CIRCULATING ENDOTHELIAL AND PROGENITOR CELLS IN CHILDREN

CIRCULATING ENDOTHELIAL AND PROGENITOR CELLS IN HEALTHY CHILDREN AND CHILDREN WITH JUVENILE IDIOPATHIC ARTHRITIS: ROLE OF FITNESS, PHYSICAL ACTIVITY, AND ACUTE EXERCISE

By JOYCE OBEID, H.B.Sc. Kinesiology

A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2015) McMaster University
(Kinesiology) Hamilton, Ontario

TITLE: Circulating progenitor and endothelial cells in healthy children and children with juvenile idiopathic arthritis: role of fitness, physical activity, and acute exercise

AUTHOR: Joyce Obeid, H.B.Sc. Kinesiology (McMaster University)

SUPERVISOR: Dr. Brian W. Timmons

NUMBER OF PAGES: xvii, 246

LAY ABSTRACT

Fitness and physical activity are critical for maintaining and improving cardiovascular health in children and adults. We don't know exactly how they do this but the evidence in adults suggests it may be related to rare cells in the blood involved in repairing damaged blood vessels. The main objective of this thesis was to learn more about these cells, called circulating endothelial cells and endothelial progenitor cells, in children. We found that only circulating endothelial cells were related to fitness and physical activity. We also found that endothelial progenitor cells increased when healthy children performed 60 minutes of cycling. On the other hand, these cells did not change when children with juvenile idiopathic arthritis performed the same exercise. More research is needed to determine exactly why these cells responded to exercise in healthy but not sick children, and to help us identify the optimal exercise to improve these cells in youth.

ABSTRACT

Circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) are sensitive markers of cardiovascular damage and repair, respectively. The aim of this thesis was to advance the state of knowledge regarding CECs and EPCs, and the factors affecting their concentrations, in children and adolescents.

The first and second studies demonstrated that CECs and EPCs were similar when participants were split by sex, chronological age (8-10 vs. 14-16 years), and biological age (≤ -1 vs. ≥ +1 year from peak height velocity). Moreover, CECs, but not EPCs, were positively associated with aerobic fitness and negatively related to daily moderate-to-vigorous physical activity. Neither CECs nor EPCs were related to level of adiposity. Although there was a large degree of inter-individual variability in both cell types, most of our data were clustered towards one end of the reported range. These observations highlighted the need to examine these cells in children with chronic conditions associated with an increased risk of poor cardiovascular health. Therefore, CECs and EPCs were examined at rest and in response to acute exercise in juvenile idiopathic arthritis (JIA) and healthy controls. Resting levels of CECs and EPCs were similar in both groups, which may be attributable to the low disease activity in the participants with JIA. High intensity, intermittent exercise (HIIE) and moderate intensity, continuous exercise (MICE) had no effect on CECs in

both groups. Conversely, MICE led to a robust increase in EPCs in healthy controls; no such change was observed in youth with JIA.

This thesis represents the first comprehensive assessment of CECs and EPCs in the context of fitness, physical activity, and acute exercise in children and adolescents. Future research should examine the function and fate of these cells in youth, as well as the potential mechanisms underlying the blunted EPC response to exercise in JIA.

ACKNOWLEDGEMENTS

I have been very fortunate to be surrounded by incredible people throughout my life, and the last seven years are no exception. I would like to thank several individuals who have helped make graduate studies one of the most memorable and rewarding experiences of my life so far.

First, to my supervisor, Dr. Brian Timmons, there aren't enough words to express my gratitude and indebtedness to you. Thank you for every challenge and every opportunity you've given me since the day I ran after you in the halls of the Ivor Wynne Centre! Thank you for being there when it mattered most, and for always believing in my potential as a researcher. You lead by example and I am honoured to have learned from you.

I would also like to express a sincere thank you to the members of my supervisory committee who have invested a tremendous amount of time and energy into helping me develop as an independent scientist. Dr. Martin Gibala, thank you for helping me see the big picture in both my research and career. Dr. Gianni Parise, thank you for challenging me to understand both sides of every argument, and for all of your thoughtful advice. Dr. Maggie Larché, thank you for your constant support in the clinic, for your enthusiasm and interest in our exercise research, and most importantly, for your friendship over the last eight years.

I am most grateful to the faculty, staff, and graduate students in the Department of Kinesiology for helping to make McMaster my home. I would also like to thank the staff with the Children's Exercise & Nutrition Centre/Child Health & Exercise Medicine Program for making me feel welcome from my very first day as an undergraduate student volunteer. To Shirley Lampman, thank you for all of your hard work, administrative support, and for your interest in my research. And to Dr. Bogdan Wilk, thank you for sharing the most wonderful stories of your research experience and travels with me, and for teaching me that just about everything can be fixed, it just takes a bit of patience and a lot of creativity!

I am blessed to have the support of a fantastic group of family and friends. Thank you to my Leamington family: Lindsey and Dylan Langlois, Andrea Obeid, Jenny Khoury, Myriah Diab, Jessica and Lamar Hanna, Sharon and Sam Zad, Bobby Peters, Anthony Obeid, Amanda and Marcello Mastronardi, Ana-Teresa Obeid, Nicole Zakaria, Mary-Jane Nohra, Margo Youssef; and to my Hamilton/international family: Hilde Ploeger, Lisa Viniika-Timmons, Peter Breithaupt, Jenna Gillen, Rachel Polito, Jules Pocsai, Kim Volterman, Hilary Caldwell, Natascja Di Cristofaro, Taralyn Schwering, Gabi Leites, Yasmeen Mezil, Maddy McDonald, and Conor Fanning. It goes without say that I am forever grateful to each of you for your endless support, encouragement, and willingness to listen (even when I'm not making sense).

I would especially like to recognize a remarkable group of women that I am privileged to call my colleagues and dear friends: Rachel Walker, Nicole Proudfoot, Thanh Nguyen, Sara King-Dowling, and Lisa Chu. Thank you for being my sounding board, for always keeping me in check, for cheering me up on the worst days, and for celebrating with me on the best days.

I would also like to extend a very special thank you to the most amazing group of children who never fail to remind me of the really important things in life: Aiden, Carson, and Rowan Timmons; Finlay and Tristan Larché; Adam Zad, Ellie Obeid, and Christopher Hanna.

Last but most certainly not least, to my family, this thesis is truly a product of your never-ending love and faith in me from the very start. Gidou and Sitou, thank you for every candle you have lit and prayer you have said in my intention. Dad, thank you for finding a way to make me laugh every single day. Leo, you are my rock. Thank you for keeping me grounded, for reminding me to lighten up when I get too serious, and for being there for me through the good and bad. Finally, Mom, thank you for being my best friend, and for knowing exactly what to say every time I've been down or stuck along the way. Thank you for believing in me, and for encouraging me to pursue my dreams wholeheartedly; I would never have made it this far without your support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ANA	Antinuclear Antibody
ANOVA	Analysis of Variance
BMI	Body Mass Index
CAC	Circulating Angiognic Cell
CD	Cluster of Differentiation
CEC	Circulating Endothelial Cell
CFU	Colony Forming Unit
CI	Confidence Interval
CRP	C-Reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
CXCR4	CXC Receptor 4
EMP	Endothelial Microparticle
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
ES	Effect Size
ESR	Erythrocyte Sedimentation Rate
FLK-1	Fetal Liver Kinase-1
FLT-3	FMS-Like Tyrosine Kinase 3
FMD	Flow-Mediated Dilation
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HbA1c	Glycated Hemoglobin
HDL	High Density Lipoprotein
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxic-Inducible Factor 1
HIIE	High Intensity Interval Exercise
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule-1
ICC	Intraclass Correlation Coefficient
IFN-y	Interferon Gamma
IL	Interleukin
IQR	Interquartile Range
JIA	Juvenile Idiopathic Arthritis
KDR	Kinase Insert Domain Receptor
LDL	Low Density Lipoprotein
MICE	Moderate Intensity Continuous Exercise

mKitL	Membrane-Bound Kit Ligand
MMP	Matrix Metalloproteinase
MVPA	Moderate-to-Vigorous Physical Activity
NO	Nitic Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
PBMC	Peripheral Blood Mononuclear Cells
RA	Rheumatoid Arthritis
SD	Standard Deviation
SDF-1	Stromal-Derived Factor-1
sKitL	Solubile Kit Ligand
SMD	Standarized Mean Difference
TNF	Tumor Necrosis Factor
UEA-1	Ulex Europaeus Lectin-1
VCAM-1	Vascular Cellular Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
$\dot{V}O_{2peak}$	Maximal Volume of Oxygen Consumed
VSMC	Vascular Smooth Muscle Cell
vWF	von Willebrand Factor
W_{peak}	Peak Mechanical Power

FORMAT AND ORGANIZATION OF THIS THESIS

This thesis was prepared in the "sandwich thesis" format outlined in the McMaster University School of Graduate Studies Guide for the Preparation of Master's and Doctoral Theses, published in November 2014. The first chapter is an introduction, which sets the context for the complete body of research. Chapter 2 is a brief description of important study design and methodological details that were omitted due to journal-specific restrictions in subsequent chapters. Chapters 3, 4, and 5 consist of three original research papers, which are either published or under peer-review. These chapters are formatted in accordance with the requirements of the journal in which they are published or submitted for publication. A concluding chapter (Chapter 6) summarizes and discusses the main findings of this thesis and includes future research directions. Finally, appendices are also included to supplement Chapters 1, 2, and 6.

CONTRIBUTIONS TO MULTI-AUTHORED PAPERS

Chapter 3:

Publication: Obeid J, Nguyen T, Walker RG, Gillis LJ, and Timmons BW (2014). Circulating endothelial cells in children: role of fitness, activity, and adiposity. *Medicine & Science in Sports & Exercise* 46: 1974-1980.

Contributions: The experiment was designed by B.W. Timmons, assisted by J. Obeid. J. Obeid recruited all participants, assisted by L.J. Gillis. J. Obeid collected the data, assisted by T. Nguyen. J. Obeid analyzed the data, assisted by R.G. Walker and B.W. Timmons. J. Obeid drafted the manuscript with assistance from B.W. Timmons; all co-authors reviewed and approved the final manuscript. The primary supervisor for this study was B.W. Timmons.

Chapter 4:

Publication: Obeid J, Nguyen T, and Timmons BW. Circulating endothelial progenitor cells in youth: fitness, physical activity, and adiposity. *International Journal of Sports Medicine* (In Review).

Contributions: The experiment was designed by B.W. Timmons,

assisted by J. Obeid. J. Obeid recruited all participants. J. Obeid collected the data, assisted by T. Nguyen. J. Obeid analyzed the data, assisted by R.G. Walker and B.W. Timmons. J. Obeid drafted the manuscript with assistance from B.W. Timmons; all co-authors reviewed and approved the final manuscript. The primary supervisor for this study was B.W. Timmons.

Chapter 5:

Publication: Obeid J, Nguyen T, Cellucci T, Larché MJ, and Timmons BW. Effects of exercise on circulating endothelial and progenitor cells in children and adolescents with juvenile idiopathic arthritis and healthy controls. *Pediatric Rheumatology* (In Review).

Contributions: The experiment was designed by B.W. Timmons and J. Obeid. T. Cellucci and M.J. Larché provided clinical expertise with selection of inclusion/exclusion criteria, and identified eligible study participants. J. Obeid collected the data, assisted by T. Nguyen. J. Obeid analyzed the data. J. Obeid drafted the manuscript with assistance from B.W. Timmons; all co-authors reviewed and approved the final manuscript. The primary supervisor for this study was B.W. Timmons.

CHAPTER 1: INTRODUCTION

Cardiovascular disease is the leading cause of mortality worldwide (Pagidipati and Gaziano 2013, Shanthi et al. 2014) and it begins in childhood (Raitakari et al. 2003, Charakida et al. 2007). Given that the incidence of cardiovascular disease is on the rise (Heidenreich et al. 2011), monitoring and detection of early changes in cardiovascular health are critical so as to minimize and potentially prevent the development of overt cardiovascular disease. This has led to the identification and tracking of a number of traditional risk factors and emerging biomarkers of cardiovascular health in pediatric and adult populations (Balagopal et al. 2011, Manuel et al. 2014). Among these are circulating endothelial cells (CEC) and endothelial progenitor cells (EPCs), which are sensitive markers of damage and repair, respectively, to the vascular endothelium (Asahara et al. 1997, Hill et al. 2003, Werner et al. 2005, Erdbruegger et al. 2006, Boos et al. 2007). Recent data suggests that these cells are responsive to physical exercise, and may represent one mechanism translating physical activity and exercise into improved cardiovascular outcomes, at least in adults (Silva et al. 2012, Volaklis et al. 2013). Unfortunately, we know very little about these cells in the pediatric years. A better understanding of both CECs and EPCs holds a number of important implications for children today, who are reportedly less fit (Tremblay et al. 2010), and more overweight/obese (Shields 2006,

Janssen et al. 2012, Roberts et al. 2012) compared with previous generations of children and adolescents. These trends, together with the fact that approximately 90% of Canadian children are not meeting the daily physical activity recommendations, suggest that these youth may be at an increased risk of developing early cardiovascular pathologies (Rizzo et al. 2007, Tanha et al. 2011). Understanding CECs and EPCs in pediatric clinical populations is equally, if not more, important given that children with chronic medical conditions are now living much longer than before but with an increased burden of secondary, disease-related health complications, including cardiovascular disease (Kavey et al. 2006). Indeed, identification and characterization of CECs and EPCs as early markers of cardiovascular health in youth may help to optimize physical activity and exercise prescriptions so as to maximize benefits to cardiovascular health. This thesis represents the first efforts towards a comprehensive assessment of the naturally occurring levels of EPCs and CECs, as well as the factors affecting their concentrations, in healthy children and adolescents, as well as those with juvenile idiopathic arthritis (JIA), who may be at an increased risk of cardiovascular disease (Argyropoulou et al. 2003, Vlahos et al. 2011).

1.1 Overview of the Vascular Endothelium

1.1.1 Structure and Function

The endothelium is a monolayer of simple squamous cells that lines the inner surface of the entire vascular system. Together with the basement membrane and internal elastic lamina, it makes up the tunica interna or the intima layer of the artery (Tortora and Grabowski 2002). Generally, the endothelium is the only tissue in direct contact with circulating blood, and as such, is the first to be influenced by physical, hormonal, or chemical changes in the blood (Deanfield et al. 2007). Endothelial cells respond to these changes by producing or regulating the release of a number of biologically active molecules (e.g., nitric oxide [NO], prostacyclin, endothelin-1), which in turn modify blood vessel wall tone, inflammation, as well as coagulant and thrombotic properties (Jaffe 1987, Deanfield et al. 2007). Thus, the endothelium plays a critical role in the maintenance of vascular homeostasis.

1.1.2 The Role of the Endothelium in the Pathogenesis of Atherosclerosis

The pathogenesis of atherosclerosis is a complex process involving the interaction of a number of growth factors, cytokines, and vasoregulatory molecules, and it begins in the endothelium. More specifically, physical or chemical stress to the endothelium will trigger

endothelial activation, a state characterized by increased uptake of monocytes, T lymphocytes, and lipids (low density lipoprotein, LDL) into the arterial wall (Ross 1995, Blann 2000). Monocytes will then differentiate into macrophages, which engulf oxidized LDL to become foam cells. Together with T lymphocytes, they form the first visible sign of atherosclerosis: the fatty streak. Over time, T lymphocytes initiate a cascade of inflammatory and growth mediators that stimulate the migration and proliferation of vascular smooth muscle cells from the media to the intima. Vascular smooth muscle cells interact with foam cells to form the intermediate or fibrofatty lesion that may then progress to an advanced lesion or fibrous plaque. This cascade of events is commonly referred to as the “response-to-injury” hypothesis of atherosclerosis (Ross 1993, 1995). In its initial stages, this response is both protective and reversible (Deanfield et al. 2007); however, repeated and/or prolonged exposure to various pathologic stimuli or cardiovascular risk factors will exhaust this protective mechanism, resulting in accumulation of atherosclerotic lesions, endothelial cell injury and detachment into the peripheral circulation, and overt cardiovascular disease (Ross 1995, Blann 2000, Endemann and Schiffrin 2004, Deanfield et al. 2007).

