a significant decrease in NK cell lysis. However, lysis was restored to baseline values when the assay was either depleted of monocytes or treated with indomethacin, an inhibitor of prostaglandin production[147]. Since our cytotoxic assay

was not depleted of monocytes, these findings provide a possible explanation for the observed decrease in specific lysis at REC2.

It is possible that prostaglandin release may have also affected NKCA per cell. However, we did not observe a significant decrease in NKCA per cell at any time. Instead, we saw that NKCA per cell was enhanced at REC2. This implies that in the overall balance of factors at play during recovery, an increase in the cytotoxicity of each NK cell was favoured. This is a particularly exciting finding because it aligns with previous unpublished observations in our lab. Using the same participants averaged across their four exercise visits, we found that the density (measured as median fluorescence intensity, MFI) of activating receptors DNAM-1 and NKG2D significantly increased on NK cells from PRE to REC2 (**Figure 25**)[154]. Given that K562 express ligands for these receptors, our data suggest that NK cells may have been more readily activated and cytotoxic during 60-minute recovery[155].

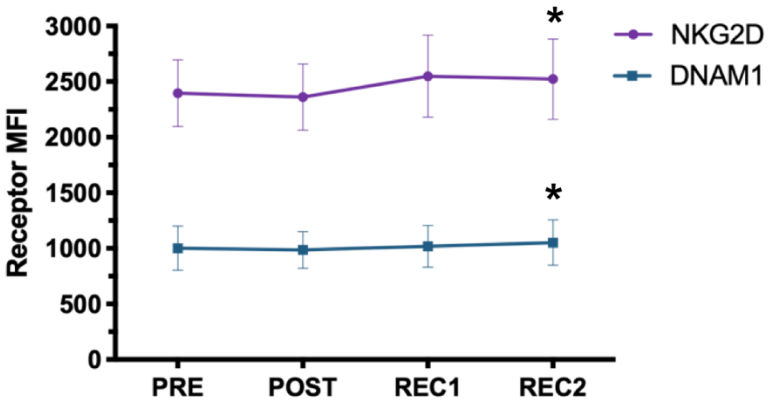

Figure 25. Activating receptor MFI. * = REC2 > PRE and POST, p<0.001. Data presented as mean ± SD. *Modified from Ushcatz (unpublished results)*[154].

Our results may also be explained by the recruitment of highly differentiated, $CD57^+$ NK cells. CD57 is a marker of maturity and terminal differentiation on NK cells[156–158]. Previous research has shown that $CD57^+$ NK cells have higher levels of perforin and enhanced cytotoxic abilities compared to $CD57^-$ cells[159,160]. Notably, $CD57^+$ NK cells may be preferentially recruited in response to acute exercise[159]. For example, Bigley et al. had participants (~30 years) cycle for 30 minutes at three different intensities (-5% LT, +5% LT, and +15% LT) and found significantly greater mobilization of $CD57^+$ vs. $CD57^-$ NK cells at each intensity[68]. While we did not quantify CD57 expression, it is possible that a similar effect occurred in our participants and facilitated the increase in NKCA per cell.

Another factor to consider is the recruitment of $CD11a^+$ NK cells in response to exercise. CD11a is part of the LFA-1 heterodimer, and is an adhesion molecule on NK cells that facilitates engagement with target cells, and polarization of the cytolytic machinery towards a target[161]. LFA-1 binds ICAM-1, which is ubiquitously expressed on K562 cells, and forms an immunological synapse for efficient lysis[162–164]. Previous studies have shown that physiological stress, caused either by an EPI infusion or public-speaking task, can preferentially mobilize $CD11a^+$ lymphocytes and also lead to a greater surface density of CD11a[41,165]. More specific to NK cells, one group of researchers showed that both moderate and high intensity exercise increased the number of $CD11a^+$ NK cells post-exercise[166]. This study did not evaluate $CD11a^+$ expression at 1-hour recovery from exercise. However, it is possible that our exercise protocols elicited a similar increase in $CD11a^+$ cells or

densities, and that these remained in circulation at REC1 and REC2. This could have enhanced NK cell conjugation with targets and led to more efficient lysis on a per cell basis.

A final factor that may have influenced NKCA per cell are cytokines such as IL-2 and IL-12. IL-2 is a proinflammatory cytokine important for repairing muscle damage elicited by exercise, and IL-12 may be important for muscle strength and performance[167]. Both of these cytokines can activate NK cells, possibly facilitating their cytotoxicity and engagement with targets[168–170]. When NK cells are incubated overnight with IL-2 or IL-12, they demonstrate significantly greater % binding to K562 and % specific lysis the next day[171]. Similar effects have also been seen after shorter incubations with different target cell lines[168,172]. Exercise can increase IL-12 and may also increase IL-2, though the literature on IL-2 is less consistent and little is known in children[167,173–175]. While acute exercise may not increase IL-2 concentrations, it is possible that NK cells have increased responsiveness to this cytokine after exercise[35]. It's not clear how these effects translate to our findings given that we did not measure the concentration of either cytokine, and NK cells would have only been exposed to IL-2 and IL-12 for a short period of time. However, the potent effects of these cytokines are a promising area for future research on the effects of exercise on NKCA per cell in youth.

5.3 Effects of Exercise Intensity on NK Cytotoxicity

We did not observe any differences in specific lysis or NKCA per cell based on exercise intensity (i.e., HI-INT = MI-INT, and HI-CONT = MOD-CONT). This aligns with our observation that there were no differences between $CD56^{dim}/CD16^{+}$ proportions after moderate vs. high intensity exercise protocols. To our knowledge, no previous study has directly compared the effect of exercise intensity on NK cytotoxicity in youth. However, our findings conflict with those reported in the adult literature. On separate testing days, Nieman et al. had young adults (age 17-31 years) complete a high (80% VO_2max) and moderate (50% VO_2max) intensity 45-minute cycling protocol[3]. A subsequent K562 chromium release assay demonstrated that high intensity exercise significantly enhanced NK cytotoxicity post-exercise and NKCA per cell during recovery, while moderate intensity exercise did not. The authors also reported that NK ($CD3^{-}/CD56^{+}/CD16^{+}$) cell counts and EPI concentrations were significantly greater after high vs. moderate intensity exercise. Though the aforementioned study tested only male participants, similar intensity-dependent effects have been found in women[49].

In the context of specific lysis, it is plausible that our children may have experienced a greater post-exercise spike in EPI after high vs. moderate-intensity exercise, as previously reported in adult literature[3]. Given that β-adrenergic receptor density increases with age, there may be a ceiling effect of exercise intensity in our participants[143,144]. Our moderate intensity protocol may have

maximally saturated NK cell β-adrenergic receptors, meaning that exercise at a higher intensity was unable to further affect NK cell recruitment or cytotoxicity.

Alternatively, it is possible that our high and moderate intensity exercise stimuli produced similar catecholamine responses in youth, and thus, similar effects on $CD56^{dim}/CD16^{+}$ cell proportions and specific lysis. While this is not supported by literature in adults, it remains plausible given that children experience an overall attenuated immune response to exercise[94]. Previous studies have shown that children have a ~40% dampened immune cell response to exercise compared to adults, and this attenuation may extend to their hormonal response as well[93,95]. Along these lines is previous work showing that children have a ~50% lower IL-6 response to exercise compared to adults[176]. These reduced responses in children may be protective of their growth, as IL-6 can inhibit growth mediators such as IGF-1[139,177,178]. Notably, post-exercise increases in IL-6 are also dependent on exercise intensity in adults, and IL-6 works in concert with EPI to facilitate $CD56^{dim}/CD16^{+}$ cell mobilization[179]. Given children's overall dampened response to exercise, it is possible that neither EPI nor IL-6 concentrations differed by exercise intensity, and that resulted in no difference in specific lysis between our protocols.

In one of the only other papers evaluate NKCA per cell after different exercise intensities in adults, Brenner et al. found no difference in NKCA per cell after either cycling for 5-minutes at 90% VO_2max vs. 2-hours at 60% VO_2max[53]. However, the comparison of these results to ours is limited by two factors. First,

exercise duration was not matched between visits, and secondly, NKCA per cell was not measured at either 30- or 60-minute recovery, which is where we saw effects in our study. Much like what may be occurring with specific lysis, there may be a ceiling effect of exercise intensity on NKCA per cell in youth. Nonetheless, we are the first study to investigate the effects of exercise intensity on specific lysis and NKCA per cell in youth, and these effects require confirmation and elucidation by future studies.

5.4 Effects of Exercise Structure on NK Cytotoxicity

We observed no difference in specific lysis or NKCA per cell after intermittent and continuous exercise. To our knowledge, no study to date has directly compared the effects of exercise structure on NKCA per cell. One study has evaluated these effects on specific lysis, and found no difference in specific lysis between intermittent and continuous exercise protocols[61]. However, in this study specific lysis was not significantly increased after either exercise, which limits the comparison of their results to ours. Given that $CD56^{dim}/CD16^{+}$ cell proportions were greater after our intermittent vs. continuous protocols, we would have expected a greater increase in specific lysis for these exercises as well. We must therefore consider what other factors may be differentially affected by intermittent and continuous exercise, and how these interact with specific lysis.

In a previous evaluation of exercise structure, Bally et al. had 12 young males with well-controlled T1D (~26 years) complete both a 90-minute continuous and intermittent cycling protocol that were matched for energy expenditure[140].

Interestingly, they found that lactate concentrations were significantly higher and pH was significantly lower during intermittent exercise. Similar findings have been reported in healthy young men (age 20-30 years)[180,181]. These effects are notable because lactate has negative effects on NK cytotoxicity. Previous research has shown that in an attempt to evade lysis by NK cells, tumor cells release lactate to acidify the tumor microenvironment[182]. Lactic acid then decreases the intracellular pH of NK cells, causing mitochondrial dysfunction and possibly apoptosis[182]. Conversely, animal models have found that blocking lactate efflux from tumors reverses these effects and restores NK cytotoxicity against breast carcinoma[183]. Taken together, these findings suggest that increased lactate production during intermittent vs. continuous exercise may have mitigated the effect of enhanced $CD56^{dim}/CD16^{+}$ cell proportions on specific lysis.