Although cardiovascular disease typically presents clinically in adulthood, damage to the endothelium begins as early as the first decade of life (Stary 1989). Indeed, post-mortem assessments of the left coronary

artery of 565 humans who died between full-term birth and 29 years of age demonstrated evidence of thickening of the intima and formation of foam cells in 45% of infants (< 1 year of age) (Stary 1989). The presence of fatty streaks was observed in 21% of the children examined between the ages of 5 and 9 years, and up to 53% of youth around the age of puberty (10 to 14 years of age) (Stary 1989). Moreover, a second post-mortem study in adolescents and young adults demonstrated that a risk factor score based only on modifiable factors (serum lipids, smoking, hypertension, obesity, and hyperglycemia) was associated with the prevalence and severity of lesions in the left and right coronary arteries and abdominal aorta (McMahan et al. 2006). Thus, minimizing exposure to risk factors in the pediatric years may slow the progression of vascular lesions (McMahan et al. 2006). Taken together, these findings suggest that monitoring endothelial health throughout childhood may provide us with critical information on the early stages of vascular damage, thereby allowing for early intervention to slow or prevent the progression of atherosclerotic lesions, and ultimately, cardiovascular disease.

1.1.3 Markers of Endothelial Health

The realization that the endothelium plays a critical role in the initiation of cardiovascular disease has led to the development of a number of techniques to evaluate endothelial health. Monitoring the progression of endothelial damage is of particular interest in the pediatric

population given that the traditional clinical endpoints (e.g. cardiovascular morbidity and mortality) are extremely rare in childhood. The gold standard for assessment of endothelial health is coronary artery intravascular ultrasound, which allows for direct assessment of the arterial wall (Nissen et al. 1991). However, this technique involves cardiac catheterization, and is therefore too invasive for use in the pediatric population. More readily used minimally- or non-invasive techniques include ultrasound-based measures of endothelial function and structure, as well as blood-based markers of endothelial activation, damage, and repair.

Endothelial function can be assessed by various methods; with flow-mediated dilation (FMD) of the brachial artery being the most common and reproducible technique used in children (Donald et al. 2006, Charakida et al. 2007). FMD was first described by Celeremajer et al., and is based on the principle that increased blood flow through the artery (i.e. hyperemia) will increase shear stress on the endothelium, leading to a local release of endothelial derived relaxing factors (e.g. NO) and subsequent dilation of the artery (Celermajer et al. 1992). In this assessment, the blood vessel is occluded by an inflated sphygmomanometer cuff and hyperemia is induced by its deflation. The change in the diameter of the artery in response to cuff deflation is measured by ultrasound. Little to no change in the diameter of the artery is indicative of an impairment in endothelial function, or endothelial

dysfunction (Endemann and Schiffrin 2004). Interestingly, endothelial dysfunction is thought to precede the development of early morphological signs of cardiovascular disease including the formation of fatty streaks (Celermajer et al. 1992, Ross 1993, 1995, Charakida et al. 2005). Indeed, impaired FMD is associated with the presence of cardiovascular risk factors in adults without clinical symptoms of cardiovascular disease (Campuzano et al. 2006). It has also been reported in asymptomatic children presenting with risk factors for cardiovascular disease, including JIA (Vlahos et al. 2011), familial hypercholesterolemia (Celermajer et al. 1992), and type 1 diabetes (Peña et al. 2012).

The assessment of circulating factors produced or regulated by the endothelium, including inflammatory cytokines, adhesion molecules, and modulators of vasoconstriction and thrombosis, can also shed some light on the state of the endothelium. Among the factors measured to date are markers of endothelial activation, including E-selectin, P-selectin, vascular adhesion molecule 1, and intracellular adhesion molecule 1 (Hwang et al. 1997, Wojakowski and Gminski 2001, Glowinska et al. 2005, Meyer et al. 2006); and modulators of hemostasis such as plasminogen activator (Thögersen et al. 1998), plasminogen activation inhibitor-1 (Wiman et al. 2000), and von Willebrand factor (vWF) (Meyer et al. 2006, Tkaczyk et al. 2008). It is important to note that although studies in adults support the link between these markers, cardiovascular risk factors, and the development

of clinical cardiovascular disease, their prognostic value in the pediatric population is still largely unknown (Deanfield et al. 2007).

More recently, circulating biomarkers of endothelial damage and repair have emerged as sensitive predictors of endothelial health and overall cardiovascular risk. CECs are mature endothelial cells shed from the intima into the circulation following irreversible damage to the endothelium (Erdbruegger et al. 2010). Conversely, EPCs are a rare population of immature cells that respond to this damage and are involved in the maintenance and repair of the endothelium (Asahara et al. 1997, Hill et al. 2003). A growing body of evidence suggests that assessment of both of these cells may provide unique insight into the balance between injury and repair in the endothelium (Blann and Pretorius 2006, Fabbri-Arrigoni et al. 2012). Much like the aforementioned circulating factors, our knowledge of CECs and EPCs in the pediatric population remains limited. The primary focus of this thesis is on CECs and EPCs in children and adolescents.

1.2 Circulating Endothelial Cells

1.2.1 Overview

In order to maintain vascular health the endothelium is renewed at a rate of <1% per day (Erdbruegger et al. 2006). Damaged endothelial cells shed from the vasculature as part of this turnover process are known

as CECs, and their concentrations in the peripheral blood are thought to reflect the degree of injury to the vessel wall (Blann et al. 2005). Indeed, individuals with chronic conditions associated with cardiovascular pathology commonly present with elevated CECs, with the highest levels observed in those with the most severe or active disease (Del Papa et al. 2004, Foster et al. 2009). Unlike much of the available markers that only predict risk of cardiovascular disease, measurement of CECs may provide valuable insight into the mechanisms underlying cardiovascular pathology, and may allow for sensitive monitoring endothelial health, as well as earlier detection and intervention to manage vascular damage (Blann et al. 2005). Despite the fact that CECs have been measured and studied for over five decades, our understanding of these cells, particularly in pediatric and healthy populations, remains limited. This is partly related to unresolved issues surrounding CEC measurement techniques and protocols, as well as the overwhelming focus on the clinical utility of these cells as biomarkers of cardiovascular damage in adults with severe chronic disease. These limitations will be discussed in the following sections, along with an overview of our understanding of CECs across the lifespan, emphasizing the small body of literature available on CECs in youth.

1.2.2 Discovery and Identification of Circulating Endothelial Cells

In 1934, CECs were mistakenly identified as cancer cells using light microscopy of May Grünwald Giemsa stained peripheral blood smears (Pool and Dunlop 1934, Long et al. 1960). However, it wasn't until 1964 that Herbeuval and Fourot suggested that these 'cancer cells' may in fact be of an endothelial lineage and are likely derived from the vasculature (Herbeuval and Fourot 1964). The authors attributed this misclassification to similarities in the morphology of circulating cancer and endothelial cells. Specifically, both populations commonly presented as clusters of large cells with a polyhedron or fusiform shape, most often with a single, faint red nucleus and pale blue cytoplasm with Giemsa staining (Herbeuval and Fourot 1964). A subsequent study highlighted the heterogeneity CECs in blood smears, with reports of bi-nuclear or polynuclear (up to 10 nuclei) CECs presenting as single cells, clusters of cells or even small fragments of the endothelium (Herbeuval et al. 1965). Gaynor et al. then used both light and electron microscopy to confirm that peripheral blood samples of CECs were indeed similar in appearance and structure to endothelial cells harvested from the aortic intima of rabbits (Gaynor, E. et al. 1968). Moreover, experimental rabbit and rat models demonstrated that, as hypothesized, CECs were released into the peripheral circulation from the endothelium in response to endotoxin-induced vascular lesions (Gaynor, E. et al. 1968, Hladovec 1973, Hladovec, J. 1978). These findings laid the

foundation for further research examining CECs in health and disease, with consistent reports of abundant CECs in various conditions including myocardial infarction, hypertension, cancer, and rickettsial infection (Bouvier, C.A. et al. 1970, Hladovec, J. 1978, Hladovec 1978). While these early studies provided novel insight into CECs as potential markers of endothelial health, the use of Giemsa staining and light or electron microscopy was neither sufficiently specific nor sensitive for enumeration of this rare cell population.

Scientific and technological advances have allowed for the development of more sophisticated techniques for CEC identification, each with distinct advantages and disadvantages. Immunomagnetic bead capture is arguably the most readily used method for CEC isolation, owing in part to the proposal and consensus on a standardized protocol for sample preparation (Woywodt et al. 2006). In brief, paramagnetic particles coated with anti-CD146 antibodies (S-Endo 1 or P1H12) are incubated with whole blood and upon application of a magnet, cells tagged with the anti-CD146 antibodies (mostly CECs) are retained and the remaining cells are discarded. These cells are then stained and counted using fluorescence microscopy (Erdbruegger et al. 2006). Modifications to this protocol include the addition of a stain with Ulex Europaeus Lectin-1 (UEA-1), a marker to confirm the endothelial cell phenotype (Holthöfer et al. 1982, Woywodt et al. 2004, 2006). Although the immunomagnetic bead

protocol is relatively well-established and widely accepted, it is also cumbersome, requires a large time commitment as well as significant expertise in identification of CECs by morphology, which introduces an element of subjectivity. Moreover, there is some evidence to suggest that the CD146 surface marker used in the magnetic isolation of CECs may not be unique to mature endothelial cells. This may lead to inadvertent enumeration of non-CEC cells including EPCs, activated T lymphocytes, trophoblasts, mesenchymal stem cells, and malignant cells (Woywodt et al. 2006, Erdbruegger et al. 2006). While secondary stains can be incorporated, this only adds to the significant time required for sample preparation. Taken together, these limitations highlight the importance of timely, multi-parametric analyses of CECs, a unique advantage offered by flow cytometry.

1.2.3 Characterizing CECs by Flow Cytometry

Flow cytometry is becoming increasingly popular for CEC identification (Khan et al. 2005, Erdbruegger et al. 2006). This method allows for relatively rapid detection of distinct cell populations with overlapping antigen expression. For example, both CECs and EPCs are $CD34^+$; however, cells can be stained simultaneously with the monoclonal antibody against CD45 and distinguished on the basis that CECs are $CD45^-$, while EPCs are $CD45^+$ (Duda et al. 2007). In addition to identifying cells that are positive and negative for specific antigen expression, it is

also possible to distinguish between those expressing low (dim) and high (bright) levels of antigens (Shapiro 2005, Khan et al. 2005). This highlights the sensitivity of flow cytometry in rare cell analysis.

To date, a number of combinations of cell surface markers (i.e., cell definitions) have been proposed for CEC identification (Table 1.1). Unfortunately, there is still no consensus as to the optimal markers and cell definitions of CECs; therefore, this multi-parametric approach has introduced significant variability in cell staining protocols between studies (Blann et al. 2005, Khan et al. 2005, Erdbruegger et al. 2006). Compared with the immunomagnetic bead technique, enumeration of resting CECs by flow cytometry generally yields higher cell counts (Goon et al. 2006). Goon et al. reported reasonable agreement between the two techniques in patients with cardiovascular disease and cancer, but poor agreement in the healthy control group, as well as at higher and lower absolute CEC counts (Goon et al. 2006). The authors reasoned that the small protocol differences might have more of an impact on CECs at the extremes. This finding is further supported by the fact that the normal range of flow cytometry-measured CECs in healthy individuals can vary by thousands of degrees of magnitude (0 to 7,900 cells/mL) from one research group to the next (Blann et al. 2005).

The large variability in CEC counts can be explained by a number of factors related to either technical limitations or the inherent

characteristics of the population. More specifically, we have yet to identify a surface marker that is unique to CECs. Even the most commonly used CEC marker, CD146, is also expressed on subsets of T lymphocytes, trophoblasts, mesenchymal stem cells, and malignant tissue (Blann et al. 2005, Duda et al. 2006). In addition, CECs may be resting or activated (Mancuso et al. 2001, Khan et al. 2005), viable (Lin et al. 2000, Mutunga et al. 2001), apoptotic or necrotic (Woywodt et al. 2003); however, the impact of these different cellular states on CEC function is not yet established and therefore not often considered (Khan et al. 2005). Finally, CECs may present as single or aggregate cells, as sheets of cells, and even small fragments of cells, which are commonly referred to as endothelial microparticles (Herbeuval et al. 1965, Mutin et al. 1999, Dignat-George and Sampol 2000). This is particularly relevant in flow cytometry since the first gating step is often identifying the cells based on their size (forward scatter) and granularity (side scatter). There is some evidence to suggest CEC presentation (i.e., state and size) may be affected by type and severity of the pathology (Mutin et al. 1999, Dignat-George and Sampol 2000), as well as the originating vascular bed of the cells (Solovey et al. 1997, Mutin et al. 1999, Lin et al. 2000).

Table 1.1 *Select sources of heterogeneity in CEC and EPC measurement by flow cytometry.*

	CEC	EPC	
Cell Sources[a]	▪ Whole blood ▪ PBMCs ▪ Cord blood ▪ Endothelium (for microscopy)	▪ Whole blood ▪ PBMCs ▪ Monocytes ▪ Cord blood ▪ Bone Marrow	
Cell Definitions[b]	▪ $CD31^+CD45^-$ ▪ $CD34^+CD31^+CD133^-$ ▪ $CD34^+CD146^+$ ▪ $CD34^+CD11c^-CD105^+CD146^+$ ▪ $CD34^+CD45^-CD133^{-*}$ ▪ $CD34^+$ $CD45^-CD146^+$ ▪ $CD34^+CD45^-CD133^-CD146^+$ ▪ $CD34^+CD3^-CD31^+CD133^-$ $CD146^+$ ▪ $CD45^-CD133^{-*}$ ▪ $CD45^-CD31^+CD133^{-*}$ ▪ $CD45^-CD133^-CD146^{+*}$ ▪ $CD146^+Syto16^+$ ▪ $CD146^+$ ▪ $CD146^+CD31^+$ ▪ $CD146^+CD3^-CD45^-$ ▪ $CD146^+CD31^+CD36^+CD45^-$	▪ $CD34^+$ ▪ $CD34^+CD31^+$ ▪ $CD34^+CD31^-$ $CD133^+CD309^+$ ▪ $CD34^+CD31^-CD146^-$ $CD309^+$ ▪ $CD34^+CD45^+$ ▪ $CD34^+CD45^{dim}$ ▪ $CD34^+CD45^-$ ▪ $CD34^+CD45^+CD133^+$ ▪ $CD34^+CD45^-CD133^{+*}$ ▪ $CD34^+CD45^+$ $CD133^+CD309^+$ ▪ $CD34^+CD45^+$ $CD146^+$ ▪ $CD34^+CD62L^+$ ▪ $CD34^+CD133^+$	▪ $CD34^+CD133^+$ $CD309^+$ ▪ $CD34^-CD133^+$ $CD309^+$ ▪ $CD34^+CD144^+$ $CD3^-$ ▪ $CD34^+CD309^+$ ▪ $CD34^+CD309^+$ $CD3^-$ ▪ $CD34^+VEGFR\text{-}1^+$ ▪ $CD45^-CD133^{+*}$ ▪ $CD45^-CD31^+CD133^{+*}$ ▪ $CD45^-CD133^{+*}$ ▪ $CD45^-$ $CD133^+CD146^{+*}$ ▪ $CD133^+$ ▪ $CD133^+CD45^-$ ▪ $CD133^+$ $CD309^+$
Units of Expression[b]	▪ # of cells ▪ Cells per µL, mL or L blood ▪ % of PBMCs ▪ % of live events	▪ Cells per µL, mL or L blood ▪ Cells per µL, mL or L lymphocytes ▪ Cells per µL, mL or L PBMCs ▪ Cells per 10^6 events ▪ % of PBMCs ▪ % of lymphocytes ▪ % of live events	
Other Methods of Enumeration	▪ Light or fluorescence microscopy ▪ Immunohistochemistry ▪ Immunomagnetic beads	▪ Immunohistochemistry ▪ Immunomagnetic beads ▪ Cell culture – colony forming units	

[a] Cells enumerated from the listed source, but the protocols employed for isolation and staining varied between studies; [b] Identified from studies discussed in this thesis (not inclusive); [*] Included a DNA or $Syto16^+$ gate.

1.1.1 Mechanisms of Endothelial Cell Detachment and CEC Fate

In healthy individuals, over 99% of endothelial cells are quiescent and tethered by integrin bonds to adhesion molecules expressed on the basement membrane including vitronectin, fibronectin, and VE-cadherin (Davis and Senger 2005, Yurchenco 2011). The mechanisms involved in breaching these bonds are not entirely clear but have been linked to

defective adhesive properties, cleavage by cytokine or proteinase activity, and mechanical injury (Woywodt et al. 2002).

In addition to their role in anchoring endothelial cells, integrins and adhesion molecules are responsible for mediating cell survival (Stromblad, 1999). In an in vitro model, Oguey et al. used recombinant adenoviruses to modify the alpha-V/beta-3 integrins expressed by human umbilical vein endothelial cells (HUVEC) to a defective beta phenotype, and demonstrated that 50-70% and 90% of the cells detached from the substrate by 24 and 48 hours, respectively (Oguey et al. 2000). They also reported that the defective integrins triggered apoptosis, with 10% of detached cells demonstrating annexin-V binding, a common indicator of apoptosis, by the 48-h time point (Oguey et al. 2000). These findings were similar to an earlier study that reported <5% survival of HUVEC cultured on plates with non-adhesive substrates, with >80% of cells showing morphologic signs of apoptosis, compared with >98% viability in control plates (Re et al. 1994). Interestingly, when non-adherent HUVEC were coated with vitronectin and fibronectin, they retained >90% viability (Re et al. 1994). More recently, beta integrin expression in an in vivo rat carotid artery model led to increased endothelial cell detachment, which was followed by cell death (Hasmim et al. 2005). Thus, not only does impaired function of adhesion molecules lead to increased endothelial cell detachment, but it also activates an apoptotic program in CECs.

Acute and chronic inflammation are tightly linked with peripheral blood concentrations of CECs (Woywodt et al. 2003, Blann et al. 2005). This may be explained by the fact that neutrophils, which are among the first cells to migrate to sites of inflammation, release a host of proteinases (i.e., matrix metalloproteinase-2, matrix metalloproteinase-9, cathepsin G, and proteinase 3) that act to disrupt integrin bonds (Wright et al. 2010, Korkmaz et al. 2010). Neutrophils also induce a pro-inflammatory response, producing and recruiting cytokines that also play a role in endothelial cell detachment (Rüegg et al. 1998, Woywodt et al. 2002, Wright et al. 2010). Rüegg et al. demonstrated that tumor necrosis factor (TNF) and interferon gamma (IFN-γ) impaired activation of the alpha-V/beta-3 integrin, thereby decreasing endothelial cell survival signals and triggering apoptosis in HUVEC. Administration of a combination of melphalan (chemotherapy), TNF, and IFN-γ in patients with melanoma resulted in increased detachment and apoptosis of endothelial cells in biopsies of the tumour tissue, which was not observed in those treated with only melphalan (Rüegg et al. 1998).

Studies examining optimal CEC sampling protocols provide the strongest evidence for CEC detachment by mechanical injury to the vasculature (Woywodt et al. 2006). More specifically, Boos et al. reported 32% higher CECs in the first 4-mL sample collected by venipuncture compared with the next 4-mL collected from the same needle (Boos et al.

2006a). Whether CECs dislodged from the endothelium by mechanical injury (i.e., traumatic venipuncture) share similar characteristics as those detached secondary to pathologic insult is unknown.

The function and fate of CECs in the peripheral circulation is poorly understood. There is some evidence to suggest that CECs are pro-inflammatory; characterized by expression of chemokines including macrophage inflammatory protein 1-alpha and interleukin-8 (IL-8), and increased neutrophil migration (Holmén et al. 2005). They were also found to inhibit EPC proliferation, migration, and endothelial nitric oxide synthase (eNOS) expression, which are critical steps in the initiation of EPC-mediated vascular repair (Section 1.3.5) (Holmén et al. 2005). Importantly, endothelial cell detachment results in exposure of the basement membrane, thereby triggering a pro-inflammatory response that can initiate the “response-to-injury” cascade described previously (Section 1.1.2), as well as EPC mobilization, described below (Section 1.3.4) (Erdbruegger et al. 2006). The fact that endothelial cell detachment appears to be an initiating step in both the development of atherosclerosis and EPC-mediated vascular repair suggests that CEC concentration may be a better indicator of endothelial health than other commonly reported markers of endothelial activation.

1.2.4 CECs Across the Lifespan

Very few studies have attempted to characterize the natural history of CECs in healthy populations, likely because they are exceedingly rare in individuals without chronic medical conditions (Erdbruegger et al. 2006). In healthy young (mean ± SD, 33 ± 8 years) and older (66 ± 8 years) adults, Shaffer et al. reported no between group differences in CECs, quantified as $CD146^+$, $CD146^+CD34^+$, $CD146^+CD31^+$, $CD146^+CD34^+CD31^+$, or $CD34^+CD31^+CD133^+$ (Shaffer et al. 2006a). Both Strijbos et al. and Wang et al. also reported no association between CEC levels and age in young to older adults (Wang et al. 2005, Strijbos et al. 2008). Only one study to date has extended the lower age range to the pediatric years, and observed lower CECs quantified by immunomagnetic bead isolation in children (10-18 years; median [IQR]: 16 [14-32]) compared with adults (19-67 years; 44 [17-80]). The authors also reported a significant positive relationship between CECs and age ($r = 0.44$, $p<0.005$), which was maintained after adjustment for cardiovascular risk factors including gender, waist circumference, blood pressure, fasting glucose, and blood lipids (Fabbri-Arrigoni et al. 2012). Whether CECs change across the pediatric years has not yet been investigated; however, monitoring early changes may allow for more effective interventions at the first signs of severe damage to the endothelium.

1.2.5 The Relationship between CECs and Other Risk Factors and Biomarkers of Cardiovascular Disease

The associations between CECs and other well-established markers of vascular health have been examined extensively in the adult literature. Given that CEC levels are thought to reflect an injured endothelium, it is not all together surprising that most studies have reported a moderate-to-strong negative association between CECs and endothelial function, measured by FMD (Rajagopalan et al. 2004, Chong et al. 2004, Lee et al. 2006). This relationship is further supported by the relatively consistent finding that individuals with elevated levels of CECs also present with high serum- and plasma-based concentrations of non-traditional biomarkers of vascular health which include, but are not limited to, vWF (Makin et al. 2004, Chong et al. 2004, Lee et al. 2006, Boos et al. 2008a), thrombomodulin (Strijbos et al. 2008), sE-selectin (Del Papa et al. 2004, Boos et al. 2008a), IL-6 (Lee et al. 2005, 2006), TNF-α , and C-reactive protein (CRP) (Wang et al. 2005). Moreover, acute changes in vWF and sE-selectin were moderately-strongly correlated with changes in CECs (r_{vWF}= 0.45 and 0.76; $r_{sE\text{-}selectin}$ = 0.41; $p<0.05$) in patients with chest pain or diagnosed acute coronary syndromes (Lee et al. 2005, Boos et al. 2008b). It must be noted that the majority of the available research has examined these associations in adult clinical populations alone (primarily cardiovascular diseases), or a combination of clinical populations and

healthy controls. Therefore, the extent to which these relationships exist in healthy adults or children presenting with smaller ranges of both CECs and other markers of vascular health is unclear.

Since most studies examine CECs in individuals with diagnosed cardiovascular diseases, a limited number of studies have assessed the relationships between CECs and traditional risk factors of cardiovascular disease, and have reported mixed results in both adult and pediatric samples (Wang et al. 2005, Kelly et al. 2010, Dias et al. 2013, Eltayeb et al. 2014). Dias et al. examined activated CECs in a sample of obese adolescents and healthy weight controls (13 to 17 years of age), and failed to demonstrate any associations with body mass index (BMI) z-score, waist circumference, or percentage body fat, even when examined by sex (Dias et al. 2013). Conversely, Kelly et al. reported that elevated CECs were linked with BMI ($r = 0.21$, $p = 0.30$), waist circumference ($r = 0.24$, $p = 0.014$), systolic and diastolic blood pressure ($r = 0.28$, 0.25, $p<0.01$), and triglycerides ($r = 0.20$, $p = 0.043$); but not with total, LDL or high density lipoprotein (HDL) cholesterol, glucose, or insulin levels ($r = -0.14$ to 0.17, $p > 0.1$) in a larger sample of obese and control youths (Kelly et al. 2010). More recently, a positive association was reported between CECs and glycated hemoglobin (HbA1c), a marker of glucose control, in children with type 1 diabetes and healthy controls ($r = 0.516$, $p = 0.004$) (Eltayeb et al. 2014). Although these data highlight the early origins of vascular damage,

it is not clear whether these relationships also extend to the healthy, typically developing pediatric population.

1.2.6 CECs in Disease

Both flow cytometry and immunomagnetic beads isolation techniques consistently identify elevated concentrations of CECs in clinical conditions associated with poor cardiovascular outcomes. Compared with healthy controls, CECs are 1.2 to 73.3 times higher in adults with diagnosed cardiovascular (Mutin et al. 1999, Makin et al. 2004, Chong et al. 2004, Boos et al. 2006b, 2007), metabolic (El Amine et al. 2010, Asicioglu et al. 2010), autoimmune (Woywodt et al. 2003, Rajagopalan et al. 2004, Holmén et al. 2005, Foster et al. 2009, 2012), infectious (George et al. 1993), and hematologic conditions (Lefevre et al. 1993, Solovey et al. 1997). Moreover, CEC levels can predict the occurrence of major coronary events in patients with acute coronary syndrome (Lee et al. 2005) and those on hemodialysis (Koc et al. 2005). The range of values observed in studies that measure CECs by flow cytometry is large, with some studies reporting as few as 10 cells/mL up to 39,000 cells/mL (Erdbruegger et al. 2006). Aside from differences in protocols, disease activity (i.e., flare up vs. remission, Woywodt et al. 2003, Foster et al. 2009) and disease severity (Del Papa et al. 2004, El Amine et al. 2010) are positively associated with CEC levels. Conversely, the use of therapeutic agents to manage disease activity led to steady declines in CECs. This effect was observed after 3

months of statin therapy in individuals with systemic sclerosis (Del Papa et al. 2008), following 6 months of immunosuppressive therapy in individuals with anti-neutrophil cytoplasmic antibodies-associated small-vessel vasculitis (Woywodt et al. 2003), as well as 6 months of TNF-alpha inhibitors in patients with psoriasis (De Simone et al. 2014). There is also some evidence to suggest that individuals with well-managed, low disease activity can demonstrate a return to similar resting CEC concentrations as healthy controls (De Simone et al. 2014).

Only a handful of studies have examined CECs in youth and have primarily focused on those with severe chronic medical conditions. More specifically, elevated CECs were reported in children (age range: <1 to 19 years) with familial hypercholesterolemia, type 1 diabetes, and vasculitis (Brogan et al. 2004, Clarke et al. 2010, Fabbri-Arrigoni et al. 2012, Eltayeb et al. 2014). These findings might be expected given that the aforementioned conditions are often characterized by chronically high levels of systemic inflammation, which may lead to increased injury to the endothelium, and subsequent detachment of endothelial cells (Section 1.2.5). Much like their adult counterparts, Clarke et al. found higher CEC levels in children with active systemic vasculitis compared with those in remission, who presented with similar CEC levels as healthy controls (Clarke et al. 2010). When 14 patients were followed longitudinally from the time of diagnosis (pre-treatment), they demonstrated a remarkable

decline in CECs with treatment at 3- and 12-months of follow-up. Interestingly, a 3-fold increase in CECs was observed in 1 patient with a disease flare at 13 months (Clarke et al. 2010). The fact that indicators of a serious degree of endothelial damage are elevated in children as young as 1 year of age highlights the importance of further exploring CECs, as well as the factors that can influence their concentrations, in the pediatric years.

1.3 Circulating Endothelial Progenitor Cells

1.3.1 Overview

Circulating EPCs are a family of rare, bone marrow-derived cells that are thought to play an important role in the maintenance endothelial integrity. Since their discovery, EPCs have been examined extensively in both animal and human models to determine their potential as markers of cardiovascular health, and importantly, explore their effectiveness as a therapeutic agent in those with cardiovascular pathologies. However, a number of limitations associated with the measurement of these cells, particularly as they relate to identification and isolation protocols, have precluded their integration into the clinical setting. Despite these limitations, a growing body of evidence suggests that EPCs are strong predictors of cardiovascular outcomes, even in healthy individuals, and are tightly associated with other markers of cardiovascular health (Hill et al. 2003,

Werner et al. 2005). Moreover, EPCs are reduced in disease states, particularly in conditions associated with cardiovascular impairments (Vasa et al. 2001b, Herbrig et al. 2006, Głowińska-Olszewska et al. 2013). The majority of the research to date has focused on EPCs in adult populations; therefore, we know considerably less about these cells throughout the pediatric years. The following sections will highlight the evolving state of knowledge regarding EPCs and their links with cardiovascular health. Although most of the evidence presented will be from human and animal adult-based studies, findings from the pediatric population will be highlighted where available.

1.3.2 Discovery and Identification of Endothelial Progenitor Cells

In 1997, Asahara and colleagues isolated EPCs from human adult peripheral blood and demonstrated that these rare, primitive cells were capable of differentiating into mature endothelial cells both in vivo, and in vitro. Importantly, in vitro differentiated endothelial cells were found to incorporate into the vasculature in an animal model of hind limb ischemia, providing the first line of evidence that vasculogenesis was not solely limited to embryonic development (Asahara et al. 1997). Indeed, before this discovery, both post-natal repair of damaged endothelium (i.e., reendothelialization) and de novo blood vessel formation were thought to occur exclusively via the activation of fully differentiated endothelial cells

derived from preexisting blood vessels (i.e., angiogenesis) (Folkman and Shing 1992, Flamme et al. 1997).

In general, EPCs are isolated by cell culture and colony assays, or based on the expression of a combination of fluorescently labeled cell surface markers using flow cytometry (Fadini et al. 2012). There is evidence to suggest very little agreement between these techniques (George et al. 2006). This may be attributed to the heterogeneity of the EPC population as well as the difficulty in distinguishing cells of endothelial and hematopoietic lineage, which are thought to be derived from the same precursor cell: the hemangioblast (Pelosi et al. 2002, Timmermans et al. 2009). Culture-based studies have identified three EPC subtypes with unique appearance and functions (Hill et al. 2003, Hur et al. 2004, Hirschi et al. 2008, Medina et al. 2010, Van Craenenbroeck et al. 2013). Early EPCs, also referred to as circulating angiogenic cells, are isolated following 4 to 7 days of culture of peripheral blood mononuclear cells (PBMCs), demonstrate low proliferative capacity, and do not differentiate into mature endothelial cells. Early EPCs support vascular health by secreting and stimulating the release of a host of pro-angiogenic cytokines (e.g., IL-8, granulocyte macrophage colony-stimulating factor [GM-CSF]) and growth factors (e.g., vascular endothelial growth factor [VEGF], hepatocyte growth factor [HGF]) (Sieveking et al. 2008, Medina et al. 2010). Colony forming unit endothelial cells (CFU-Hill) represent the

fraction of non-adherent PBMCs that form colonies of hematopoietic cells and myeloid progenitors following 7 days of culture (Hill et al. 2003). Unlike early EPCs, CFU-Hill are thought to demonstrate a largely hematopoietic phenotype with signs of commitment to a monocyte or T lymphocyte lineage (Yoder et al. 2007, Hirschi et al. 2008). Finally, late EPCs or endothelial colony forming cells emerge after 2 to 4 weeks of PBMC culture, present with a high proliferative potential, and are capable of forming blood vessels in vivo (Lin et al. 2000, Yoder et al. 2007, Hirschi et al. 2008). The link between the three EPC subtypes is not fully known; however, it has been suggested that a small fraction of early EPCs can give rise to late EPCs (Fadini et al. 2012). More importantly, studies examining these subtypes in vitro suffer from the challenge of replicating their findings in vivo. Thus, it is unclear whether these cells exists in the circulation or if they are solely a product of specific cell culture conditions (Urbich and Dimmeler 2004, Fadini et al. 2012). Flow cytometry can circumvent this inherent limitation of cell culture by allowing for the direct assessment of EPCs from peripheral blood, umbilical cord blood, and bone marrow.

1.3.3 Characterizing EPCs by Flow Cytometry

Flow cytometry allows for relatively rapid and reproducible multi-parametric measurement of EPCs. Although it is considered the most appropriate technique to quantify EPCs in the clinical setting (Fadini et al.

2012), there is still no consensus as to the optimal protocol or combination of cell surface markers for EPC identification (Asahara et al. 1997, Peichev et al. 2000, Gehling et al. 2000, Crosby et al. 2000, Duda et al. 2007, Van Craenenbroeck et al. 2008a, 2013, Timmermans et al. 2009, Schmidt-Lucke et al. 2010, Fadini et al. 2012). Indeed, the wide array of available protocols and cell definitions (Table 1.1) has led to large discrepancies between studies with respect to normal concentrations of EPCs in the circulation, as well as the strength of their association with various markers of cardiovascular health.