Another similar possibility is that our two exercise structures differentially affected TGF-β concentrations. To date, no study to date has examined this cytokine in youth following continuous and intermittent exercise. However, Zebrowska et al. found that in young males (~24 years) who completed two exercise protocols (40-minutes of continuous cycling vs. 4 x 5-minute cycling bouts separated by 5-minute rest periods), there was a significant difference between TGF-β concentrations after the two exercises[141]. TGF-β significantly increased after only intermittent exercise, and this is notable because TGF-β is known to inhibit the activities and function of NK cells[184–186]. More specifically, TGF-β interferes with the mTOR pathway that is essential for NK cell metabolism, and that

controls the production of perforin and granzyme B[187]. In vitro experiments have previously shown that TGF-β can inhibit normal mTOR activation to IL-15 and reduce granzyme B content[187,188]. Conversely, the deletion of TGFBR2 (a TGF-β receptor on murine NK cells) restores NK cytotoxicity and reduces tumor metastasis in mice[184].

It is possible that both lactate and TGF-β concentrations are greater immediately after intermittent vs. continuous exercise. Since these both have negative effects on NK cytotoxicity, their enhanced levels could explain why we did not see a difference in specific lysis after intermittent or continuous exercise, despite a greater proportion of $CD56^{dim}/CD16^{+}$ cell proportions after the former. While these factors could have also interfered with NKCA per cell, it is likely that they had returned to baseline values by REC1 and REC2. Little is known about post-exercise TGF-β in youth; however, young children have a faster lactate recovery from exercise than adults[189,190]. We recognize that we did not measure either lactate or TGF-β concentrations in our participants, and as such, our explanations are speculative but point to an area for future research.

5.5 Sex, Puberty, and NK Cell Cytoxicity

In our participants, we found no significant effect of pubertal status or sex on baseline or post-exercise specific lysis and NKCA per cell. This is surprising because previous research in adults and animal models suggest that NK cytotoxicity is affected by sex hormones, which we would expect to differ between

our male and female participants, as well as our pre- and post-pubertal participants.

While few studies have examined the effects of puberty on NK cytotoxicity, our results do align with findings from Boas et al. Both at baseline and after the Wingate Anaerobic Test, there was no difference in NK cytotoxicity between pre- and post-pubertal boys[99]. In a unique study that assessed the effects of exercise on gene expression in PBMC, Radom-Aizik et al. had early and late-pubertal females complete 10 x 2-minute bouts of cycling at 90% VO_2max[191]. Exercise altered a similar number of genes involved in NK cell-mediated cytotoxicity in both groups. Post-exercise, early- and late-pubertal girls had a ~2 fold increase in the expression of perforin and granzyme B genes in PBMC. If these pathways were similarly upregulated in our participants, this may help explain why specific lysis and NKCA per cell did not differ between pubertal groups. Timmons et al. also reported no effect of pubertal status on the increase in density of CD69 (a proxy marker for NK cytotoxicity) expression on NK cells after a 60-minute bout of cycling at 70% VO_2max in boys[31,192]. Importantly, unpublished results in our lab found no difference in the MFI of activating receptors NKG2D or DNAM-1 between the pre- and post-pubertal participants in the present study[154]. Thus, the NK cells of pre- and post-pubertal children may have been similarly activated by targets. Taken together, our findings and the limited evidence in the literature suggest that in children, irrespective of pubertal status, each individual NK cell is more active during recovery from exercise.

We are not aware of any studies in children that have examined the effects of sex on specific lysis and NKCA per cell after acute exercise. However, a large scale study in children (~9 years) showed no relationship between sex and NK cell cytotoxicity at rest[92]. Additionally, while not a direct comparison of boys and girls, Radom-Aizik et al. examined the effects of exercise on gene expression in two separate publications for males and females[193]. Comparing these studies in late-pubertal boys and girls, we can see that exercise caused many similar changes in PBMC gene expression, most notably a 2.1 vs. 2.08 fold increase in perforin gene expression and 2.3 vs. 2.25 fold increase in granzyme B gene expression in males vs. females, respectively[191,193]. Though perforin and granzyme B content were not directly measured in NK cells, these data align with our finding of no sex-related differences in NK-mediated K562 lysis, for which perforin and granzyme B are essential.

Several studies in adults have examined the effects of exercise on NK function by sex. In perhaps the best parallel of our own study design, Brahmi et al. evaluated NK specific lysis in males and females (21-37 years) after a progressive cycling exercise test and reported no significant difference in post-exercise specific lysis between males and females[32]. However, these researchers did not control for contraceptive use or the menstrual cycle. The female menstrual cycle is known to influence NK cells where NK cytotoxicity may be greater in women during the follicular vs. luteal phase, though these effects are not unanimously reported[118,194,195]. It has also been shown that female contraceptive users have

significantly lower NK cytotoxicity than men[118]. Participants were taking estrogen-based contraceptives, which is a hormone known to inhibit NK cytotoxicity[117,196]. It is important to point out that participants taking contraceptives were excluded from our study and we attempted to mitigate effects of menstrual cycle by testing our post-pubertal females in each of the 4 menstrual phases.

Although our results do align with a number of exercise-related studies in children and adults, they are nonetheless surprising given the known effect of sex hormones on NK cells. For example, estradiol has been correlated with reduced NK cell cytotoxicity in vitro[117,196,197]. Conversely, prolactin may enhance NK cytotoxicity against K562 targets, though the evidence for this hormone is mixed[198–200]. These effects are notable because both estradiol and prolactin are generally higher in females than males[201,202]. It is possible that the positive and negative effects of these female sex hormones in our participants effectively balanced out, resulting in similar lysis between our males and females. However, this explanation is likely too simplistic as there are many more hormones and interactions to consider in vivo. It seems more likely that our small sample, with a limited number of post-pubertal participants, restricted our ability to definitively evaluate the effect of sex and puberty on the exercise-induced changes in NK function.

The above rationale can help explain why we did not see differences in specific lysis or NKCA per cell between pre- vs. post-pubertal children, or boys vs. girls. However, we recognize that our smaller sample size limits our ability to make definitive conclusions. Given the small to medium effect size for some of our

comparisons (e.g. $\eta^2_p = 0.100$ for the main effect of pubertal status on specific lysis), it will be interesting to reassess these effects once in a larger sample of participants.

6.0 Limitations

The most notable limitation of our study is the small sample size. We aimed to recruit 24 participants; however, only 11 had completed the study and were included in this thesis. This meant that we were likely underpowered to address all of our objectives. Exercise caused many changes of a large magnitude, for example in specific lysis and NKCA per cell, that we can be confident in. However, we also observed trends in our data that may have emerged as statistically significant if we had achieved our target sample size. In an effort to accommodate our smaller sample size, we did run separate two-way ANOVAs to for puberty and sex, as well as report effect sizes for our data. Our sample size was primarily limited due to COVID-19 restrictions, which halted all recruitment and study visits in March 2020. It is promising that 8 participants are currently enrolled and awaiting safe return to the lab; however, their eligibility based on pubertal development will need to be reassessed.

Another limitation of our study is the inability to characterize sites of NK cell infiltration. Blood samples allowed us to analyze the phenotype and cytotoxicity of circulating NK cells; however, we cannot say with certainty how this translates to actions at peripheral tissues. Animal models may provide a glimpse into lymphocyte trafficking post-exercise, for example suggesting an increase in lymphocytes at the lungs. However, the inability to perform similar biopsies in humans, especially young children, limits the extent to which we can explore NK trafficking. Thus, we cannot provide a direct link between our observations and

functional/clinical outcomes in vivo (i.e., decreased rates of upper respiratory tract infections with moderate exercise)[109,203].

A third limitation of our study is that we evaluated NK cell cytotoxicity using only one target cell line. K562 cells are MHC deficient, and previous work suggests that exercise may differentially affect the ability of NK cells to lyse MHC-expressing targets[68]. Lysis against MHC-expressing targets depends on an array of activating and inhibitory signals, and as such we were unable to evaluate how exercise may affect more complex mechanisms of NK cell cytotoxicity. Notwithstanding, our study was an important first step towards characterizing post-exercise NK cell cytotoxicity in youth. Future studies may focus on determining the relationship between exercise-induced changes in receptor expression and cytoxicity in children.

A final limitation of our study is that we did not evaluate the expression of CD57 on NK cells. CD57 is a marker of terminal differentiation and is associated with increases in cytotoxicity. Studies in adults have shown that NK cells expressing CD57 are preferentially recruited in response to exercise[68]. Thus, quantifying CD57 expression in our children may have allowed for a more concrete explanation of why we observed an increase in specific lysis and/or NKCA per cell. While this would have strengthened the interpretation of our results and may be an interesting future step, it does not reduce the importance of our findings.

7.0 Novelty of Findings

The primary goal of this thesis was to examine the effects of exercise on NK cell cytotoxicity in healthy pre- and post-pubertal boys and girls. Our secondary aim was to investigate changes in NK cell proportions with exercise, with reference to how these may help explain alterations in cytotoxicity. We have produced several novel findings outlined below.

1. We showed that an acute bout of aerobic exercise in children can promote an immediate increase in total NK cell-mediated lysis, followed by an increase in the cytotoxicity of each NK cell. This effect was seen after both high and moderate intensity exercise and did not differ based on the structure of the exercise protocol. These findings suggest that NK cell cytotoxicity can be enhanced with as little as 5-minutes of all-out exercise. This is a particularly exciting because it demonstrates that children's natural activity patterns are physiologically relevant and can produce important immune responses. Additionally, it suggests that feasible exercise programs could be used to enhance NK cell cytotoxicity, which may be of particular benefit in children who are, for example, immunocompromised.
2. We reported, for the first time, no differences in resting or post-exercise NK cell cytotoxicity in pre- and post-pubertal children, and in boys and girls.
3. $CD56^{dim}/CD16^{+}$ NK cell proportions increased to a significantly greater extent following intermittent vs. continuous exercise. These observations

imply that exercise structure may be a more important determinant of NK cell recruitment than exercise intensity.

4. Post-pubertal children have a significantly greater proportion of $CD3^{-}/CD56^{+}$ NK cells after exercise than pre-pubertal children. These recruitment-related differences did not translate to similar effects on specific lysis. Thus, we have demonstrated that while NK cell mobilization may drive changes in specific lysis, other factors are clearly at play and the measurement of NK cell mobilization is insufficient to infer function.