At a minimum, the phenotypic definition of EPCs should include one marker of immaturity and one marker of endothelial lineage (Fadini et al. 2012). However, the selection of these markers is complicated by the heterogeneous nature of the EPC population. Indeed, there is some evidence to suggest that expression of phenotypic markers varies according to the developmental stage of the cells (Werner and Nickenig 2006, Timmermans et al. 2009, Tongers et al. 2010). The identification of EPCs is further complicated by the fact that there are no known antigens that are unique to this cell population, regardless of developmental stage. For example, Asahara et al. defined EPCs as $CD34^{+}CD309^{+}$ cells (Asahara et al. 1997); however, CD34 is also expressed on a number of other cell types including hematopoietic stem/progenitor cells (Sutherland and Keating 1992, Sutherland et al. 1996, Sidney et al. 2014), while

CD309 (also known as VEGFR-2, kinase insert domain receptor [KDR] or fetal liver kinase-1 [Flk-1]) is present on both hematopoietic stem/progenitor cells and mature endothelial cells (Ziegler et al. 1999). A number of additional markers have been proposed to allow more sensitive identification of EPCs, including CD133 and dim/negative expression of CD45 as markers of immaturity, along with CD31, vWF, and CD144 (VE-cadherin) as markers of endothelial commitment. Much like the aforementioned markers, these antigens are not unique to EPCs and as such, their combinations cannot distinguish EPCs from either hematopoietic stem/progenitor cells or more mature endothelial cells (Hristov et al. 2003, Case et al. 2007). Thus, the exact phenotypic definition of EPCs continues to be a matter of debate and has served to limit our understanding of these cells, as well as their therapeutic application in the clinical setting.

1.3.4 Mechanisms of EPC Mobilization

The exact pathways leading to EPC mobilization are not entirely known; however, a better understanding of the factors that influence mobilization may hold important implications for interventions aimed at increasing EPCs. The mechanisms of EPC mobilization overlap significantly with those involved in hematopoietic stem/progenitor cell mobilization (Papayannopoulou 2004, Aicher et al. 2005), which is not all together surprising given their shared ancestry (Pelosi et al. 2002). In

resting, healthy states, the majority of EPCs are tethered to stromal cells in the stem cell niche, a microenvironment within the bone marrow (Tilling et al. 2009). The interaction between EPCs and stromal cells is primarily mediated by integrins, as well as stromal cell-expressed membrane-bound Kit Ligand (mKitL) and stromal-derived factor (SDF-1) that bind to EPC-expressed cKit (or CD117) and CXC receptor 4 (CXCR4), respectively (Tilling et al. 2009). Broadly speaking, mobilization of EPCs begins with disruption of these bonds, which in turn allows the cells to migrate to the peripheral circulation via the sinusoidal endothelium of the bone marrow (Aicher et al. 2005).

Physical or chemical stress, most often in the form of tissue damage, ischemia or inflammation, is associated with an increase in both hypoxic-inducible factor 1 (HIF-1) and VEGF, which in turn upregulate circulating levels of SDF-1 in the peripheral circulation (Asahara et al. 1999, Ceradini et al. 2004). Upon entry to the bone marrow, SDF-1 activates the phosphoinositide 3-kinase/Akt/eNOS pathway thereby increasing bone marrow levels of NO, which then activates matrix metalloproteinase 9 (MMP-9), an extracellular proteinase produced by stromal cells (Aicher et al. 2003, Zheng et al. 2007, Tilling et al. 2009). MMP-9 is responsible for the conversion of mKitL to its soluble form (sKitL), which competes with and prevents stromal cells from binding with the cKit receptor found on EPCs (Heissig et al. 2002). It is important to

note that activation the MMP-9 pathway is largely dependent on the bioavailability of NO in the bone marrow (Aicher et al. 2003, Lu et al. 2014a). In fact, eNOS knockout mice demonstrate reduced MMP-9 activity, and lower levels of EPCs that were comparable to those observed in MMP-9 knockout mice; however, infusion of sKitL (downstream of MMP-9) rescued EPC mobilization (Aicher et al. 2003). In addition to the MMP-9-mediated impairment of mKitL-cKit bonds, a concurrent local increase proteinases like cathepsin G and elastase allows for cleavage of integrins and the SDF-1 to CXCR4 bonds, thereby further weakening the stromal cell-EPC interaction (Lévesque et al. 2003, Chavakis et al. 2005). The disrupted bonds between stromal cells and EPCs in the bone marrow together with the increased levels of SDF-1 in the circulation establish a concentration gradient that forces the $CXCR4^+$ EPCs to exit the bone marrow (Hattori et al. 2001, Everaert et al. 2010).

Although SDF-1 is reportedly the most potent chemoattractant of EPCs (Wright et al. 2002), a host of growth factors and cytokines are also known to mobilize EPCs via, and potentially independent of, the MMP-9 pathway including VEGF (Asahara et al. 1999), GM-CSF (Takahashi et al. 1999), G-CSF (Lévesque et al. 2003), erythropoietin (Heeschen et al. 2003), IL-8 (van Pel et al. 2006), HGF (Aicher et al. 2005, Tilling et al. 2009), and leukemia inhibitory factor (Kucia et al. 2004). Moreover, various exogenous agents such as anti-hypertensive, anti-inflammatory,

and anti-diabetic medications, are also known to increase circulating levels and function of EPCs by acting on one or many of the aforementioned factors in the EPC mobilization pathway (Lee and Poh 2014). Much of the available literature has focused on using pharmacotherapies to increase EPCs in individuals with diagnosed cardiovascular disease; however, exercise may represent an additional avenue to stimulate EPC mobilization in both clinical and healthy populations (Custodis and Laufs 2007, Silva et al. 2012, Krüger et al. 2014b). Indeed, a non-invasive exercise-induced increase in EPCs may be more appropriate for use in the pediatric population, and is discussed in detail in subsequent sections.

1.3.5 The Role of EPCs in the Maintenance of Vascular Health

Although EPC involvement in reendothelialization and vasculogenesis is widely accepted, a proof-of-concept model to definitively outline their role in these complex processes is lacking. More specifically, there are no known techniques to isolate and systematically modify the EPC population without concurrently affecting other cellular components involved in vascular repair, including hematopoietic stem/progenitor cells and mature vessel wall endothelial cells (Timmermans et al. 2009). The available evidence suggests that the EPC response to endothelial damage recapitulates the inflammatory response to endothelial activation (i.e., Section 1.1.2). In brief, damaged endothelial cells demonstrate increased expression of a host of adhesion factors and chemokines, such as

integrins, P- and E-selectin, and SDF-1, that regulate homing and adhesion of mobilized EPCs to the injured endothelial cells and basement membrane (Urbich and Dimmeler 2004, Chavakis et al. 2005, Zampetaki et al. 2008). Proteinases including Cathepsin L and MMP-2 mediate transmigration of EPCs into the damaged tissue (Urbich et al. 2005b). The specific cytokine environment will then determine whether EPCs will continue to differentiate into endothelial cells (Asahara et al. 1997), or secrete cytokines and growth factors that initiate vascular repair or angiogenesis by local resident endothelial cells (Urbich et al. 2005a).

It is important to note that there remain a number of unresolved issues with respect to the nature of the EPC contribution to neovascularization. First, the specific environments regulating the EPC differentiation and function have yet to be determined (Lu et al. 2014b). Indeed, Urbich et al. demonstrated that when exposed to VEGF, $CD34^+$ cells differentiated into endothelial-like cells, while exposure of the same cells to GM-CSF and inflammatory cytokines led to differentiation to macrophages (Urbich et al. 2003). Second, the interactions and contributions of distinct subsets of EPCs and hematopoietic stem/progenitor cells to neovascularization remains unknown (Urbich and Dimmeler 2004). Finally, Timmermans et al. questioned the extent to which EPC functions vary by vascular bed, and suggested that some sites may be more susceptible to competition between EPCs and mature

endothelial cells in vascular repair (Timmermans et al. 2009). Each of these limitations may serve to explain the discrepancies between human and animal studies that have reported up to 90% incorporation of genetically labeled transplanted EPCs into sites of neovascularization (Shi et al. 1998, Takahashi et al. 1999, Peichev et al. 2000, Kalka et al. 2000, Crosby et al. 2000), and others that failed to observe any direct EPC contribution to endothelial regeneration (Ziegelhoeffer et al. 2004, Kinnaird et al. 2004, Urbich et al. 2005a, Korf-Klingebiel et al. 2008, Hagensen et al. 2012, Jiang et al. 2013). Despite these limitations, the overwhelming majority of the literature supports a critical role of EPCs in the maintenance of vascular homeostasis, with clear links between EPC levels and endothelial function, reendothelialization, cardiovascular-related hospitalization, and mortality (Kalka et al. 2000, Crosby et al. 2000, Hill et al. 2003, Werner et al. 2005, Werner and Nickenig 2006, Lu et al. 2014b).

1.3.6 EPCs Across the Lifespan

Aging is associated with a decline in markers of cardiovascular health, even in the absence of overt cardiovascular disease (Weinsaft and Edelberg 2001, Hill et al. 2003). As such, it is not surprising that studies also report age-related declines in both EPC number (Rauscher et al. 2003, Thijssen et al. 2006, Zaldivar et al. 2007, Jie et al. 2009, Yang et al. 2013) and function (Edelberg et al. 2002, Heiss et al. 2005, Xia et al. 2012). Studies in animal and human models suggest these changes are

linked to a decline in proliferation of EPCs (Rauscher et al. 2003, Heiss et al. 2005, Hoetzer et al. 2007), impaired recruitment or mobilization of EPCs from the bone marrow (Heiss et al. 2005, Hoetzer et al. 2007, Sugihara et al. 2007), and decreased survival of circulating EPCs (Heiss et al. 2005).

To date, most of the literature in humans is focused on comparisons of EPCs in young adults and the elderly; very few studies have directly examined EPCs from childhood to adulthood or even across the pediatric years. In a cross-sectional study of 1- to 81-year-old participants with no history of cardiovascular disease, Jie et al. demonstrated a significant negative association between EPC counts, measured by flow cytometry, and age ($r = -0.37$, $p<0.01$) (Jie et al. 2009). When grouped by age, children (ages 1 to 17 years) presented with EPC levels three times higher than their adult counterparts (>20 years; 72 ± 60 vs. 22 ± 22 cells/100,000 granulocytes, respectively) (Jie et al. 2009). These findings were consistent with a second, more recent study that reported a negative association between CFU-Hill counts and age ($r = -0.29$, $p = 0.04$) (Fabbri-Arrigoni et al. 2012). Importantly, the variability within the pediatric group was also three times higher than that observed in the adult group, which may be indicative of changes in EPC levels as a result of growth and maturation (Jie et al. 2009). Only one study has examined changes in EPCs across the pediatric years and reported

significantly higher EPCs in pre-/early-pubertal compared with late-pubertal males (112 ± 21 vs. 63 ± 8 cells/μL blood, respectively) (Zaldivar et al. 2007). The reduction in EPCs in the late-pubertal group was also accompanied by significantly lower levels of G-CSF, which is involved in the mobilization of EPCs from the bone marrow (Zaldivar et al. 2007). While these findings suggest that EPCs decrease with chronological and/or biological age in children, they are based on a relatively small sample (n = 27 total, 14 pre-pubertal) of male participants, and as such, may not be generalizable to the pediatric population.

1.3.7 Relationships between EPCs and Other Risk Factors and Biomarkers of Cardiovascular Disease

The links between EPCs and well-established risk factors and emerging biomarkers for cardiovascular disease have been examined extensively in healthy adults and those with medical conditions. Traditional risk factors including obesity (Müller-Ehmsen et al. 2008, MacEneaney et al. 2009), hypertension (Vasa et al. 2001b, Schmidt-Lucke et al. 2005, Fernandes et al. 2012), hyperglycemia (Kränkel et al. 2005, Chen et al. 2007), hypercholesterolemia (Werner and Nickenig 2006), smoking (Vasa et al. 2001b, Hill et al. 2003, Schmidt-Lucke et al. 2005), and physical inactivity (Volaklis et al. 2013) have generally been associated with reduced EPCs, as measured by cell culture or flow cytometry. Similarly, the majority of studies examining emerging biomarkers such as CRP

(Verma et al. 2004), IL-6 (Herbrig et al. 2006), TNF-α (Ablin et al. 2006), as well as endothelial dysfunction (Hill et al. 2003), also demonstrate negative associations with EPCs. Interestingly, improvements in cardiovascular risk factors through either lifestyle modification (e.g., smoking cessation, weight loss, physical activity) or pharmacotherapies (e.g. anti-hypertensives, TNF- α inhibitors) consistently result in increases, and in some cases restoration of EPCs to healthy concentrations (Vasa et al. 2001a, Croce et al. 2006, Werner and Nickenig 2006, Ablin et al. 2006, Lee and Poh 2014).

The relationship between risk factors, biomarkers, and EPCs is more tenuous in children. For example, Bruyndonckx et al. recently reported significantly lower levels of EPCs, defined as $CD34^{+}CD309^{+}CD45^{dim/-}$, in obese youth compared with healthy weight controls, and found that EPC levels were negatively associated with BMI z-score ($r = -0.270$, $p = 0.013$) (Bruyndonckx et al. 2014). Conversely, EPCs defined as either $CD34^{+}CD144^{+}$ or $CD34^{+}CD309^{+}$ were positively associated with BMI in children with type 1 diabetes and healthy controls ($r = 0.320$ and 0.300, respectively, $p < 0.05$) (Głowińska-Olszewska et al. 2013). This finding was in line with the work of Jung et al. that demonstrated that $CD34^{-}CD309^{+}CD133^{+}$ EPCs were roughly 60% higher in overweight youth compared with healthy controls. However, these differences disappeared when EPCs were defined as

$CD34^+CD309^+CD133^+$ (Jung et al. 2009). Similarly, no differences in $CD34^+$ EPCs were observed between overweight or obese youth and healthy controls (Kinik et al. 2005), and neither BMI z-score, body fat percentage, nor waist circumference were associated with $CD34^+$, $CD34^+CD133^+$, or $CD34^+CD117^+$ EPCs (Kinik et al. 2005, Arnold et al. 2010). The discrepancies observed with body size also extend to other traditional and non-traditional risk factors, including blood pressure (Jung et al. 2009, Fabbri-Arrigoni et al. 2012), blood glucose levels (Fabbri-Arrigoni et al. 2012, Głowińska-Olszewska et al. 2013, Hörtenhuber et al. 2013), blood lipids levels (Jung et al. 2009, Fabbri-Arrigoni et al. 2012, Głowińska-Olszewska et al. 2013), direct or secondhand smoke exposure (Jung et al. 2009, Groner et al. 2015), and markers of inflammation (Jung et al. 2009, Głowińska-Olszewska et al. 2013, Hörtenhuber et al. 2013, Groner et al. 2015). It is likely that the poor agreement in the pediatric literature is in part due to small sample sizes, and largely due to differences in EPC definitions between studies. In fact, of the available studies, only 2 studies measuring the same outcomes also employed the same simple $CD34^+$ definition of EPCs (Kinik et al. 2005, Arnold et al. 2010).

1.3.8 EPCs in Disease

In healthy individuals, EPCs represent between 0.01 and 0.2% of PBMCs (Duda et al. 2007). Most, but not all, studies suggest that both

adults and children with chronic medical conditions present with even lower resting levels of EPCs as well as impaired EPC function (Kränkel et al. 2005, Herbrig et al. 2006, Avouac et al. 2008, Egan et al. 2008, Kim et al. 2010, Fabbri-Arrigoni et al. 2012, Povsic et al. 2013). Among the most commonly examined conditions in youth are those that are classically associated with increased risk of cardiovascular disease, including obesity (Bruyndonckx et al. 2014, 2015), type 1 diabetes (Hörtenhuber et al. 2013), familial hypercholesterolemia (Fabbri-Arrigoni et al. 2012), and more recently, JIA (Rusak et al. 2015). Based on the adult literature, alterations in EPC profiles in disease states are thought to primarily be linked to defective mobilization and homing of EPCs from the vascular niche to sites of injury (Dimmeler and Zeiher 2004, Paleolog 2005). Moreover, prolonged exposure to vascular insult in the form of inflammation or altered hormonal profiles may lead to exhaustion of the bone marrow pool, and an inability to produce additional EPCs in response to ongoing vascular injury (Tepper et al. 2002, Loomans et al. 2004, Urbich and Dimmeler 2004, Hörtenhuber et al. 2013). It is also plausible that alterations to the cytokine milieu due to systemic inflammation might impair EPC survival and differentiation (Dimmeler and Zeiher 2004, Paleolog 2005, Pitchford et al. 2009). Interestingly, management of disease symptoms with anti-inflammatory medications (Rusak et al. 2015) and maintenance of glycemic control (Hörtenhuber et al. 2013) in children

was associated with an increase in EPC levels. These data suggest that much like in adult populations, external factors may be used to improve or correct potential EPC impairments in youth.

1.3.9 CECs and EPCs: Differences, Similarities, and Interactions

The general consensus that CECs and EPCs represent two distinct populations is based on relatively well-established differences in their origins, morphology, proliferative capacity, and phenotype. To examine cell origin, Lin et al. assessed CECs and late EPCs (so-called endothelial outgrowth cells) in the peripheral blood of recipients of a gender-mismatched bone marrow transplant. Using fluorescent in situ hybridization analysis for the X and Y chromosomes, they demonstrated that 95 ± 6% of the CECs measured in fresh blood were derived from the recipient, suggesting that these cells primarily originate from the vessel walls (Lin et al. 2000). Conversely, by 27 ± 4 days of culture only 17 ± 4% of the late EPCs were from the recipient, indicating that the majority of circulating EPCs were derived from cells originating in the donor bone marrow (Lin et al. 2000). Unlike EPCs, which are commonly studied using cell culture (Asahara et al. 1997), CECs are mature cells, most of which are apoptotic or necrotic, and therefore demonstrate limited proliferative capacity in cell culture (Solovey et al. 1999, Woywodt et al. 2003, Holmén et al. 2005). Differences in cell maturity are also reflected in cell surface marker expression; for example, less mature EPCs are $CD133^{+}$, while

mature CECs lose CD133 expression (Yin et al. 1997, Gehling et al. 2000). However, there remains considerable debate and overlap in CEC and EPC antigen expression (Table 1.1), which makes it difficult to definitively distinguish the two populations based on single cell surface markers (Erdbruegger et al. 2006).

Theoretically, a simple reciprocal relationship between CECs and EPCs is plausible given their respective roles in endothelial damage and repair (Figure 1.1); however, very few studies have concurrently examined these cells in the same sample of individuals. Holmen et al. examined CECs and EPCs in adults with vasculitis and kidney disease and as expected, reported elevated CECs and reduced EPCs in patients with active disease compared with those in remission, and healthy controls (Holmén et al. 2005). This finding is in line with the majority of studies that have independently examined CECs and EPCs in adults and children with chronic conditions compared with healthy controls (Sections 1.2.7 and 1.3.8). They also reported that CECs isolated from patients with active disease inhibited proliferation of EPCs isolated from healthy controls, and impaired their migration toward VEGF compared with EPCs that were not exposed to CECs (Holmén et al. 2005). On the contrary, increased levels of EPCs have been reported following acute myocardial infarction (Shintani et al. 2001, Leone et al. 2005, 2006), which has also been linked with elevated levels of CECs (Li et al. 2013). Although these findings were

derived from independent samples, they may indicate that specific factors or hormonal/cytokine environments can overcome the inhibitory effect of CECs on EPC mobilization and migration.

In a sample of 10- to 18-year old children, Fabbri-Arrigoni et al. found that those with familial hypercholesterolemia presented with reduced EPCs (CFU-Hill) and elevated CECs compared with age-matched healthy controls (Fabbri-Arrigoni et al. 2012). The authors did not find any relationship between EPC and CEC levels in the patient group, and did not examine this association in the pooled sample of participants. Together with the adult data, this small body of evidence highlights the complex and poorly understood relationship between CECs and EPCs.

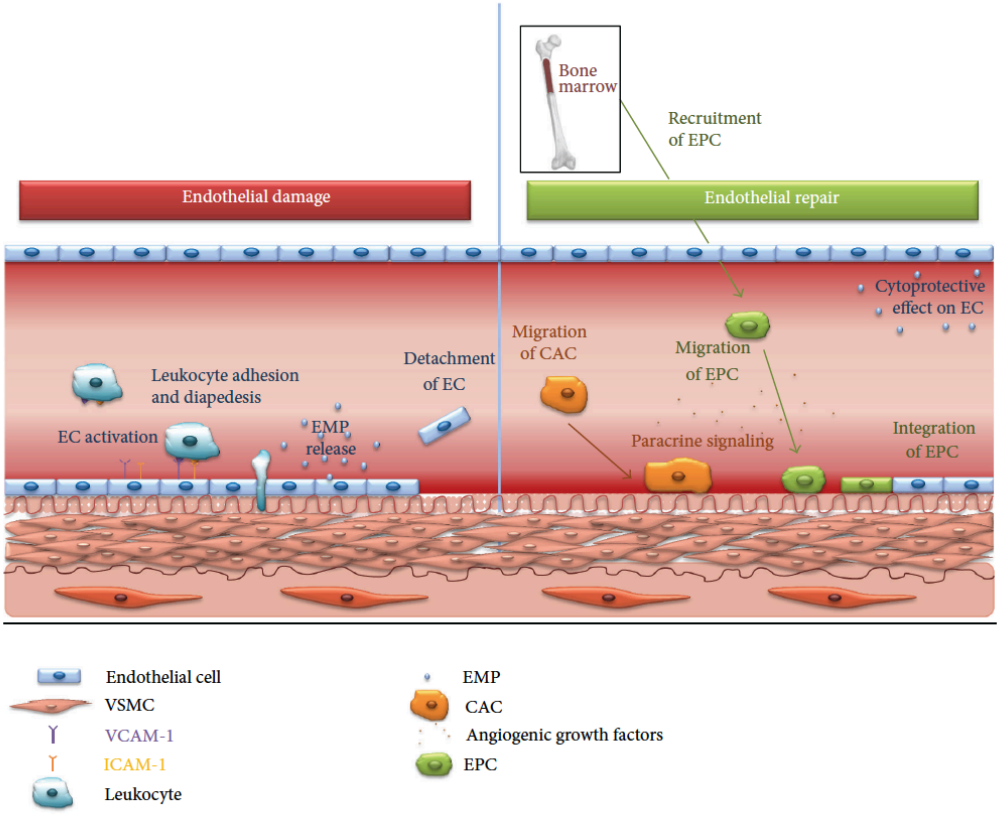

Figure 1.1 *CECs and EPCs in the maintenance of the vascular endothelium.* In the simplest terms, vascular injury leads to endothelial cell activation and eventual detachment of damaged endothelial cells into the peripheral circulation (depicted above as Detachment of EC, which then become known as CECs). Together, the denuded endothelium and CECs trigger a cascade of events culminating in mobilization and migration of EPCs from the bone marrow to the site of injury, where they play a central role in endothelial repair. The exact pathways and factors involved in the interaction between CECs and EPCs remain unclear. VSMC: vascular smooth muscle cell; VCAM-1: vascular cellular adhesion molecule-1; ICAM-1: intracellular adhesion molecule-1; EMP: endothelial microparticle; CAC: circulating angiogenic cell (early EPC); EPC: endothelial progenitor cell. Reproduced with permission under Creative Commons from Bruyndonckx et al., 2013, Copyright Bruyndonckx et al.

1.4 Effects of Exercise and Physical Activity on Circulating Progenitor and Endothelial Cells

1.4.1 Overview

Both physical activity and aerobic fitness are well-known predictors of cardiovascular health, and have been identified as modifiable risk factors for cardiovascular disease in children and adults (Fletcher et al. 1992, Shiroma and Lee 2010, Balagopal et al. 2011, Eckel et al. 2014). Interestingly, a growing body of evidence suggests that both CECs and EPCs are responsive to exercise, and may represent one mechanism underlying the relationship between fitness, physical activity, and cardiovascular health, at least in adults. Nevertheless, the aforementioned limitations in the assessment of these cells have made it difficult to compare studies and arrive at any general conclusions with respect to the relationship and effects of fitness, physical activity and acute exercise on CECs and EPCs. The following sections will examine these studies, with a special focus on cross-sectional and acute exercise studies performed in human adults and youth where cells were assessed by flow cytometry, since these were the primary focus of this thesis. Both cell culture-based studies and those involving an exercise training protocol are discussed where relevant.

In an effort to summarize the available literature, a series of meta-analyses were conducted to address four questions (details provided in

Appendix A): (1) Is there a relationship between fitness and resting EPCs and/or CECs in healthy individuals and those with chronic disease? (2) Is there a relationship between physical activity and resting EPCs and/or CECs in healthy individuals and those with chronic disease? (3) What is the effect of an acute bout of exercise on EPCs and/or CECs in healthy individuals? And (4) does the effect of an acute bout of exercise on EPCs and/or CECs differ between healthy individuals and those with chronic disease? The review and compilation of the literature revealed that there were too few studies for meta-analyses involving CECs, and that while a number of studies examine EPCs in the context of fitness and exercise, very few have focused on habitual levels of physical activity. These issues, along with many others are discussed in each of the subsequent sections. Finally, since there were too few studies in the pediatric population to perform the planned meta-analyses, a synthesis of the small body of evidence examining EPCs and CECs in the context of exercise in youth is provided.

1.4.2 Fitness, Physical Activity, Exercise and CECs in Adults

The first study to examine CECs in the context of exercise was performed in a small sample of 44- to 85-year-old adults with diagnosed effort angina (Mutin et al. 1999). Using the immunomagnetic bead isolation technique, the authors reported no sign of CECs in any patient at rest, and no significant change in CEC number immediately and 4-h after a

symptom-limited maximal test (30-W step per 3-min stage) on a cycle ergometer (Median [IQR]: 0 [0, 0] vs. 2 [0, 3] vs. 0 [0, 1], respectively, p = 0.417) (Mutin et al. 1999). Conversely, Boos et al. used a similar protocol and observed almost a 2-fold increase in CECs immediately after a symptom-limited maximal treadmill test (Bruce protocol) that returned to baseline values by 30-min in a group of 58.4 ± 9.8-year-old adults with symptoms of exertion-related chest pain (Boos et al. 2008b). Neither baseline nor change in CEC levels were associated with exercise time or $\dot{V}O_{2max}$, which led the authors to conclude that exercise-related changes in CECs were "somewhat unpredictable" (Boos et al. 2008b).

The only other study to assess CECs as a primary outcome in the context of exercise examined the effect of fitness level and exercise training on CECs in healthy young men (O'Sullivan 2003). CECs were identified by microscopy using cresyl violet- and vWF-stained smears, and were elevated in the high-fit (Mean ± SD, $\dot{V}O_{2max}$ = 71 ± 2 ml/kg/min) vs. low-fit group ($\dot{V}O_{2max}$ = 53 ± 2 ml/kg/min; CECs: 71 ± 13 vs. 41 ± 5 cells/mL blood, $p < 0.05$). Unlike the findings of Boos et al., baseline CECs were positively associated with $\dot{V}O_{2max}$ ($r = 0.56$, $p<0.05$) (O'Sullivan 2003). Moreover, 5 weeks of supervised 30-min cycling sessions in the low-fit group led to an increase in resting CECs to levels that were comparable to the high-fit group (53 ± 7 cells/mL, p = 0.26). Consistent with the work of Mutin et al., no change was seen in CECs in

either group following an acute maximal exercise test (30-W step per 3-min stage). The author suggested that the elevated CECs in the high-fit group and post-training are likely related to increased shear stress with chronic exercise, and may be indicative of increased endothelial cell turnover; however, markers of endothelial regeneration (i.e., EPCs) were not assessed (O'Sullivan 2003).

An additional 4 studies have examined the effects of acute exercise on CECs; however, these are often overlooked as CECs are reported as a secondary outcome, with the primary focus being on EPCs (Shaffer et al. 2006b, Möbius-Winkler et al. 2009, Sandri et al. 2011, Schier et al. 2014). Much like the findings of both O'Sullivan and Mutin et al., these studies have reported no effect of a maximal bout of exercise on CECs in individuals with diagnosed cardiovascular disease (Shaffer et al. 2006b, Sandri et al. 2011, Schier et al. 2014). On the other hand, a maximal exercise test in healthy controls was found to increase CECs at 4-hours post-exercise (Sandri et al. 2011), while prolonged exercise (240-min at 70% anaerobic threshold) led to an increase at 2-hours post-exercise, with a peak at 3.5 hours (Möbius-Winkler et al. 2009). Interestingly, Möbius-Winkler et al. also observed that the increase in CECs was followed by an increase in VEGF and EPCs, and postulated that the increase in EPCs may occur to counteract and maintain an intact endothelium. This finding lends some support the theory of increased endothelial turnover with

exercise proposed by O'Sullivan (O'Sullivan 2003, Möbius-Winkler et al. 2009). Taken together, the mixed findings in the adult population suggest that exercise may have a more marked effect on CECs in healthy individuals; however, this effect requires further investigation. Indeed, it is not clear whether the increase in CECs in healthy adults is also met with subsequent increase in the rate of clearance of CECs, nor is there any indication of whether exercise can affect the total levels or proportions of viable, necrotic, or apoptotic CECs in health or disease.

1.4.3 Fitness and Resting Concentrations of EPCs in Adults

Indicators of fitness are the most consistently reported descriptive outcomes in exercise-related EPC studies; however, they are seldom examined in the context of their association with EPC number or function. Results of the meta-analysis that included any correlations between markers of fitness and EPCs performed in human, adult, and healthy and/or disease populations (n = 5; details of analysis reported in Appendix A) suggested a moderate, positive link between fitness and EPCs (Fixed effects pooled correlation coefficient [lower, upper 95% confidence interval] = 0.457 [0.367, 0.538], $p < 0.001$; Figure 1.2). Although this meta-analysis only included studies that quantified EPCs by flow cytometry, the findings are consistent with cell-culture based studies that have reported increased EPC migratory capacity (Van Craenenbroeck et al. 2010a, Yang

et al. 2013), proliferative capacity (Yang et al. 2013), and colony forming units (Sarto et al. 2007) in trained vs. untrained individuals.

It is important to note that of the 5 studies examined in the meta-analysis, 3 either failed to report results pertaining to the correlation of specific subsets of EPCs with fitness or reported negative findings but did not provide any statistical details. For example, two separate studies by Van Craenenbroeck et al. examined $CD34^+$ and $CD34^+CD309^+$ cells in adults with congestive heart failure and healthy controls, and reported a significant association between $CD34^+$ cells and fitness (4a/b, and 5a/b in Figure 1.2), defined as $\dot{V}O_{2max}$ and maximal workload; however, these associations were not reported for $CD34^+CD309^+$ cells (Van Craenenbroeck et al. 2010a, 2010b). Similarly, while Manfredini et al. reported the complete results of the observed positive association between $CD34^+CD133^+CD309^+$ cells and both 6-minute walk distance and maximal treadmill speed (2a-d in Figure 1.2), they also reported that the $CD34^+$ subset of cells was not associated with either marker of fitness but did not provide Pearson correlation coefficients or probability values. Aside from the included studies, Povsic et al. found that EPCs were positively associated with measures of mobility and endurance (gait speed, 6-minute walk distance, chair stand time, and physical function score) in older adults with impaired glucose tolerance, even after adjustment for age, BMI, presence of medical conditions that affect function, and inflammation;

however, the authors did not report correlation values (Povsic et al. 2013). As such, the exclusion of the aforementioned findings has likely biased the pooled analysis, and perhaps more importantly, the overall body of literature, highlighting the need for appropriate and complete reporting of statistical results. Despite these limitations, it is plausible that more fit individuals would also present with higher EPCs, which may be secondary to the effects of repeated bouts of exercise and/or increased levels of habitual physical activity.