8.0 Future Directions

Our study was an important first step towards determining if exercise can affect the NK cell function in children. Future work in our lab will focus on further elucidating the mechanisms that contribute to this response. The supernatant from our incubation assays were preserved, and analysis of these samples will contribute to our understanding of how exercise may affect NK cell cytotoxicity. For example, we can evaluate the presence of cytokines such as TNFα and IL-2 to determine their role in modifying NK cytotoxicity at various post-exercise timepoints. Furthermore, we will be able to investigate if the cytokine milieu in response to immune challenge differs between pre-and post-pubertal children, or boys and girls.

Another important next step in our study will be to investigate whether NK cell cytotoxicity is enhanced post-exercise because of changes to the phenotype of the actual NK cell, or alterations in the cellular microenvironment. After each exercise visit, we stored plasma and serum from children at rest, post-exercise, and 30- and 60-minutes recovery. These samples will allow us to perform cross-culture experiments where, for example, NK cells from a participant's baseline can be incubated with their post-exercise serum. Cytotoxicity could be evaluated following this incubation, and compared cytotoxicity following the incubation of post-exercise NK cells with serum from rest. This would help us determine if changes in NK cell cytotoxicity are driven by exercise-induced alterations to the NK cell itself or the signalling environment. Additionally, it would be interesting to

measure perforin and granzyme B content in NK cells at the various post-exercise timepoints, and after cross-culture experiments, to determine if/how exercise affects cytotoxic molecules. Ultimately, the goal of these next steps are to improve our understanding of mechanisms underlying the post-exercise change in NK cytotoxicity in children.

Our findings could be better contextualized by performing a maximum activation assay with our stored PBMC. For example, PMA and ionomycin are potent stimulators of NK cell cytokine production and degranulation[204,205]. While we showed that exercise can increase the cytotoxicity of NK cells, we did not compare these results to a positive control. By stimulating NK cells from the various post-exercise timepoints with PMA and ionomycin, we can determine the maximum capability for NK cell activation at these timepoints. Comparing these results to the measures of cytotoxicity provided in this thesis will allow us to determine what percent of maximum NK cell activation is elicited by exercise and provide knowledge on the potency of exercise as a stimulus for immune function.

9.0 Implications and Conclusions

We have shown that an acute bout of exercise can transiently increase NK cell cytotoxicity in youth. Notably, these effects occur in pre- and late/post-pubertal children, and in girls and boys. Perhaps the most exciting finding of this thesis is that NK cell cytoxicity can be enhanced by a variety of different exercise protocols. As little as 5-minutes of intermittent, all-out exercise is sufficient to cause an approximate 1.2 fold increase in the percent of target cells lysed, and an approximate 1.9 fold increase in the number of targets lysed by each NK cell. This translates to the activity patterns of young children, which are characterized by short bursts of high intensity activity[62]. It also means that in a real-world setting, youth who participate in a variety of physical activities are likely to benefit from short-term enhancements in the cytotoxicity of their NK cells. Furthermore, our results can guide the implementation of exercise programs designed to acutely manipulate NK cell cytoxicity in youth. These may be of particular benefit in children who are immunocompromised. For example, youth receiving chemotherapy may be encouraged to engage in short bouts of exercise designed to improve their immune function, and may be more incentivized to do so when given the choice between multiple exercise intensities and structures.

While acute exercise may cause only transient increases in NK cell cytoxicity, these may still be of clinical relevance. Repeated bouts of physical activity may have a cumulative, positive effect on immune function. For example, regular physical activity in adults reduces the risk of upper respiratory tract

infections and is associated with reduced risk for developing several types of cancer[8,109,206]. Additionally, a longitudinal study in adults found that individuals with naturally higher cytoxicity of their NK cells had a significantly reduced incidence of cancer 11 years later, even after controlling for age and lifestyle factors such as alcohol consumption and cigarette smoking[207]. Thus, children should be encouraged to induce even transient increases in NK cell cytotoxicity on a regular basis, as this may benefit longer term health outcomes. Ultimately, the goal of this thesis was to generate data to support the evidence-based implementation of exercise as medicine in pediatric populations. We have provided a foundation for understanding the effects of acute exercise on natural cytoxicity in youth, and future studies should build on our results to evaluate the clinical utility of exercise programs for children.

10.0 References

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Appendix A: Supplementary Graphs

Figure A1: Specific lysis by exercise protocol

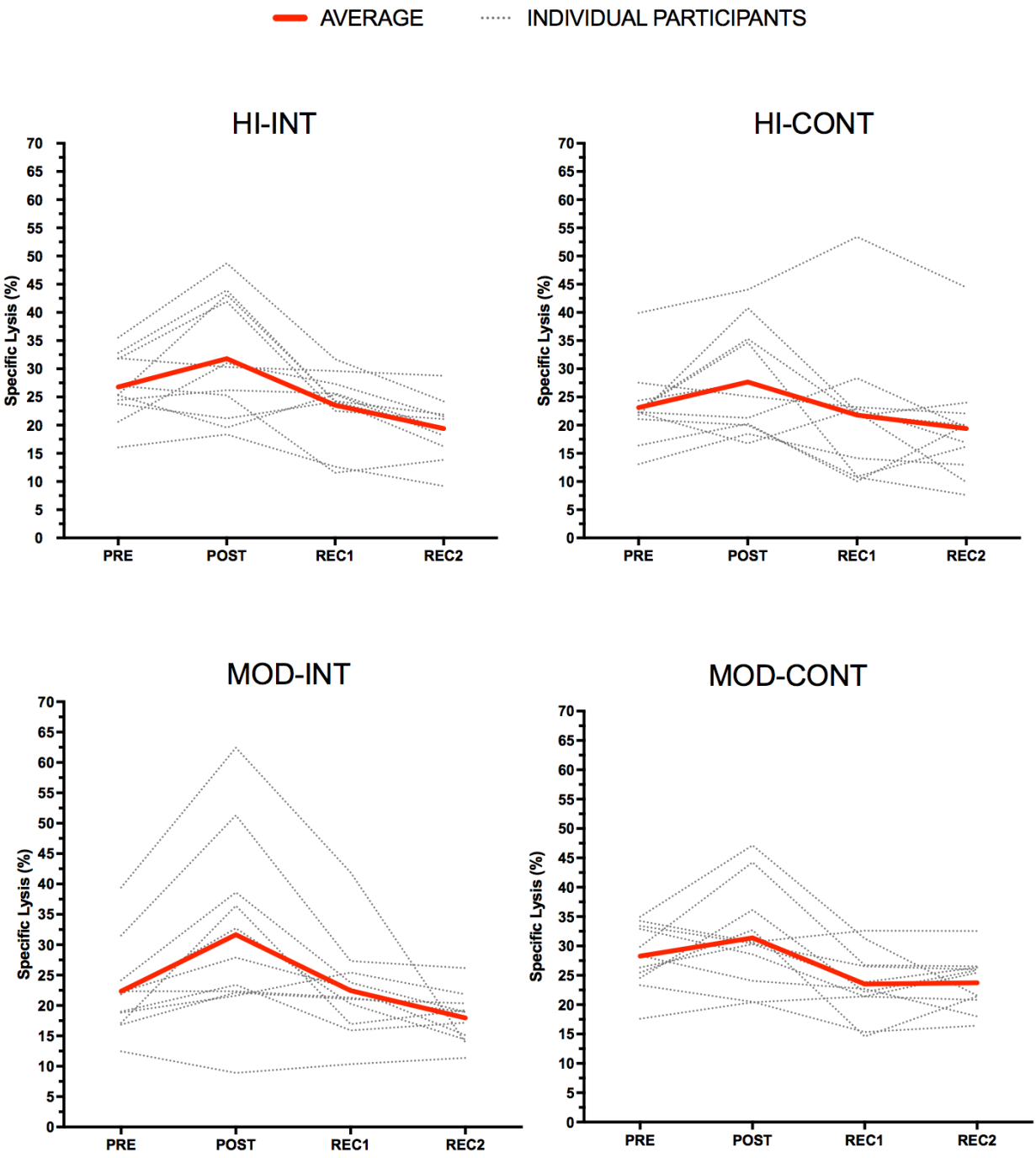


Figure A2: NKCA per cell during the 4 exercise protocols

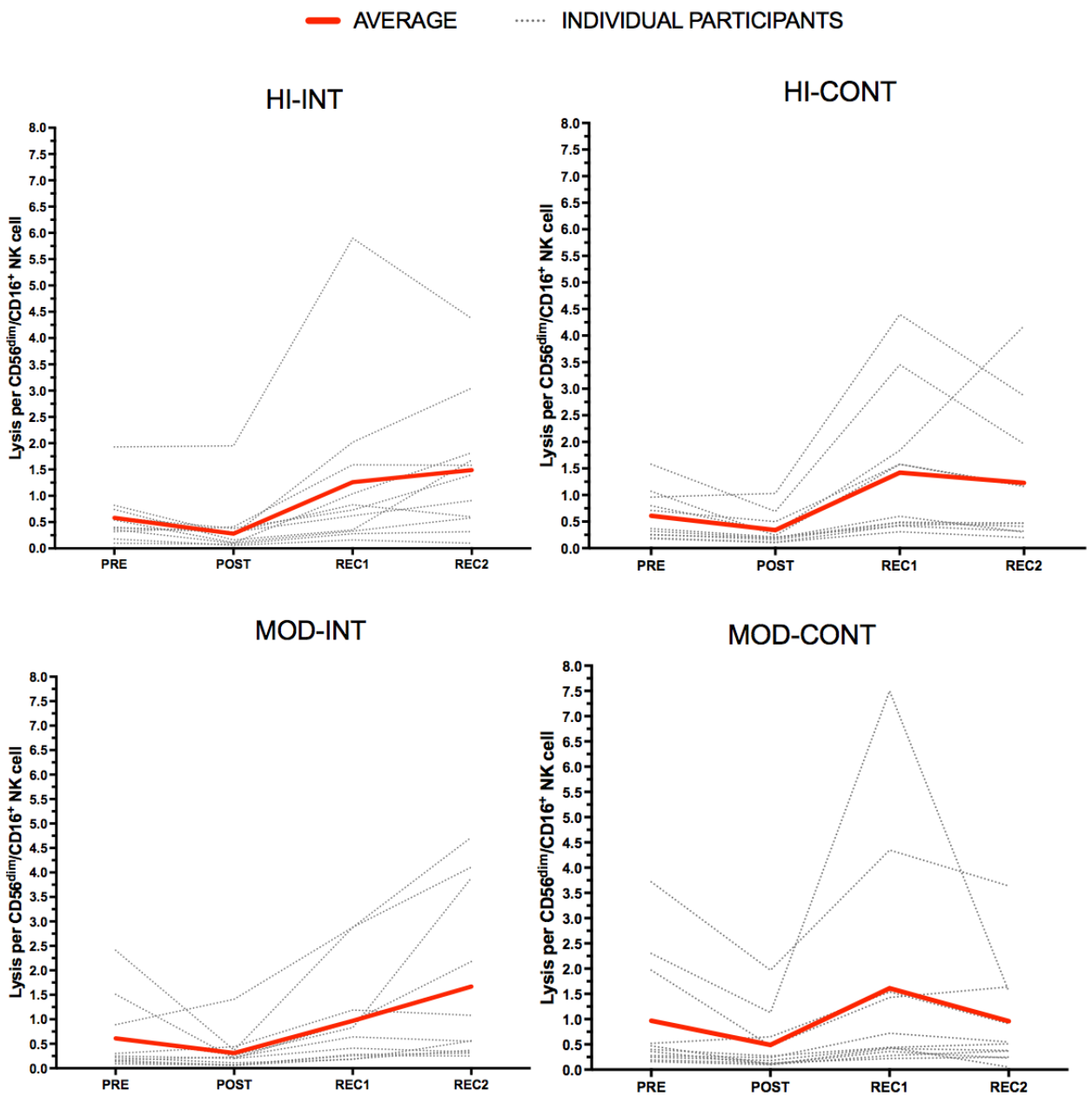


Figure A3-A: Specific lysis by participant during the 4 exercise protocols
Note: To better compare the magnitude of the response between participants, all participants are plotted using the same y-axis scale (0 to 70% range).