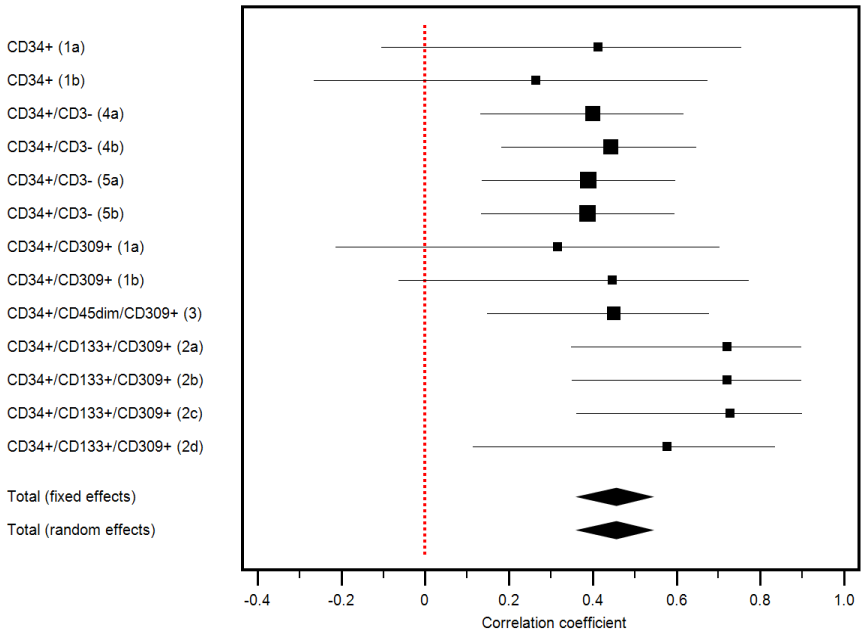

Figure 1.2 *Forest plot of correlations between fitness and EPCs in healthy adults and adults with chronic disease.* Pooled results of 13 correlations from 5 studies revealed a moderate, positive relationship between fitness and EPCs. Data are identified and sorted according to EPC cell definition followed by the reference, where (1) a: $\dot{V}O_{2max}$ in healthy young men (19-28 years), b: $\dot{V}O_{2max}$ in healthy older men (67-76 years)(Thijssen et al. 2006); (2) Patients with end-stage renal disease on hemodialysis, a: EPCs as % of collected cells and 6-minute walk distance, b: EPCs as % of collected cells and maximum treadmill speed, c: EPCs as cells/μL and 6-minute walk distance, d: EPCs as cells/μL and maximum treadmill speed (Manfredini et al. 2009); (3) $\dot{V}O_{2max}$ in patients with congestive heart failure and healthy controls (Mezzani et al. 2013); (4) Patients with congestive heart failure and healthy controls, a: $\dot{V}O_{2max}$, b: maximal workload (Van Craenenbroeck et al. 2010b); and (5) Patients with congestive heart failure and healthy controls, a: $\dot{V}O_{2max}$; b: maximal workload (Van Craenenbroeck et al. 2010a).

1.4.4 Physical Activity and Resting Concentrations of EPCs in Adults

Habitual physical activity is a well-established predictor of cardiovascular health (Balagopal et al. 2011, Conraads et al. 2014),

independent of aerobic fitness (Myers et al. 2015). Although the mechanisms underlying this relationship are complex, the observed association between habitual physical activity and endothelial function suggests that it may involve EPCs (Abbott et al. 2002, Myers et al. 2015). Only two studies have examined the link between habitual physical activity and EPCs, both in patients with coronary artery disease. Luk et al. used the self-reported International Physical Activity Questionnaire to estimate habitual physical activity levels (defined as walking, moderate-, and vigorous-intensity activity) in metabolic equivalents per week (Luk et al. 2009). They reported that while $CD133^{+}CD309^{+}$ cells were highest in individuals in the highest tertile of physical activity, neither $CD133^{+}CD309^{+}$ nor $CD34^{+}CD309^{+}$ were associated with FMD or physical activity in a multivariate regression model adjusting for age, sex, and presence of cardiovascular risk factors (Luk et al. 2009). The authors suggested that although increased physical activity is associated with an increase in endothelial function (measured by FMD), this improvement does not likely involve the action of EPCs (Luk et al. 2009). More recently, Kruger et al. asked participants with a history of myocardial infarction to report their weekly involvement in activities of daily living (e.g., walking, active commuting to work, using the stairs), leisure activities (e.g., gardening), and sport activities (e.g. swimming, tennis). Unlike Luk et al., the authors found that $CD34^{+}CD309^{+}$ concentrations were significantly and positively

associated with total physical activity time ($r = 0.60$, $p = 0.023$), time spent in activities of daily living ($r = 0.560$, $p = 0.021$), and to a lesser extent with leisure and sport active time ($r = 0.42$, 0.41, respectively. $p<0.05$) (Krüger et al. 2014a). Conversely, $CD34^+CD45^+$ concentrations were not linked with any indicator of physical activity (data and statistics not reported). Plasma G-CSF levels were positively associated with total activity and activities of daily living, and when assessed by linear regression, combinations of plasma G-CSF levels and total physical activity ($\beta = 0.41$) as well as G-CSF levels and activities of daily living ($\beta = 0.50$) were significant predictors of $CD34^+CD309^+$ but not $CD34^+CD45^+$ cells (again, data and statistics not reported) (Krüger et al. 2014a). The authors suggested that activity-related increases in G-CSF levels may play a role in mobilizing specific subsets of EPCs, namely $CD34^+CD309^+$ but not $CD34^+CD45^+$ cells.

Exercise training is a structured form of physical activity that has been shown to increase resting EPC concentrations and function in health and disease (Hoetzer et al. 2007, Sarto et al. 2007, Walther et al. 2009, Erbs et al. 2010, Van Craenenbroeck et al. 2010b, Schlager et al. 2011, Gatta et al. 2012, Cesari et al. 2012, 2013, Fernandes et al. 2012, Yang et al. 2013, Mezzani et al. 2013, Ribeiro et al. 2013, Choi et al. 2014); however, it is important to recognize that exercise training studies do not provide an accurate indication of the relationship between EPCs and

physical activity, *per se*. More specifically, these studies do not commonly quantify any physical activity performed outside of the prescribed intervention, which only represents a small fraction of waking and potentially active time. Thus, the relationship between EPCs and physical activity remains unclear. Not only are the available findings are limited by the use of subjective measures of physical activity (Shephard and Vuillemin 2003, Prince et al. 2008), but also by the confounding effect of the various EPC cell definitions.

1.4.5 Effects of Acute Exercise on EPCs in Adults

A single, acute bout of exercise is known to upregulate a number of factors involved in EPC mobilization, homing, paracrine activity, and differentiation (Lenk et al. 2011, Koutroumpi et al. 2012, Volaklis et al. 2013, Palmefors et al. 2014). As such, it is not surprising that most studies report a transient, positive effect of acute exercise on EPC number and function in both healthy adults as well as those with chronic disease (Lenk et al. 2011, Koutroumpi et al. 2012, Volaklis et al. 2013). However, there is some evidence to suggest that the degree of change in number and function may depend on a host of variables that include but are not limited to experimental factors such as exercise duration (Laufs et al. 2005), intensity (Laufs et al. 2005), timing of blood sample collection (Möbius-Winkler et al. 2009), as well as the presence and/or severity of disease (Adams et al. 2004, Van Craenenbroeck et al. 2010a, 2011).

Although most studies to date have examined changes in EPCs following a graded maximal exercise test, one study by Laufs et al. compared the effects of 3 protocols of varying durations and intensities on EPCs in healthy males. They found that 30-minutes of running at either a moderate (~68% $\dot{V}O_{2max}$) or high intensity (~82% $\dot{V}O_{2max}$) elicited a similar ~1.5- to 2.5-fold increase in EPCs; no change was observed when the high intensity exercise was performed for only 10-min (Laufs et al. 2005). The results of an exercise duration subgroup analysis from our meta-analysis suggested a graded EPC response by exercise duration (Figure 1.3; Details in Appendix A), whereby exercise of less than 20-min resulted in the largest improvements in EPCs, followed by a smaller but still significant increase with exercise between 21 and 180 minutes in length, and a negligible effect of even longer bouts of exercise (>180-min). It is important to note that the exercise intensity varied between the studies included in the meta-analysis, with most of the studies of <20-min consisting of a graded maximal exercise test. Therefore, it may be that a maximal intensity is required to induce a significant EPC response during short bouts of exercise; however, in the absence of a direct comparison, it is difficult to truly distinguish the effects of exercise intensity and duration on EPCs.

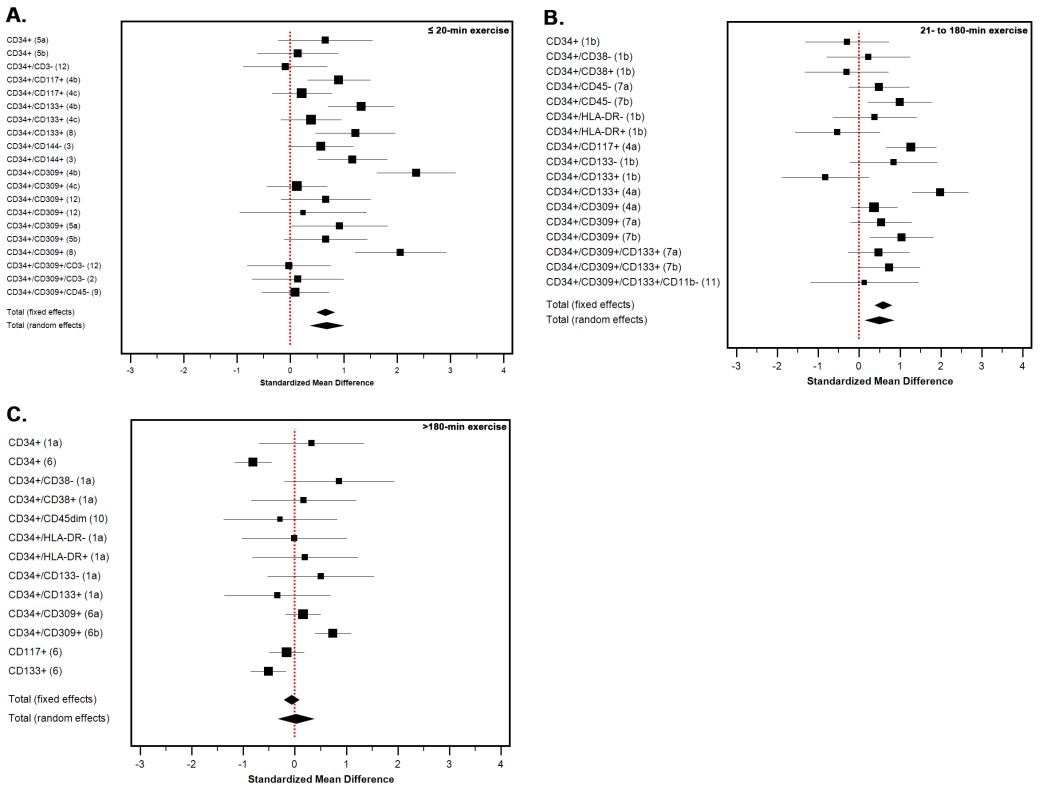

Figure 1.3 *Forest plot of the effects of an acute bout of (A) < 20-min, (B) 21- to 180-min exercise, and (C) >180-min exercise on EPCs in healthy adults.* A significant degree of heterogeneity was observed between studies for all three exercise durations (Q_A = 65.6, p<0.0001; I^2_A = 71.0 [lower, upper 95% CI: 54.5, 81.6]; Q_B = 47.4, p = 0.0001; I^2_B = 66.2 [43.8, 79.7]; Q_C = 53.7, p<0.0001; I^2_C = 77.7 [62.2, 86.8]); pooled results were examined using random effects models. Standardized mean difference (SMD) values were calculated as post-exercise EPCs – pre-exercise EPCs. The largest effect was seen with short bouts of acute exercise (A, <20-min), which led to a significant increase in EPCs (medium effect, $SMD_{pooled\text{-}A}$ = 0.681 [0.394, 0.967], p<0.001), followed by a small, but still significant positive, effect of exercise bouts between 21 to 180 minutes in length (B; $SMD_{pooled\text{-}B}$ = 0.494 [0.164, 0.824], p = 0.003). Longer bouts of exercise (>180-min) had no significant effect on EPCs when pooled ($SMD_{pooled\text{-}C}$ = 0.023 [-0.308, 0.354], p = 0.893). Data are identified and sorted according to EPC cell definition followed by the reference, where (1) a: Marathon (range: 159 – 254 min), b: half-marathon (range: 69 – 91 min) (Bonsignore et al. 2002); (2) Maximal exercise test on a cycle ergometer (25 W step per 3-min stage) (Adams et al. 2004); (3) Maximal exercise test on a cycle ergometer or treadmill (no additional details provided) (Rehman et al. 2004); (4) a: 30-min running at 100% velocity at anaerobic threshold (~82% $\dot{V}O_{2max}$), b: 30-min running at 80% of velocity

at anaerobic threshold (~68% $\dot{V}O_{2max}$), c: 10-min running at 80% of velocity at anaerobic threshold (~68% $\dot{V}O_{2max}$) (Laufs et al. 2005); (5) Maximal exercise test on a cycle ergometer (25 W step per 2-min stage) from a: PBMC samples (n = 11), and b: whole blood samples (n = 14) (Van Craenenbroeck et al. 2008b); (6) Marathon (mean ± SEM: 4 h 11 min ± 4 min), a: EPCs expressed as cells/mL blood, and b: EPCs expressed as a % of $CD309^+$ cells (Adams et al. 2008); (7) 30-min of cycling at a workload equivalent to 80% of lactate threshold in a: South Asian men, and b: white European men (Cubbon et al. 2010); (8) Maximal exercise test on a treadmill (12% incline, 3.5 km/h); (9) Maximal exercise test on a treadmill (Bruce protocol) (Scalone et al. 2013); (10) Ultra-endurance cycling race consisting of 550-km (7,000-m altitude) over 4 days, 8-h competition alternating with 8-h rest (Stelzer et al. 2014); (11) 30-min treadmill running (heart rate maintained >140 bpm) (Chang et al. 2015); (12) Maximal exercise test on a cycle ergometer (ramp protocol, 10 or 20 W/min stages) (Van Craenenbroeck et al. 2010a).

The temporal profile of the EPC response to an acute bout of exercise is not entirely clear. Möbius-Winkler et al. examined EPCs at baseline and over 15 time points during and up to 24-hours after 240-min of cycling at 70% of anaerobic threshold in a sample of young, healthy men. When EPCs were defined as $CD34^+CD309^+$, a significant increase from baseline was noted at 210 minutes, with a peak at 240-min of exercise (5.5-fold increase); EPCs returned to baseline concentrations by 30-min post-exercise (Möbius-Winkler et al. 2009). Using the $CD133^+CD309^+$ cell definition, the authors reported a smaller 3.5-fold increase and peak in EPC response at 210-min of exercise, which was maintained until 120-min post-exercise, and returned to baseline values by 24-hours (Möbius-Winkler et al. 2009). Interestingly, a rapid increase in VEGF was noted at 5 and 10 min of exercise (1.9-fold increase), which correlated with the subsequent change in $CD133^+CD309^+$ EPCs (r = 0.67,

p = 0.005), but not $CD34^+CD309^+$ EPCs (r = 0.05, p = 0.86). Sandri et al. used the same two cell definitions to examine EPCs over a 72-hr period following a maximal treadmill test, and reported a significant increase in both EPCs at 360-480 min post-exercise, with a peak at 24-h post-exercise in patients with peripheral arterial occlusive disease and healthy controls (Sandri et al. 2011). Although the EPC responses were delayed in comparison to the findings of Möbius-Winkler et al., the pattern of change was similar in that $CD34^+KDR^+$ EPCs returned to baseline before $CD133^+CD309^+$ cells, which remained elevated until the 72-h time point (Sandri et al. 2011). Still other studies have reported an increase in EPCs in as little as 10- to 30-min post-exercise; however, sampling time points were limited to 1 or 2 time points within the 1-h period following and as such, may have missed a peak in EPC response (Rehman et al. 2004, Laufs et al. 2005, Van Craenenbroeck et al. 2008b). Nevertheless, the available data from time-course studies suggest that the overall pattern of the EPC response may be similar in health and disease, but the magnitude and timing of the response may differ by group, or by the duration and intensity of the exercise bout.

Exercise may represent a potent, non-pharmacologic tool to increase EPCs, and as such, holds great potential as an adjunct therapy for individuals at risk of poor cardiovascular health, as well as those with diagnosed cardiovascular disease. Unfortunately, studies examining the

effects of acute exercise on EPCs in these populations have yielded mixed results, with some demonstrating a similar or exaggerated EPC response to exercise (Adams et al. 2004, Van Craenenbroeck et al. 2010b), and others reporting a smaller or completely blunted EPC response when compared with healthy controls (Shaffer et al. 2006b, Sandri et al. 2011, Van Craenenbroeck et al. 2011, Scalone et al. 2013). These inconsistencies were reinforced by our meta-analysis (Figure 1.4; Details in Appendix A), which revealed a large degree of variability (lower, upper 95% confidence interval: -0.468, 0.234) in the difference in EPC response to acute exercise in adults with and without diagnosed medical conditions; this led to an overall negligible difference in EPC response between groups (SMD_{pooled} = -0.117, p = 0.512). It is plausible that an exaggerated EPC response to exercise observed in select clinical populations may represent a compensatory mechanism to increase repair and prevent further damage to the endothelium (Fadini et al. 2007, Van Craenenbroeck et al. 2010a). Conversely, a blunted EPC response to exercise may be a result of exhaustion of the bone marrow, and subsequent reduced mobilization of EPCs (Kissel et al. 2007, Van Craenenbroeck and Conraads 2013). As of yet, there is no clear evidence to inform the differences in EPC responses between clinical populations; however, Van Craenenbroeck et al. suggested that these may be related to the baseline levels of oxidative stress and chronic inflammation (Van Craenenbroeck

and Conraads 2013), which are often considered to be indicators of disease severity.