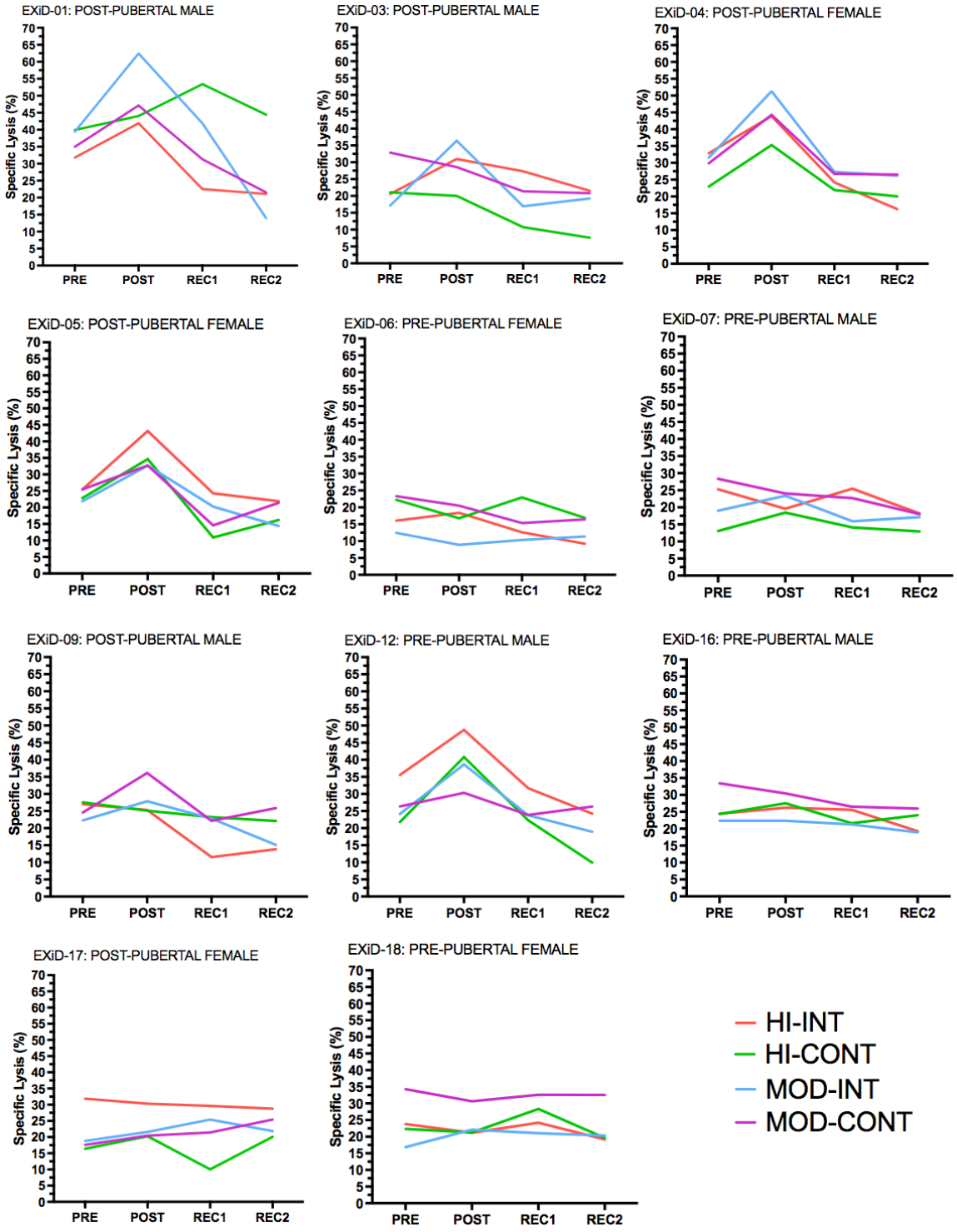


Figure A3-B: Specific lysis by participant during the 4 exercise protocols
Note: To better visualize the pattern of the NK response, participants are plotted using an individualized y-axis scale.

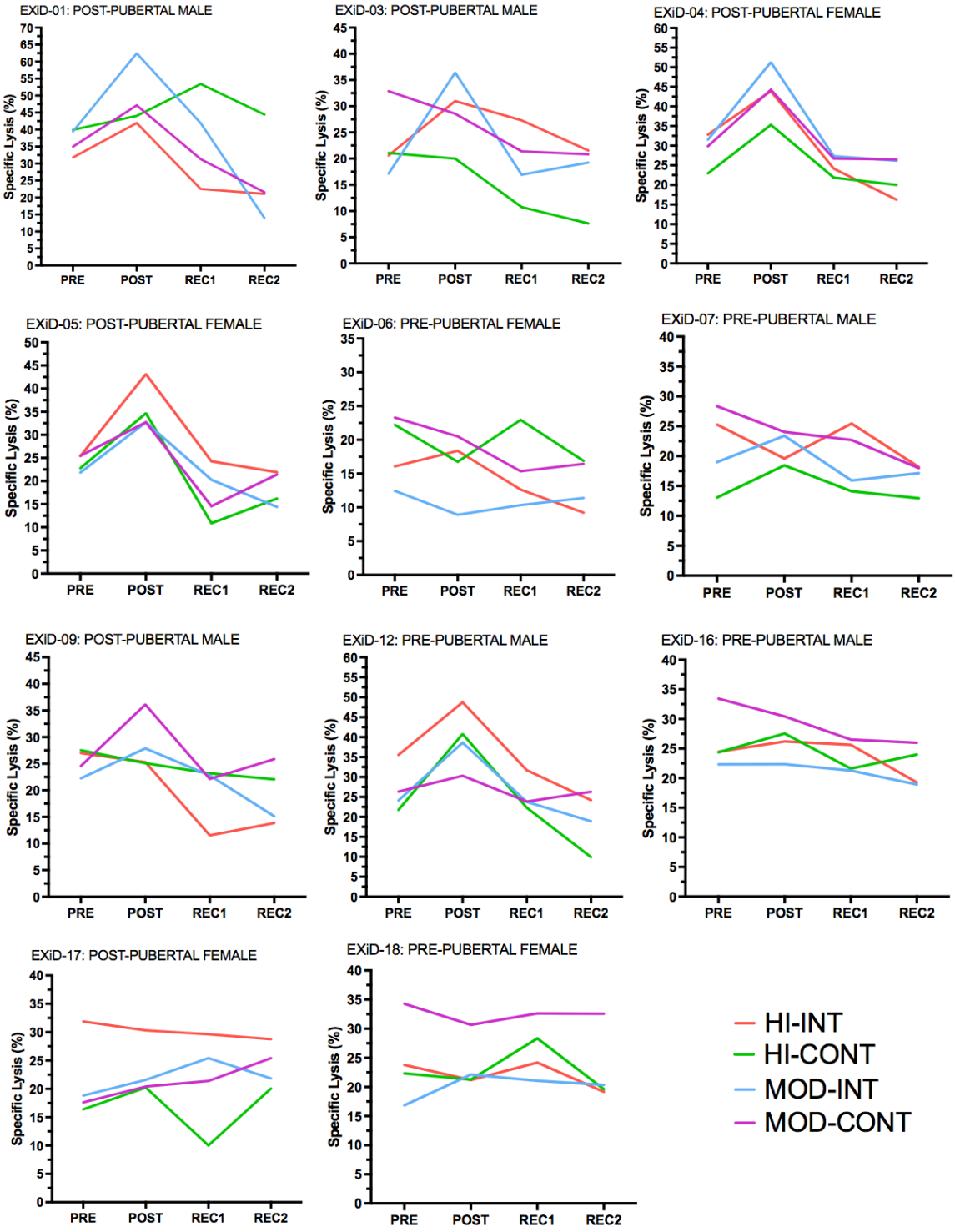


Figure A4-A: NKCA per cell by participant during the 4 exercise protocols
Note: To better compare the magnitude of the response between participants, all participants are plotted using the same y-axis scale (0 to 8 range).

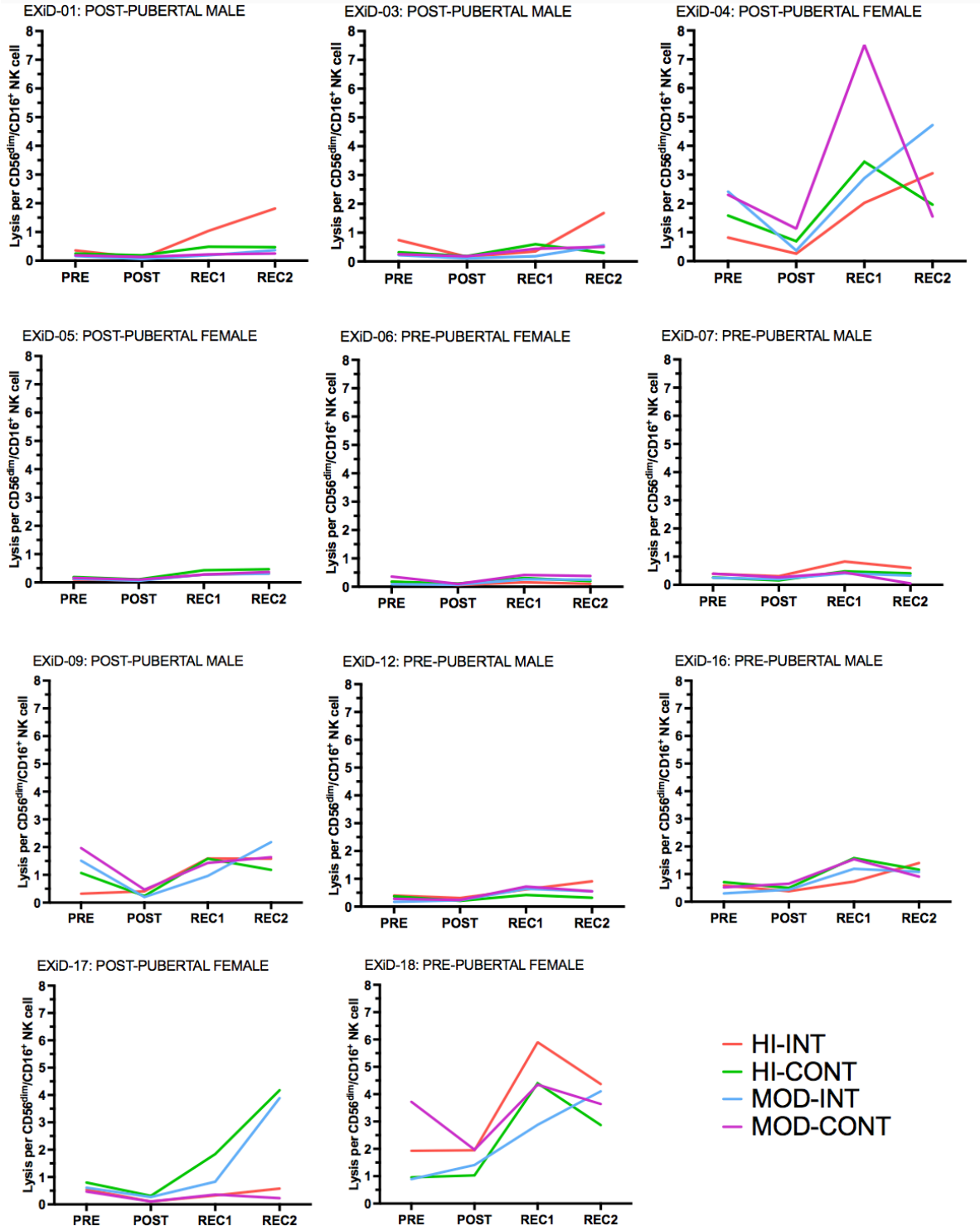
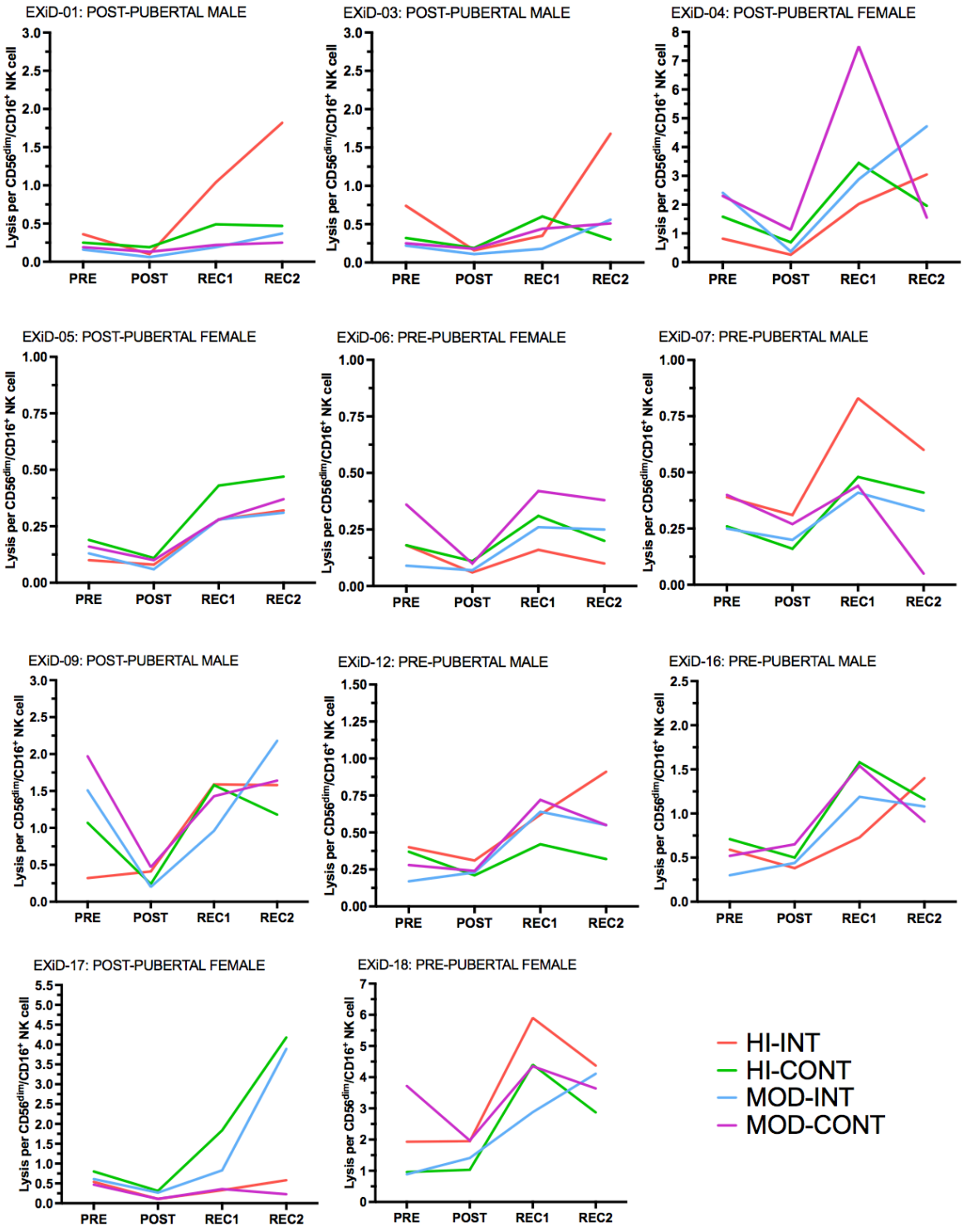


Figure A4-B: NKCA per cell during the 4 exercise protocols
Note: To better visualize the pattern of the NK response, participants are plotted using an individualized y-axis scale.



Appendix B: Effect of Target:Effector ratio

Effect of Ratio on NK Specific Lysis

A two-way repeated measures ANOVA was run to compare specific lysis obtained by 3 different T:E ratios (1:12.5, 1:25, 1:50) at different exercise timepoints (PRE, POST, REC1, REC2) in children. There was a statistically significant main effect for ratio ($F(2, 86) = 223.97$, $p < 0.001$; $\eta^2_p = 0.839$) and timepoint ($F(3, 129) = 29.84$, $p < 0.001$; $\eta^2_p = 0.410$), as well as an interaction between ratio and timepoint ($F(6, 258) = 4.94$, $p < .001$; $\eta^2_p = 0.103$). Overall, a graded effect was seen where specific lysis values obtained at the 1:50 ratio were consistently significantly higher than those obtained at 1:25, which were in turn higher than 1:12.5, regardless of timepoint ($p<0.001$ for all).

Note: All subsequent analyses were run using data obtained from incubations at the 1:50 ratio.

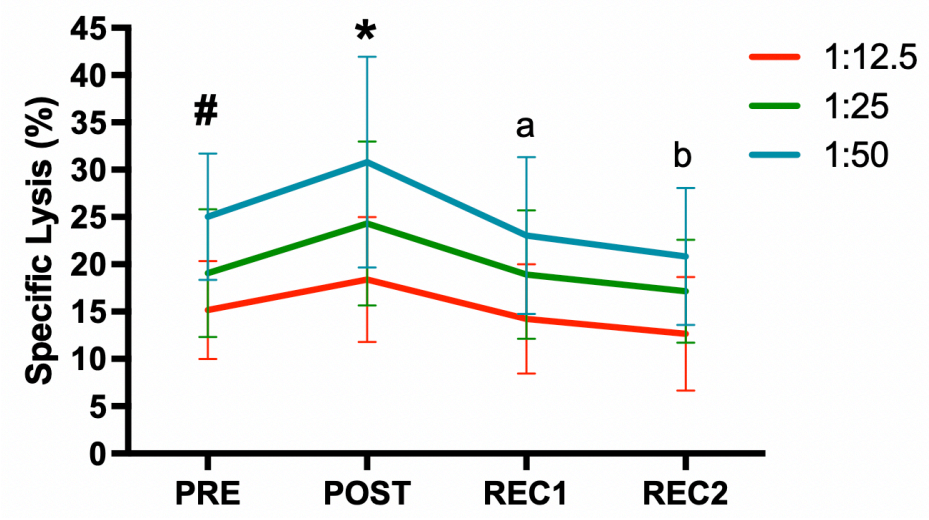


Figure 4. **Effect of Ratio on Specific Lysis**. Specific Lysis at 3 T:E ratios averaged across the 4 exercises (HI-INT, HI-CONT, MOD-INT, MOD-CONT). Specific lysis was highest for the 1:50 ratio across all timepoints. # = 1:50 > 1:25 and 1:12.5 at PRE, * = 1:50 > 1:25 and 1:12.5 at POST, a = 1:50 > 1:25 and 1:12.5 at REC1, b = 1:50 > 1:25 and 1:12.5 at REC2; all $p<0.001$. All data are presented as mean ± SD.