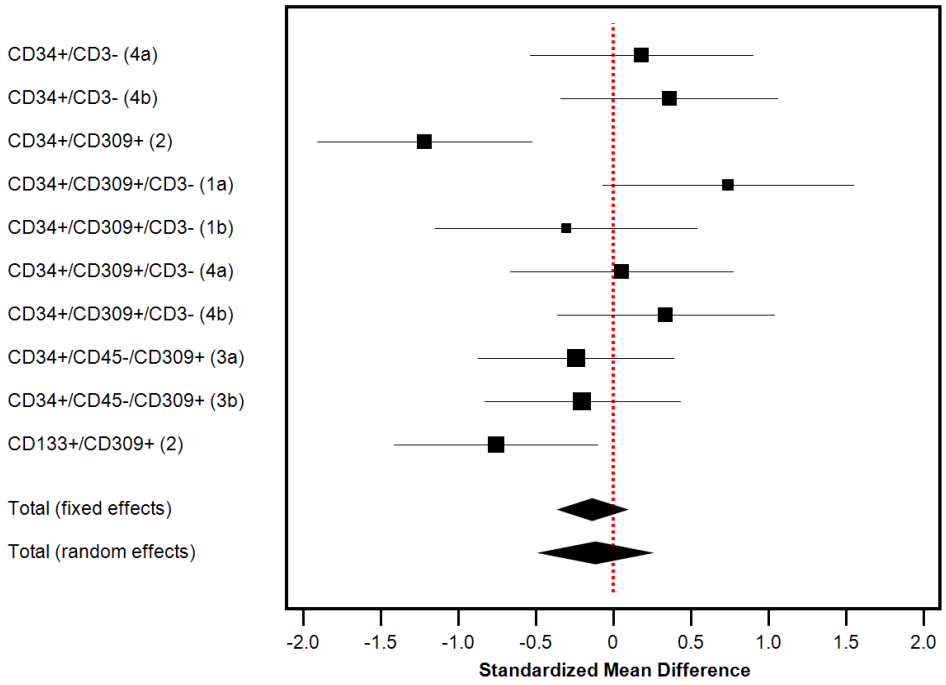

Figure 1.4 *Forest plot of the comparison of the effects of acute exercise on EPCs in healthy adults and adults with chronic disease.* Pooled results of 10 comparisons of adults with chronic disease and healthy controls from 5 studies revealed a significant degree of heterogeneity between studies ($Q = 24.0$, $p=0.004$; $I^2 = 62.55\%$ [lower, upper 95% CI: 25.7, 81.1]), thereby prompting the use of a random effect model. SMD is calculated as change in disease group – change in healthy controls, where a positive value indicates a greater exercise response in the disease group, and a negative value is a smaller exercise response in the disease group relative to healthy controls. Pooled analysis suggested a trivial effect, and no difference in EPC response to exercise between groups ($SMD_{pooled} = -0.117$ [-0.468, 0.234], $p=0.512$). Data are identified and sorted according to EPC cell definition followed by the reference, where (1) Maximal exercise test on a cycle ergometer (25 W step per 3-min stage) in a: coronary artery disease and exercise-induced ischemia, and b: coronary artery disease and no exercise-induced ischemia (Adams et al. 2004); (2) Maximal exercise test on a treadmill (12% incline, 3.5 km/h) in peripheral arterial occlusive disease (Sandri et al. 2011); (3) Maximal exercise test on a treadmill (Bruce protocol) in a: microvascular angina, and b: diagnosed coronary artery disease (Scalone et al. 2013); (4) Maximal exercise test on

a cycle ergometer (ramp protocol, 10 or 20 W/min stages) in a: mild congestive heart failure, and b: severe congestive heart failure (Van Craenenbroeck et al. 2010a).

A single bout of acute exercise leads to transient changes in a host of factors that may be involved in exercise-induced changes in EPCs (Lenk et al. 2011, Palmefors et al. 2014). In both healthy and disease populations, exercise-related increases in shear stress may increase the bioavailability of NO (via an increase in eNOS), which then stimulates an increase in MMP-9, a key regulator of EPC recruitment from the bone marrow (reviewed in Section 1.3.4). In addition, exercise-induced skeletal muscle secretion of VEGF (Gustafsson et al. 1999), SDF-1 (Ratajczak et al. 2003), and HIF-1 (Gustafsson et al. 1999, Ameln et al. 2005) into the general circulation may lead to enhanced EPC mobilization. In adults with chronic medical conditions, particularly those with ischemic cardiovascular disease, exercise-induced ischemia is thought to play a critical role in upregulating circulating HIF-1, which acts in an MMP-9-dependent manner to increase EPC mobilization (Ceradini et al. 2004, Volaklis et al. 2013). There is some evidence to suggest that aside from an increase in EPC mobilization, acute exercise may also increase the EPC proliferation, differentiation, and paracrine activity, which may be related changes in the systemic and local cytokine/hormonal environment (Chang et al. 2015). Taken together, the evidence suggests that an acute bout of exercise may modulate both EPC number and function; however, the exact pathways

involved in regulating the EPC response are not entirely understood, and may be influenced by a number of factors. Additionally, although the results of our meta-analysis suggest that a short, acute bout of maximal intensity exercise may have the most potent effect on EPCs, a more rigorous and systematic investigation is needed to determine the optimal exercise duration, intensity, and modality to maximize EPC response.

1.4.6 Exercise, CECs, and EPCs in the Pediatric Population

To date, there are no studies that have examined CECs and only 8 studies that have assessed EPCs in the context of fitness, physical activity, and/or exercise in the pediatric population. Of these, 3 presented cross-sectional data, 2 examined the effects of acute exercise, and 3 assessed the effects of an exercise intervention on EPCs. The heterogeneity in study populations, exercise protocols, and EPC definition yielded mixed results between studies, which further highlights the need for additional research to enhance our understanding of EPCs and their links with fitness and activity in healthy youth.

Walther et al. found that aerobic fitness ($\dot{V}O_{2max}$) was weakly associated with EPC concentrations, defined as $CD34^{+}CD309^{+}$ ($r = 0.33$, $p<0.001$), in a sample of 50 healthy children between the ages of 11 and 12 years (Walther et al. 2008). No such relationship with $\dot{V}O_{2max}$ was observed when the same surface markers were used in a smaller sample

(N = 24) of 8- to 16-year-old obese youth (r = 0.131, p = 0.572); however, the authors reported slightly stronger associations between $\dot{V}O_{2max}$ and EPCs when they were defined as $CD34^+$ (r = 0.458) or $CD34^+CD133^+$ (r = 0.465, $p<0.05$) (Arnold et al. 2010). Similar discrepant findings have been reported in comparisons of EPCs in athletes and healthy, non-athlete controls (Walther et al. 2008, 2009, D'Ascenzi et al. 2014). Indeed, healthy children receiving standard physical education classes (45 min, 2x/wk) but not registered in any extra-curricular sports presented with approximately 41% lower resting levels of $CD34^+CD45^{dim}CD309^+$ EPCs, and 19% lower fitness levels compared with children of the same age who were enrolled in a specialized sport school (45 min, 12x/wk and competitive events) (Walther et al. 2009). Conversely, D'Ascenzi et al. found no differences between competitive soccer players (1.5-2.5 hr, 6-8x/wk and competitive events) and healthy non-athlete controls for $CD309^+$, $CD34^+CD133^+CD309^+$, $CD34^+CD133^-CD309^+$ or $CD34^-CD133^+CD309^+$ cells (Walther et al. 2009, D'Ascenzi et al. 2014). The authors suggested that chronic training may not influence baseline levels of EPCs in healthy adolescents (D'Ascenzi et al. 2014). Training studies in both healthy children, as well as overweight and obese youth, suggest otherwise (Walther et al. 2009, Park et al. 2012, Bruyndonckx et al. 2015).

The first study to directly examine the effect of exercise training on EPCs compared students receiving an intervention of daily physical

education (45 min/session, at least 15-min aerobic exercise) in a school setting over an academic year compared with controls receiving a standard physical education curriculum of 2 × 45-min sessions/week (Walther et al. 2009). They reported a 19.3% increase in $CD34^{+}CD45^{dim}CD309^{+}$ EPCs in the intervention group, compared with a 14.6% decrease in controls (between group comparison, $p = 0.023$), but no change in EPCs defined as $CD34^{+}$ or $CD34^{+}CD309^{+}$ ($p = 0.791 – 0.891$) (Walther et al. 2009). Despite the increase in one subset of EPCs in the intervention group, their values were still 27.8% lower than the concentrations observed in children attending a specialized sport program ($p = 0.006$). Interestingly, the increased exposure to physical education classes did not have any effect on the migratory capacity of EPCs exposed to SDF-1, which was similar in the intervention and control groups, but still significantly lower than the specialized sport group (Walther et al. 2009). This finding was consistent with a previous study by the same authors (Walther et al. 2008), and in both cases they postulated that a higher training volume and intensity might be required to alter EPC function; however, they did not report any specific details about the intensity of physical education classes (Walther et al. 2008, 2009).

More recently, both Park et al. and Bruyndonckx et al. reported significantly larger improvements in EPCs of 266.7% and 62.8%, respectively, following shorter interventions (12 weeks and 5/10 months,

respectively) in overweight and/or obese youth (Park et al. 2012, Bruyndonckx et al. 2015). Whether the marked differences in the improvements in EPCs are related to the participant population and/or to the exercise interventions is difficult to determine as neither Walther et al. nor Bruyndonckx et al. provided any specific details with respect to the intensity of the exercise performed in their studies. Park et al. reported that their 3×/wk, 12-week combined aerobic (30-min at 50-70% of heart rate reserve) and resistance training (2 × 8-12 repetitions, 7 exercises, 60% of 1 repetition max) program led to significant improvements in fitness, as well as in markers of endothelial activation that may act as potential regulators of EPC mobilization, including a decrease in resting levels of sE-selectin, and increases in both VEGF and NO levels compared with the non-exercising control group (Park et al. 2012). As part of their study, Bruyndonckx et al. examined endothelial microparticles (defined as $CD34^{+}CD42b^{-}$) and reported a 21.5% decrease in their concentrations at the 10-month time point (Bruyndonckx et al. 2015). Endothelial microparticles are small fragments of cells that are shed from activated and apoptotic endothelial cells, and may therefore provide some insight into the effects of exercise training on CEC levels. However, it must be noted that endothelial microparticles are considered a distinct population from CECs, and perhaps more importantly, that this study consisted of both an exercise and dietary intervention, making it difficult to separate the

unique effects each component of the intervention on both EPCs and endothelial microparticles (Bruyndonckx et al. 2015). Taken together, these findings suggest that a chronic exercise stimulus can positively affect EPCs in healthy children, and may have an even more potent effect on overweight and obese youth with increased risk factors for cardiovascular disease.

Examining the impact of a single, acute bout of exercise on EPCs is critical for enhancing our understanding of their relationship with exercise, and the potential mechanisms underlying the exercise-induced improvements in cardiovascular health. Zaldivar et al. examined changes in EPCs following 10 × 2-min bouts of cycling at a workload corresponding to 50% of the workload between anaerobic threshold and maximal oxygen uptake in pre- and post-pubertal boys. They reported an 83 ± 19% increase in EPCs, defined as $CD34^{+}CD45^{dim}$, in pre-pubertal boys, and a trend towards a larger 170 ± 45% increase in the post-pubertal boys ($p = 0.067$) (Zaldivar et al. 2007). The change in EPCs was matched by an increase in FMS-like tyrosine kinase 3 (FLT-3), G-CSF, and SDF-1, which are each involved in upregulating EPC mobilization from the bone marrow (Zaldivar et al. 2007). The only other study to examine the effects of acute exercise on EPCs was in a small sample of youth with chronic kidney disease, and reported no change in either $CD34^{+}$ or $CD34^{+}CD31^{+}CD45^{dim}CD133^{+}$ EPCs following 20-min of continuous cycling

at 50% of the workload at maximal oxygen uptake (Lau et al. 2014). These findings were unchanged when EPCs were re-examined using the same definition as Zaldivar et al. (Obeid et al., unpublished observation). The differences in EPC responses between these studies may be due to differences in the exercise protocols, but may also be related to a reduced EPC response to exercise in participants with a chronic medical condition, as seen in adults with peripheral arterial disease (Shaffer et al. 2006b, Sandri et al. 2011) and chronic heart failure (Van Craenenbroeck et al. 2011).

The findings of the small number of studies examining EPCs and CECs in in youth serve to highlight a number of important gaps and limitations in our understanding of these cells in the pediatric population. First, there are no studies examining exercise and CECs in youth. Second, although there are data available on the differences in EPCs in “active” versus “non-active” children, no study has assessed the link between habitual levels of physical activity, *per se*, and EPCs. Third, one common factor underlying the discordant findings between studies is the difference in cell definitions of EPCs, suggesting this may be an important consideration for future work. Finally, the available literature in youth is largely incomplete and much like adult studies, biased to primarily report significant data, while under-reporting statistics surrounding insignificant findings and important details about exercise protocols. Given the ever-

growing body of evidence supporting the role of CECs and EPCs in the monitoring and maintenance of cardiovascular health in adults, and the potential beneficial effect of physical activity and exercise on these cells, additional work that more closely examines the role of CECs and EPCs, and their link with physical activity and exercise in youth seems warranted.

1.5 Objectives and Hypotheses

1.5.1 General Objective

The general objective of this thesis was to examine EPCs and CECs, and the factors influencing their concentrations, during childhood and adolescence.

1.5.2 Specific Objectives

The specific objectives of the studies in this thesis were to:

(1) Examine sex-, chronological- and biological age-related differences in CECs at rest, and assess their association with aerobic fitness, physical activity, and adiposity (Chapter 3);

(2) Examine sex- and biological age-related differences in EPCs at rest, assess their association with aerobic fitness, physical activity, and adiposity, and determine the effect of cell definition on EPC levels (Chapter 4);

(3) Conduct preliminary examination of potential differences in EPCs and CECs at rest in children with JIA and healthy controls, and compare changes in EPCs and CECs in response to acute bouts of moderate intensity, continuous exercise, and high intensity, interval exercise (Chapter 5).

1.5.3 Specific Hypotheses

The specific hypotheses of the studies in this thesis were that:

(1) CECs would vary between boys and girls, be lower in younger, less mature participants, and demonstrate a negative association with aerobic fitness and physical activity but a positive association with adiposity (Chapter 3);

(2) EPCs would vary between boys and girls, be higher in less mature participants, demonstrate a positive association with aerobic fitness and physical activity but a negative association with adiposity, and vary significantly based on phenotypic definition (Chapter 4);

(3) EPCs would be lower, and CECs higher in youth with JIA compared with healthy controls, and that both types of acute exercise would lead to similar increases in EPCs, and decreases in CECs in JIA and healthy controls (Chapter 5).

CHAPTER 2: PROJECT DESIGN, STUDY PARTICIPANTS, AND METHODOLOGICAL CONSIDERATIONS

Research in pediatric populations is challenged by a number of unique issues ranging from obvious ethical concerns, to the potentially confounding effects of growth and maturation on outcomes of interest. Indeed, inter-individual differences in body size and pubertal status can introduce significant variability in study outcomes, and potentially inflate (Type I error) or mask (Type II error) differences between participants or time points. Thus, it is important for the pediatric researcher to account for factors related to growth and maturation, and understand the impact of inter-individual differences in the timing and tempo of their development when designing studies involving children and adolescents.

In addition to the aforementioned challenges, the application of multicolour flow cytometry for immunophenotyping of rare cell populations is fraught with its own special considerations. Among these are the selection of the appropriate number and type of antibodies for assessment of rare cells, the development of robust and reliable protocols for cell isolation and staining, as well as the standardization of analysis and gating strategies to ensure objective assessment of cell populations.

The following sections will highlight the methodological considerations surrounding the study design, participant recruitment, and

flow cytometry measures used throughout this thesis. Detailed methods for each study are presented in their respective chapters.