Appendix Table B1: Specific Lysis and NKCA per cell by Target:Effector ratio

TIMEPOINT	RATIO	SPECIFIC LYSIS (MEAN ± SD)	SPECIFIC LYSIS (RANGE)	NKCA PER CELL (MEAN ± SD)	NKCA PER CELL (RANGE)
High Intensity Intermittent Exercise (HI-INT)					
PRE	1 : 12.5	15.37 ± 4.95	6.55 – 21.12	1.11 ± 0.82	0.22 – 2.74
	1 : 25	19.33 ± 8.62	4.84 – 31.97	0.88 ± 0.79	0.09 – 2.95
	1 : 50	25.97 ± 5.97	16.06 – 35.55	0.71 ± 0.61	0.10 – 1.93
POST	1 : 12.5	18.53 ± 7.43	10.39 – 30.41	0.49 ± 0.41	0.10 – 1.6
	1 : 25	25.09 ± 8.85	8.07 – 36.02	0.44 ± 0.47	0.04 – 1.72
	1 : 50	31.79 ± 11.24	18.37 – 48.73	0.28 ± 0.25	0.06 – 0.95
REC1	1 : 12.5	15.07 ± 7.21	1.13 – 24.30	2.27 ± 3.53	0.04 – 12.69
	1 : 25	20.52 ± 5.11	10.43 – 26.86	2.03 ± 3.05	0.23 – 10.78
	1 : 50	24.45 ± 6.77	11.55 – 32.416	1.24 ± 1.65	0.16 – 5.90
REC2	1 : 12.5	12.74 ± 6.71	2.02 – 23.67	2.86 ± 3.25	0.14 – 11.26
	1 : 25	17.84 ± 4.95	9.78 – 23.92	2.19 ± 2.00	0.25 – 6.52
	1 : 50	20.66 ± 6.53	9.21 – 32.47	1.47 ± 1.28	0.10 – 4.37
High Intensity Continuous Exercise (HI-CONT)					
PRE	1 : 12.5	14.08 ± 5.66	7.98 – 27.88	1.15 ± 0.86	0.27 – 2.4
	1 : 25	16.72 ± 7.76	9.73 – 37.09	0.72 ± 0.53	0.21 – 1.83
	1 : 50	23.13 ± 6.76	13.08 – 39.91	0.61 ± 0.46	0.18 – 1.58
POST	1 : 12.5	16.30 ± 5.28	7.46 – 28.-3	0.78 ± 1.03	0.17 – 3.72
	1 : 25	20.94 ± 7.03	12.68 – 33.15	0.51 ± 0.57	0.09 – 2.02
	1 : 50	27.65 ± 9.55	16.76 – 44.04	0.34 ± 0.29	0.11 – 1.03
REC1	1 : 12.5	12.90 ± 6.98	4.19 – 28.57	2.68 ± 3.33	0.42 – 12.31
	1 : 25	18.04 ± 9.80	5.98 – 42.68	1.88 ± 1.86	0.35 – 6.49
	1 : 50	21.78 ± 12.81	10.04 – 53.41	1.42 ± 1.37	0.31 – 4.40
REC2	1 : 12.5	11.19 ± 6.67	3.61 – 26.06	2.98 ± 4.23	0.35 – 12.53
	1 : 25	15.83 ± 7.76	7.11 – 31.51	1.78 ± 1.96	0.21 – 6.66
	1 : 50	19.42 ± 9.71	7.62 – 44.43	1.23 ± 1.29	0.20 – 4.18
Moderate Intensity Intermittent Exercise (MOD-INT)					
PRE	1 : 12.5	14.37 ± 5.87	4.98 – 24.60	1.38 ± 1.60	0.15 – 5.13
	1 : 25	17.69 ± 5.09	11.50 – 26.06	0.95 ± 1.14	0.12 – 3.52
	1 : 50	22.34 ± 7.47	12.45 – 39.41	0.61 ± 0.74	0.09 – 2.41
POST	1 : 12.5	18.82 ± 8.85	8.69 – 39.05	0.53 ± 0.47	0.11 – 1.60
	1 : 25	25.56 ± 12.04	16.09 – 53.36	0.45 ± 0.53	0.08 – 1.91

	1 : 50	32.43 ± 15.37	8.90 – 62.45	0.31 ± 0.39	0.06 – 1.41
REC1	1 : 12.5	12.36 ± 2.79	7.63 – 16.58	1.86 ± 1.89	0.22 – 5.47
	1 : 25	16.29 ± 5.10	6.88 – 25.96	1.35 ± 1.60	0.18 – 4.99
	1 : 50	22.45 ± 8.00	10.34 – 41.88	0.97 ± 1.00	0.18 – 2.88
REC2	1 : 12.5	11.36 ± 4.15	4.57 – 20.00	3.62 ± 4.14	0.27 – 11.88
	1 : 25	15.43 ± 3.88	11.21 – 23.99	2.45 ± 2.64	0.41 – 7.79
	1 : 50	17.95 ± 4.15	11.39 – 26.17	1.67 ± 1.75	0.25 – 4.72
Moderate Intensity Continuous Exercise (MOD-CONT)					
PRE	1 : 12.5	16.77 ± 4.57	10.38 – 25.83	1.90 ± 2.69	0.36 – 8.92
	1 : 25	21.71 ± 4.37	13.17 – 28.13	1.18 ± 1.49	0.21 – 4.32
	1 : 50	28.28 ± 5.43	17.61 – 34.98	0.90 ± 1.14	0.16 – 3.72
POST	1 : 12.5	19.96 ± 4.43	13.97 – 25.57	1.17 ± 1.72	0.18 – 5.92
	1 : 25	25.75 ± 6.38	17.35 – 38.74	0.72 ± 0.98	0.13 – 3.52
	1 : 50	31.38 ± 8.61	20.42 – 47.14	0.45 ± 0.57	0.10 – 1.97
REC1	1 : 12.5	16.53 ± 4.72	6.37 – 26.03	2.75 ± 3.51	0.38 – 10.96
	1 : 25	20.74 ± 6.07	10.61 – 30.92	1.88 ± 2.36	0.27 – 7.18
	1 : 50	23.50 ± 5.66	14.57 – 32.61	1.50 ± 2.22	0.22 – 7.50
REC2	1 : 12.5	15.30 ± 6.12	6.91 – 26.26	2.14 ± 3.26	0.34 – 11.68
	1 : 25	19.46 ± 4.09	12.53 – 26.71	1.40 ± 1.77	0.25 – 6.27
	1 : 50	25.25 ± 6.28	16.44 – 38.40	0.91 ± 0.98	0.23 – 3.64

Appendix C: ANOVA Tables

Table C1: ANOVA table for effect of exercise on specific lysis

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	381	3	127.1	1.52	.229	0.132
Timepoint	2628	3	876.1	12.80	.000*	0.561
Exercise x Timepoint	247	9	27.5	1.45	.180	0.126

Table C2: ANOVA table for puberty and specific lysis

Variable	SS	Df	MS	F	P value	Partial eta-squared
Puberty	137.6	1	137.6	1.00	.344	0.100
Timepoint	849	3	283	11.51	.000*	0.561
Puberty x Timepoint	136.5	3	45.5	1.85	.162	0.171

Table C3: ANOVA table for sex and specific lysis

Variable	SS	Df	MS	F	P value	Partial eta-squared
Sex	10.2	1	10.2	0.07	.801	0.007
Timepoint	895.4	3	298.5	10.08	.000*	0.528
Sex x Timepoint	1.1	3	0.4	0.01	.998	0.001

Table C4: ANOVA table for effect of exercise on NKCA per cell

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	0.38	3	0.13	0.2	.895	0.020
Time	30.8	3	10.27	8.33	.000*	0.454
Exercise x Time	6.57	9	0.73	1.951	.055	0.163

Table C5: ANOVA table for puberty and NKCA per cell

Variable	SS	Df	MS	F	P value	Partial eta-squared
Puberty	0.85	1	0.851	0.253	.627	0.027
Timepoint	10.52	3	3.507	6.574	.002*	0.422
Puberty x Timepoint	0.79	3	0.265	0.496	.688	0.052

Table C6: ANOVA table for sex and NKCA per cell

Variable	SS	Df	MS	F	P value	Partial eta-squared
Sex	1.52	1	1.516	0.462	.514	0.049
Timepoint	10.92	3	3.64	6.966	.001*	0.436
Sex x Timepoint	1.09	3	0.364	0.697	.562	0.072

Table C7: ANOVA table for effect of exercise on total NK proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	106	3	35.5	4.49	.010*	0.310
Timepoint	2074	3	691.3	25.76	.000*	0.720
Exercise x Timepoint	167	9	18.5	5.19	.000*	0.342

Table C8: ANOVA table for puberty and total NK proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Puberty	235.5	1	235.5	8.05	.020*	0.472
Timepoint	698	3	232.7	36.37	.000*	0.802
Puberty x Timepoint	193.2	3	64.4	10.07	.000*	0.528

Table C9: ANOVA table for sex and total NK proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Sex	25.3	1	25.3	0.48	.505	0.051
Timepoint	738.8	3	261.3	20.07	.000*	0.690
Sex x Timepoint	14.6	3	4.9	0.37	.773	0.040

Table C10: ANOVA table for effect of exercise on $CD56^{bright}$ cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	1.033	3	0.344	2.134	.117	0.176
Timepoint	1.697	3	0.566	8.313	.000*	0.454
Exercise x Timepoint	0.54	9	0.06	1.368	.215	0.120

Table C11: ANOVA table for puberty and CD56^{bright} cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Puberty	1.46	1	1.46	1.517	.249	0.144
Timepoint	0.58	3	0.193	1.907	.152	0.175
Puberty x Timepoint	0.071	3	0.024	0.234	.872	0.025

Table C12: ANOVA table for sex and CD56^{bright} cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Sex	1.235	1	1.235	1.251	.292	0.122
Timepoint	0.584	3	0.195	1.958	.144	0.179
Sex x Timepoint	0.124	3	0.041	0.416	.743	0.044

Table C13: ANOVA table for effect of exercise on CD56^{dim}/CD16^{+} cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	51	3	17	2.60	.070	0.206
Timepoint	841.3	3	280.4	16.86	.000*	0.628
Exercise x Timepoint	85.2	9	9.5	4.14	.000*	0.293

Table C14: ANOVA table for puberty and CD56^{dim}/CD16^{+} cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Puberty	46.4	1	46.37	1.87	.204	0.172
Timepoint	275.5	3	91.83	20.71	.000*	0.697
Puberty x Timepoint	68.9	3	22.97	5.18	.006*	0.365

Table C15: ANOVA table for sex and CD56^{dim}/CD16^{+} cell proportion

Variable	SS	Df	MS	F	P value	Partial eta-squared
Sex	20.4	1	20.4	0.74	.413	0.076
Timepoint	310.1	3	103.4	15.51	.000*	0.633
Sex x Timepoint	8.7	3	2.9	0.44	.73	0.046

Table C16: ANOVA table for effect of T:E ratios on specific lysis

Variable	SS	Df	MS	F	P value	Partial eta-squared
Timepoint	4211	3	1404	29.8	.000*	0.410
Ratio	8494	2	4247	224	.000*	0.839
Timepoint x Ratio	265	6	44	4.9	.000*	0.103

Table C17: ANOVA table to compare % Max HR during exercise protocols

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	2756	3	919	234.9	.000*	0.777

Table C18: ANOVA table to compare RPE during exercise protocols

Variable	SS	Df	MS	F	P value	Partial eta-squared
Exercise	96	3	32	5.0	.007*	0.357

Appendix D: Parent/Guardian Consent Form and Assent Form

PARENT CONSENT FORM

Title of Study:	Characterizing the effects of **EX**ercise **i**ntensity and **D**uration on natural killer cell response at distinct stages of growth and development **(EXiD Study)**
Local Principal Investigator:	Dr. Joyce Obeid, PhD (Department of Pediatrics)
Principal Investigator:	Emily Hauck, MSc Candidate (Faculty of Health Sciences)
Funding Source:	Natural Sciences and Engineering Research Council of Canada

INTRODUCTION

Your child is being invited to participate in a research study conducted in Dr. Joyce Obeid's lab, and led by Emily Hauck. We are inviting your child because they are healthy. In order to decide whether or not you and your child want to be a part of this research study, you should understand what is involved and the potential risks and benefits. This form will give you detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you and your child wish to participate. Take your time to make your decision. Feel free to discuss it with your family.

WHY IS THIS RESEARCH BEING DONE?

The benefits of exercise have been studied for a long time in adults. In fact, many studies show that regularly exercise can improve a person's ability to fight off infection. This is because exercise can help improve Natural Killer cells, which are a type of immune cell in your body that are fast at recognizing invaders and killing them. However, studies in adult have also shown that natural killer cells can behave differently depending on how intensely or how long people exercise. What is surprising is that we don't know much about how these cells act in children and adolescents and so we are unable to develop a way to use exercise to improve the ability of the natural killer cells to do their job. As children grow and mature, their bodies change and with that, their immune system matures as well. The goal of the study is to understand the relationship between exercise and the body's ability to protect itself at different stages of development.

WHAT IS THE PURPOSE OF THIS STUDY?

We have a couple of questions to answer in this study. Our main goal is to see how different types of exercise affect the number and activity of the Natural Killer cells. We are also interested in understanding how growth and development play a role in this process by looking at differences in pre-pubertal and pubertal boys and girls.

WHAT WILL MY RESPONSIBILITIES BE IF THEY TAKE PART IN THE STUDY?

If your child volunteers to participate in this study, we will ask them to come to our lab at McMaster University on **5 occasions over a 5-week period**. These visits will be to conduct exercise testing in the laboratory. We will schedule these visits at your convenience.

Visit #1 will take about 2 hours to complete, and will include the following:

1) We will measure your child's height and weight. We will also ask your child to stand on a machine that will help us measure how much muscle and fat they have in their body. This machine does not hurt, it just requires your child to stand still for 2 minutes.
2) We will ask your child to assess their puberty status using a questionnaire.
3) We will assess your child's fitness level on a special bicycle. This exercise test (called a "VO_{2max} test") lasts 8 to 12 minutes and feels like your child is riding up a hill. During the test, we will ask your child to wear a face mask over their mouth and nose, this will help us measure how much oxygen they are breathing during the test. We will also ask your child to wear a heart rate monitor on their chest so we can monitor their heart rate throughout the test.
4) Before going home, we will give your child a small belt to take home for the week. This device is called an accelerometer, and it will help us measure your child's movement – your child's physical activity and sedentary time. It's like a Fitbit, except your child will wear it around their waist, and take it off any time they sleep or do any water activities (swimming or shower). Your child will be asked to wear this belt for 7 days, and to return it at their next study visit.

Visits #2-5 will take about 2.5 hours each to complete. We will book these visits at least 4 days apart. Each visit will be identical except for the type of exercise your child will do:

1) When your child comes into the lab, we will get them to lie down for 10 minutes. Then we will collect a resting blood sample. To do this we will put in a small, flexible plastic tube in their vein – this is called a catheter, and it will help us collect blood samples throughout your child's visit without having to poke your child with a needle each time.
2) After their resting sample, we will get your child set up on our bike and they will be asked to perform one of 4 exercises. The order of these exercises will be determined by chance, like picking a random number out of a hat:
    a. High intensity all-out intermittent cycling, where your child will pedal as hard as they can for 15-seconds, then get a 1-minute break. Your child will be asked to repeat the15-sec cycling/1-minute break a total of 24 times.
    b. Moderate intensity all-out intermittent cycling, where your child will pedal as hard as they can for 15-seconds, then get a 1-minute break. Your child will be asked to repeat the15-sec cycling/1-minute break a total of 24 times. For this test, the workload on the bike will be lower than the high intensity session.
    c. High intensity, continuous cycling, where your child will pedal at a comfortable pace for 30 minutes. The workload on the bike will feel challenging, but it will stay the same throughout the test.

d. Moderate intensity, continuous cycling, where your child will pedal at a comfortable pace for 30 minutes. The workload on the bike will feel easier than the high intensity session, and it will stay the same throughout the test.

3) We will collect blood samples before your child exercises, right after they exercise, 30-minutes after they stop exercising, and again 1 hour after they stop exercising. We will collect a total of 80 mL of blood. This is about 5 tablespoons of blood.
4) We will ask your child to fill out some questionnaires that tell us about their physical activity. All answers will be kept confidential.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

i) Blood sampling. An experienced investigator will insert the small needle that will be used to place the small, flexible plastic tube. Getting a poke with a needle can hurt. If your child is worried about the needle, we can use a special cream to numb the area where the needle will go in so they do not feel any pain. A small bruise might appear where the needle goes through the skin. Because we will provide water on a regular basis throughout each visit, taking this amount of blood will have no negative effects. There is also a chance your child will feel light-headed after the blood sample. We will have snacks and water on hand to minimize the risk of this happening.

ii) Exercise testing. The VO_{2max} test requires you to give a maximal effort. This means that your child will feel quite tired after they are done the test. The other exercise your child will be asked to complete during visits 2 – 5 will be very similar to what they might do as part of their daily life and may make them sweat and feel tired. There is a small chance your child could feel dizzy or nauseous when your child exercises, but this feeling will go away pretty quickly. None of the exercise tests will pose any health concern. We will monitor your child's heart rate during each exercise session.

HOW MANY PEOPLE WILL BE IN THIS STUDY?

We are asking 12 pre-pubertal and 12 pubertal boys and girls to participate in this study. Your participation is voluntary.

WHAT ARE THE POSSIBLE BENEFITS FOR MYSELF MY AND/OR FOR SOCIETY?

We cannot promise any personal benefits to your child from participation in this study. We will make each exercise session fun and enjoyable. Your child's participation will be very important for us to learn how natural killer cells work and will provide insight into how we can best utilize exercise to help strengthen the immune system. We will provide your child with a report card at the end of the study letting them know how they did.

WHAT INFORMATION WILL BE KEPT PRIVATE?

All of your child's information will be stored in locked filing cabinets or a password protected computer under the supervision of Dr. Joyce Obeid for 10 years. No one outside of our research team will have access to your information. Your child will be assigned a participant number, and this number will be used to identify them. Records identifying your child will be kept confidential. If the results of the study are published, your child's identity will remain confidential.

CAN PARTICIPATION IN THE STUDY END EARLY?

If your child volunteers to be in this study, they may withdraw at any time with no judgement by informing the investigator, Emily Hauck at 438 – 868 – 8667 by email at haucke@mcmaster.ca. The investigator may withdraw your child from this research if circumstances arise which warrant doing so.

WILL I BE PAID TO PARTICIPATE IN THIS STUDY?

We will provide your child $100 as reimbursement for their participation in this study. If your child quits the study for personal reasons, we will change the amount for the time completed. If you and your child choose to quit because of a complication from the study, we will give your child the full amount.

IF I HAVE ANY QUESTIONS OR PROBLEMS, WHOM CAN I CALL?

If you have any questions about the research now or later, or if you think you may have a research-related injury, Emily Hauck at 436 – 868 – 8667. Dr. Joyce Obeid directly at 905-521-2100 extension 75865 (Daytime) or 905-928-5538 (Nighttime). If you have any questions regarding your rights as a research participant, you may contact the Office of the Chair of the Hamilton Integrated Research Ethics Board at 905-521-2100 extension 42013.

CONSENT STATEMENT

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to allow my child to participate in this study entitled: "Characterizing the effects of **EX**ercise **i**ntensity and **D**uration on natural killer cell response at distinct stages of growth and development **(EXiD Study)"**. I understand that I will receive a signed copy of this form.

Would you like to be contacted by Dr. Obeid or a member of the Child Health & Exercise Medicine Program research team with information about future studies other than the one described in this consent form? Any future studies would be approved by the Research Ethics Board, and would require you to sign a new consent form. **Please note we will only contact you if you are eligible for a maximum of 2 times per year.**

☐ Yes, please contact me. ☐ No, please do not contact me.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____
Signature of Legally Authorized Representative Date

Consent form administered and explained in person by:

_____
Name and title

_____ _____
Signature Date

SIGNATURE OF INVESTIGATOR:

In my judgement, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to participate in this research study.

_____ _____
Signature of Investigator Date

FUTURE RESEARCH

At the end of the study, we may wish to store leftover blood sample for use in future studies which will aim to understand how exercise influences the immune system. We will not store your child's sample longer than 10 years. All records identifying your child will remain confidential. Information about your child will not be released. If the results of the study are published, your child's identity will remain confidential. No genetic testing will be performed on these samples. Hamilton integrated Research Ethics Board approval will be obtained for these future studies.

CONSENT STATEMENT FOR STORAGE OF SAMPLES (BLOOD)

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to have my child's blood stored so it can be used in future research studies approved by the Hamilton Integrated Research Ethics Board other than the one described in this information form.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____
Signature of Legally Authorized Representative Date

Consent form administered and explained in person by:

_____
Name and title

_____ _____
Signature Date

SIGNATURE OF INVESTIGATOR:

In my judgement, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to have their child's blood stored so it can be used in future research studies approved by the Hamilton Integrated Research Ethics Board other than the one described in this information form.

_____

Name and title

Signature of Investigator Date

PHOTO, AUDIO AND VIDEO RELEASE FORM (OPTIONAL)

I, _____, hereby give McMaster University's Faculty of Health Sciences my permission to take and use any photographs, movie films, audio or video tapes taken on (date) _____, and I consent to the reproduction of same in any proper manner whatsoever for possible publication and I hereby waive any rights that I may have in such photographs, movie films and audio or video tapes or reproductions of same.

I hereby release McMaster University's Faculty of Health Sciences, its employees, agents, servants and attending physicians from all actions, causes of actions, claims and demands arising out of such consent.

Notice of collection of personal information

By taking my photograph, whether by still photograph, film or video and/or taping my voice, I acknowledge that McMaster University is collecting my personal information as defined by the Freedom of Information and Protection of Privacy Act of Ontario (RSO 1990).

The personal information is collected under the authority of The McMaster University Act, (1976). The information is used for public relations purposes of the Faculty of Health Sciences including, but not limited to, publications, websites and materials promoting McMaster University. Personal information will not be used for any unrelated purpose without prior consent. This information is protected and is being collected pursuant to section 39(2) and section 42 of the Freedom of Information and Protection of Privacy Act of Ontario (RSO 1990). Questions regarding the collection or use of this personal information should be directed to the Manager, Public Relations, Faculty of Health Sciences.

Name of Participant	Signature of Participant	Date
Name of Witness	Signature of Witness	Date

CHILD ASSENT FORM

Title of Study:	Characterizing the Effects of Exercise Intensity and Duration on Natural Killer Cell Response at Distinct Stages of Growth and Development
Local Principal Investigator:	Dr. Joyce Obeid, PhD (Department of Pediatrics)
Principal Investigator:	Emily Hauck, MSc Candidate (Faculty of Health Sciences)
Funding Source:	Natural Sciences and Engineering Research Council of Canada

Why are we doing this study?
A research study is a way to learn more about people. We are doing a research study to learn about how exercise changes your immune system. Your immune system is made up of different cells that help protect you from getting sick, one type of cell is called a natural killer cell. A natural killer cell is one of the first cells to protect your body from a virus or an invader. When you exercise, signals are sent natural killer cells that help them move around your body to look for invaders. We want to see if how different types of exercise help these cells do their job.

Why am I being asked to be in the study?
We are inviting you to be in the study because you are a healthy, young person.

What if I have questions?
You can ask questions if you do not understand any part of the study. If you have questions later that you don't think of now, you can talk to me again or ask your mom or dad to call Emily at 438-868-8667 or email haucke @mcmaster.ca or contact Dr. Joyce Obeid at 905-521-2100 ext. 75865.

If I am in the study what will happen to me?
If you decide that you want to be part of this study, you will be asked to come visit our exercise lab 5 times. During each visit, we will ask you to ride a special bicycle. At your first visit, we will do a bike ride that feels like riding up a hill, you will also wear a face mask so we can measure how much oxygen your body is using. We will give you a small belt to wear at home to find out how active you are. When you come back for the rest of your study visits, we'll ask you to do different cycling activities. We will take a little bit of blood from a vein in your arm at these visits so that we can measure the natural killer cells. We will also write down how fast your heart is beating and how much air you are breathing when you are exercising.

Will I be hurt if I am in the study?
To take some blood, we need to put a special tiny plastic tube in your arm. We use a needle to make sure the tube goes in the right spot, but the needle part does not stay in your arm. The special tiny plastic tube will not hurt when it is in your arm. There is a small chance that you will get a bruise on

your arm where the needle first went in, but this will go away after a few days. The exercise you will do might make you feel a little tired, and some kids feel a bit dizzy, but it will not hurt you.

Will the study help me?
If you are in the study, it may not benefit you directly. You will learn about your fitness and physical activity level. We will also learn how exercise affects your immune system.

Do I have to be in this study?
You do not have to be in this study, if you do not want to be. If you decide that you don't want to be in the study after we begin, that's OK too. You can tell me, and nobody will be angry or upset. It is your choice. We are discussing the study with your parents and you should talk to them about it too.

What happens after the study?
When we are finished this study, we will write a report about what we learned. This report will not include your name or that you were in the study.

Assent:
If you decide you want to be in this study, please print/write your name in the spot below. If you decide that you don't want to be in the study, even if you have started in the study, then all you have to do is tell Dr. Obeid or Emily that you don't want to be in the study anymore. You can call Dr. Obeid at 905-521-2100 extension 75865 or Emily at 438-868-8667 at any time.

If you have questions regarding your rights as research participant, you may contact office of the Chair of Hamilton Integrated Research Ethics Board at 905-521-2100 ext. 42013.

I, _____ (Print your name) would like to be in this research study.

_____ (Date of assent)

_____ (Name of person who obtained assent)

_____ (Signature of person who obtained assent and Date)

_____ (Local Principal Investigator name)

_____ (LPI signature and Date)

Appendix E: Medical and Physical Activity Questionnaires

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

Date of Birth: _____

1. Does your child have any of the following conditions (circle each of the appropriate):
   a) Heart disease
   b) High blood pressure
   c) Loss of consciousness
   d) Asthma
   e) Intestinal disease
   f) Surgery or fractures
   g) Allergies
   h) Diabetes
   i) Epilepsy
   j) Others: _____
   k) None

2. Present health:
   a) Good
   b) Complaints: _____

3. Is your child currently taking any medications? If yes, how frequently?

Medication	Frequency
_____	_____
_____	_____
_____	_____
_____	_____

☐ My child is not currently taking any medications

4. Has your child taken any medications in the past month? If yes, how frequently?

Medication	Frequency
_____	_____
_____	_____
_____	_____
_____	_____

☐ My child has not taken any medications in the past month

5. When thinking of prior exercise involvement, has your child experienced (circle the appropriate):
   a) An inability to keep up with other children
   b) Chest pain
   c) Fainting
   d) Dizziness
   e) Irregular heart beat
   f) Wheezing
   g) Other: _____
   h) None of the above

6. Has your physician ever suggested that your child restrict their levels of physical activity?
   ☐ Yes
   ☐ No

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

7. Do you know of any medical reason that would prevent your child from participating in physical activity?

   ☐ Yes. Please specify: _____
   ☐ No

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

Menstrul Cycle Questionnaire

1. Have you had your first period yet?
   - ☐ Yes (please complete questions #2-6)
   - ☐ No (you do not need to complete the rest of the questionnaire)

2. Approximately how old were you when you had your first period?

3. When is the last time you had your period?
   - ☐ I can't remember
   - ☐ The first day of my last period was on: _ _ - _ _ _ - _ _ _ _ (day – month – year)
   - ☐ I don't remember the first day, but I know I had my period on: _ _ - _ _ _ - _ _ _ (day – month – year)

4. On average, how many days does your period last?
   - ☐ 1 – 2 days
   - ☐ 3 – 4 days
   - ☐ 5 – 7 days
   - ☐ More than 7 days
   - ☐ It's different each time
   - ☐ I'm not sure

5. Do you get your period every month? If not, how often do you think you get your period?
   - ☐ Yes
   - ☐ No, I get my period: _____

6. Are you currently taking an oral contraceptive (birth control)?
   - ☐ Yes, name (if you remember): _____; I have been taking it for: _____
   - ☐ No, I never have
   - ☐ No, but I used to. I stopped taking it: _____.

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

ACTIVITY QUESTIONNAIRE

Dear Parent:
The purpose of the following questionnaire is to help us evaluate the activity habits of your child. Please be as accurate as possible in your answers. Feel free to add any details that seem relevant.

1. How would you compare the physical activity of your child to that of her/his friends?
   - ☐ My child is as active as her/his friends
   - ☐ My child is more active than her/his friends
   - ☐ My child is less active than her/his friends
   - ☐ Not sure

2. How would you compare the activity of your child with that of her/his sibling(s)?
   - ☐ My child is as active as her/his sibling(s)
   - ☐ My child is more active than her/his sibling(s)
   - ☐ My child is less active than her/his sibling(s)
   - ☐ Not applicable (no siblings)
   - ☐ Not sure

3. How many hours in a typical day is your child engaged in the following activities:

Activity	Less than 1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	More than 5 hours
TV						
Video games						
Computer						
Phone						

4. Which mode of transportation does your child use to travel to and from school?
   - ☐ Car / bus
   - ☐ Walking
   - ☐ Biking
   - ☐ Other:_____

   If biking or walking; what is the total time spent actively travelling per day?
   - ☐ 0-10 minutes
   - ☐ 10-20 minutes
   - ☐ 20-30 minutes
   - ☐ More than 30 minutes

5. In your opinion, is your child as active as she/he should be?
   - ☐ Yes
   - ☐ My child is too active
   - ☐ My child is not sufficiently active
   - ☐ Not sure how much physical activity she/he needs

6. If your child is not as active as she/he should be, what, in your opinion, is the reason? (you can select more than one answer)
   - ☐ Lack of interest
   - ☐ Disease
   - ☐ Lack of suitable conditions
   - ☐ Other: _____
   - ☐ I don't know

7. In a typical week, how many days a week does your child participate in organized sport outside of school?
   - ☐ Once a week
   - ☐ 2 – 3 times a week
   - ☐ 4 – 5 times a week
   - ☐ More than 5 times a week
   - ☐ My child does not participate in sport outside of school
   - ☐ I don't know

8. How many hours a day is your child involved in organized sport?
   - ☐ Less than 1 hour a day
   - ☐ 1 – 2 hours a day
   - ☐ More than 2 hours a day
   - ☐ Not applicable

9. If your child participates in organized sport outside of school, please list the sport(s) below:

   _____

   _____

   _____

   _____

10. In a typical week, how many days a week does your child engage in spontaneous physical activity (e.g. walking the dog, going to the park, riding their bike, etc.)?
    - ☐ Once a week
    - ☐ 2 – 3 times a week
    - ☐ 4 – 5 times a week
    - ☐ 6 – 7 times a week
    - ☐ My child does not engage in spontaneous activity
    - ☐ I don't know

11. How many hours a day does your child engage in spontaneous physical activity?
    - ☐ Less than 1 hour a day
    - ☐ 1 – 2 hours a day
    - ☐ 3 – 4 hours a day
    - ☐ More than 4 hours a day
    - ☐ I don't know