2.1 Project Design

The studies in this thesis are cross-sectional in nature. To date, much of what is known about CECs and EPCs, and the factors influencing their concentrations in the peripheral blood, is based on adult literature. The studies presented in Chapters 3, 4, and 5 were designed to further our understanding of CECs and EPCs in the pediatric population. More specifically, Chapters 3 and 4 represent the first efforts towards characterizing resting CECs and EPCs in children and adolescents, and examining their associations with fitness, physical activity and body composition. Although the ranges of CEC and EPC proportions and concentrations were relatively large, most of the data was clustered toward one end of the range, which was likely related to a low prevalence of cardiovascular risk factors in healthy youth. This led to the design of a study to examine these cells in children with JIA who may be at an increased risk of CVD compared with their healthy peers (Chapter 5). The effect of acute exercise on CECs and EPCs was also examined in Chapter 5 as a potential mechanism for exercise-related improvements in cardiovascular health in children and adolescents.

2.1.1 Estimate of Biological Age

In order to examine differences in CECs and EPCs by pubertal status, biological age was estimated from anthropometric measurements as described by Mirwald et al. (Mirwald et al. 2002). Standing and sitting height, weight, and chronological age were determined to allow for calculation of years from peak height velocity (YPHV), which was used as an indicator of maturity offset. A positive YPHV value indicates that the participant is beyond PHV (i.e., late- or post-pubertal), while a negative value indicates that the participant has yet to attain PHV (i.e., pre- or early-pubertal). Values were calculated separately for males and females using the following equations:

$$YPHV_{males} = -9.236 + 0.0002708 \cdot \text{Leg Length} \times \text{Sitting Height} - 0.001663 \cdot \text{Age} \times \text{Leg Length} + 0.007216 \cdot \text{Age} \times \text{Sitting Height} + 0.002292 \cdot (\text{Weight} \div \text{Height} \times 100)$$

$$YPHV_{females} = -9.376 + 0.0001882 \cdot \text{Leg Length} \times \text{Sitting Height} + 0.0022 \cdot \text{Age} \times \text{Leg Length} + 0.005841 \cdot \text{Age} \times \text{Sitting Height} - 0.002658 \cdot \text{Age} \times \text{Height} + 0.7693 \cdot (\text{Weight} \div \text{Height} \times 100)$$

2.2 Study Participants

2.2.1 Selection of a Representative Sample

Participant recruitment for Chapters 3 and 4 was designed to capture a study sample that was generally reflective of the Canadian pediatric population, and to maximize the likelihood of inter-individual variability in fitness, physical activity, and body composition. For the most

part, participants were healthy weight and recreationally active children (i.e., not training for competitive sport). These participants (n = 59, ~63% of sample) were recruited using flyers throughout the general community, as well as through study information letters distributed to a stratified (by geographical region) and randomized selection of elementary and secondary schools in the Hamilton-Wentworth District School Board. By design, ~22% of participants (n = 21) were overweight or obese based on the Centre for Disease Control criteria (Ogden et al. 2002, Roberts et al. 2012). This group was recruited from the general community as well as through the weight management program at the Children's Exercise and Nutrition Centre, McMaster Children's Hospital. Another ~15% of the sample (n = 14) comprised of youth training for competitive sport (i.e., participating in training ≥4 times per week), recruited from local hockey, volleyball, and triathlon teams. With the exception of overweight/obesity, children were only excluded if they had a diagnosed medical condition at the time of study participation.

In order to assess differences in CECs and EPCs associated with growth and maturation, participants were deliberately recruited in two groups based on pubertal status: pre-/early-pubertal and late-/post-pubertal. For the purposes of participant recruitment, these groups were defined by chronological ages of 8 to 10 years and 14 to 16 years, respectively. The decision to exclude 11- to 13-year-old children was

based on the wide range of pubertal stages observed in this age range (Rowland 1996, Armstrong et al. 1999a, 1999b). Moreover, given that puberty is a time of rapid physiological and behavioural changes (Bar-Or and Rowland 2004, Rowland 1996), and very little is known about how these changes impact CECs and EPCs, we hypothesized that the inclusion of a mid-pubertal group would increase the variability of our findings, and thereby require a significantly larger sample to address our objectives. Thus, children in two distinct age groups, 8 to 10 and 14 to 16 years, were recruited in an effort to maintain a reasonable sample size and sufficient statistical power to examine CECs and EPCs by chronological age and pubertal status.

2.2.2 Juvenile Idiopathic Arthritis and Healthy Controls

Specific details of the inclusion and exclusion criteria for the participants with JIA and healthy controls are described in detail in Chapter 5. Importantly, due to the demanding requirements of this study with respect to time commitment, exercise intensity, and blood sampling, we recruited children and adolescents in a broad age range of 7 to 17 years to participate. While children with JIA were asked to invite a friend or sibling to participate as a healthy control, it became difficult to recruit female participants in the control group to match the JIA participants. As such, participants are only matched by age, and not sex. However, this is not expected to have a significant impact on the study findings given that

no sex-based differences were observed for either CECs or EPCs in Chapters 3 and 4, respectively.

2.3 Flow Cytometry

2.3.1 Measurement of EPCs and CECs

Both CECs and EPCs in this thesis were quantified by flow cytometry according to the protocol of Duda et al. (Duda et al. 2007). The lyse-no wash protocol was performed on PBMCs isolated from 10 mL of whole blood. The monoclonal antibodies and bound fluorochromes used for cell enumeration were: CD34-APC, a marker of hematopoietic precursor and endothelial cells; CD31-FITC, a marker of monocytes and endothelial cells; CD45-PerCP and CD133-PE, markers of hematopoietic precursors that are not expressed on mature endothelial cells (Woywodt et al. 2004, Burger and Touyz 2012). Cells of interest were expressed as both:

1. A proportion of PBMCs (% of PBMCs):

$$\%\ \text{of PBMCs} = \frac{\text{EPC or CEC count}}{\text{PBMC count}} \times 100$$

2. A concentration in whole blood:

$$[\text{CEC or EPC}] = \%\ \text{of PBMCs} \times \frac{\text{PBMC count}}{\text{L of whole blood}}$$

where PBMC count per litre of whole blood was calculated from the sum of lymphocyte and monocyte counts from the same sample, determined using a Coulter counter by the McMaster Core Facility.

2.3.2 Comparison of Flow Cytometers

In Chapter 5, two flow cytometers were used for assessment of CECs and EPCs: a BD LSRII (n = 2 JIA and 2 controls) and a Miltenyi Biotec MACSQuant (n = 5 JIA and 4 controls) flow cytometer. This was done for practical reasons associated with the limited availability of the BD LSRII unit. Importantly, participants with JIA and their matched control were assessed on the same unit.

In order to determine if it was appropriate to pool our results, CECs and EPCs were examined on both units with 10 blood samples taken from a convenience sample of 5 participants. Dependent sample t-tests were calculated to determine group level differences between units, and Bland-Altman plots were generated to assess agreement and potential systematic bias in CECs and EPCs as a percentage of PBMCs (Bland and Altman 1986). Mean bias and 95% limits of agreement (LoA) were calculated and corrected for multiple observations per individual, as previously described by Bland and Altman (Bland and Altman 2007). Acceptable agreement was based on the recommendations of Duda et al., which suggest that the standard deviation between duplicate samples should be no more than 0.2% for CECs and 0.01% for EPCs (Duda et al.

2007). These values were used as the upper threshold for the estimated bias between units.

No significant differences were observed between the MACSQuant and BD LSRII units for CECs ($t = 2.17$, $df = 9$; $p = 0.06$, Figure 2.1A) or EPCs ($t = 0.16$, $df = 9$; $p = 0.88$, Figure 2.1B). For the agreement analysis, differences between units were calculated as MACSQuant – BD LSRII. Mean bias (LoA) for CECs was 0.011 (LoA = -0.022, 0.045; Figure 2.1C), and EPCs was 0.001 (LoA = -0.067, 0.070; Figure 2.1D). These bias values fell well below the recommended threshold, as did the calculated standard deviations of the mean bias (CECs = 0.0002; EPCs = 0.001). Taken together, these results suggest that CEC and EPC levels determined using the MACSQuant and BD LSRII were similar.

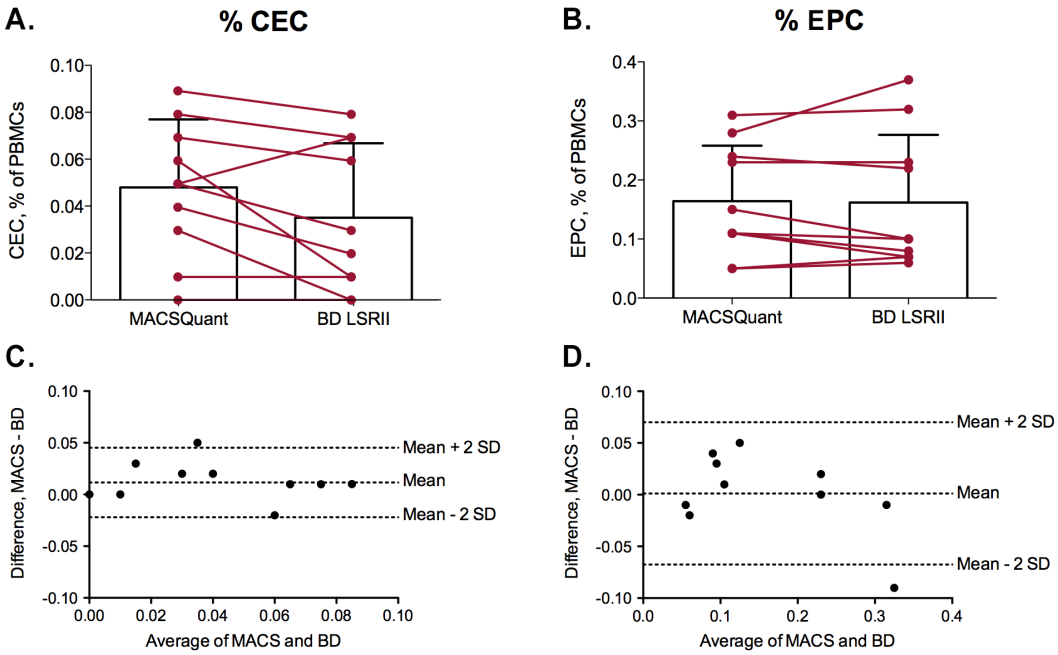

Figure 2.1 *Comparison of the MACSQuant (MACS) and BD LSRII (BD) flow cytometers*. Analysis suggested comparable measurement of CECs (A and C) and EPCs (B and D) expressed as a percentage of PBMCs. Mean = weighted mean bias, calculated as the mean of the difference between the MACS and BD, adjusted for multiple observations per individual; Mean - 2SD and + 2SD = 95% lower and upper limits of agreement, adjusted for multiple observations per individual.

2.3.3 Repeatability, Reliability and Reproducibility of Measurement

Both CECs and EPCs are rare cell populations (Woywodt et al. 2002, 2004, Duda et al. 2007). In adults, CECs and EPCs are estimated to account for approximately 0.1 – 6.0% and 0.01 – 0.20% of PBMCs, respectively (Duda et al. 2007). As such, it is not uncommon to detect fewer than 50 events in a given sample, which highlights the need for repeatable, reliable and reproducible measurement of these cells. Repeatability and test-retest reliability of CEC and EPC enumeration were

determined in duplicate with 10 samples collected at random. Blood samples were collected and processed in accordance with the protocol described in Chapters 3 and 4. Cells were enumerated on either the MACSQuant (n = 5) or the BD LSRII (n = 5) flow cytometer, and data were pooled for all statistical analyses. Reproducibility of CEC and EPC enumeration was determined from resting blood samples taken from Chapter 5. Intraclass correlation coefficients (ICC, model: two-way mixed) were calculated, and interpreted according to Landis and Koch, where poor agreement was defined as 0.0 – 0.20, 0.21 – 0.40 as fair, 0.41 – 0.60 as moderate, 0.61 – 0.80 as substantial, and 0.81 – 1.00 as almost perfect agreement (Landis and Koch 1977).

Repeatability was established by examining a single sample on two occasions, separated by approximately 1 hour. Samples were stored at 2-8°C until analysis, and compensation settings were kept constant between assessments. The repeatability coefficients for CECs and EPCs suggested that the estimated absolute differences between duplicate measurements were no greater than 0.001% and 0.002% on 95% of occasions, respectively. The ICC for single measures of CECs was 0.958 (0.829, 0.990; $p<0.001$, Figure 2.2A), and the ICC for EPCs was 0.920 (95% upper, lower confidence intervals (CI): 0.708, 0.981; $p<0.001$, Figure 2.2B).

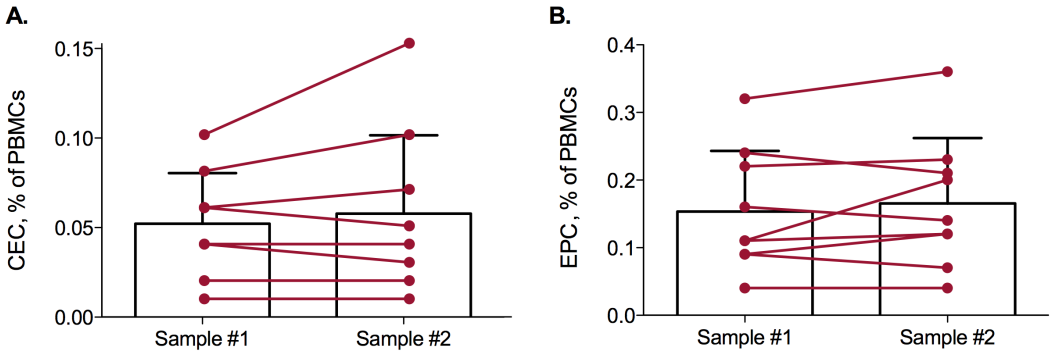

Figure 2.2 *Repeatability of CEC and EPC assessments*. Almost perfect repeatability was observed for CECs (A) and EPCs (B). Data are presented as group mean and standard deviations for sample #1 and sample #2 (bars), with an overlay of paired individual samples.

Test-retest reliability was established by collecting and staining two separate samples from each individual. Flow cytometry assessments of duplicate samples were performed consecutively to avoid potential differences due to sample degradation over time; however, the cytometer and compensation settings were reset between assessments. The ICC (lower, upper 95% CI) for single measurement of CECs was 0.823 (0.464, 0.952; p=0.001, Figure 2.3A), and 0.975 (0.907, 0.994; p<0.001, Figure 2.3B) for EPCs.

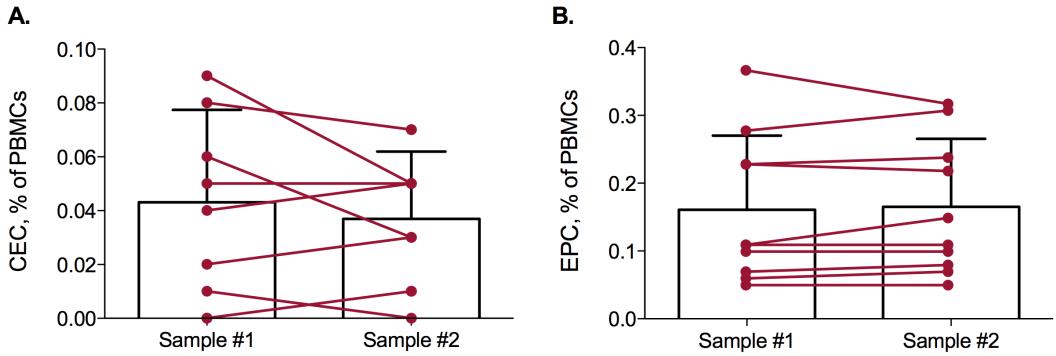

Figure 2.3 *Reliability of CEC and EPC assessments.* Almost perfect levels of agreement were found for both CECs (A) and EPCs (B) in 2 blood samples collected from the same individual, suggesting reliable enumeration of both cell populations. Data are presented as group mean and standard deviations for sample #1 and sample #2 (bars), with an overlay of paired individual samples.

Reproducibility was examined using resting blood samples collected on two separate days from the same participants. Blood samples were collected approximately 1 week apart, at the same time of day. Participants were asked to replicate their diet and activity behaviours on both days for 24 hours prior to sample collection, and to refrain from eating and drinking for 3 hours before the blood sample. The reproducibility of CEC measurement was poor, with a single measurement ICC (95% CI) of -0.042 (-0.441, 0.532; p=0.562, Figure 2.4A). Conversely, the ICC for single measurement of EPCs was substantial at 0.653 (0.067, 0.908; p=0.021, Figure 2.4A), although this was lower than the ICCs reported for the repeatability and reliability assessments. There is very little data to inform the typical lifespan and turnover rates of CECs and EPCs; however,

the relatively high levels of repeatability and reliability together with the low levels of reproducibility, especially with the CEC cells, may be more indicative of day-to-day variability in these cell populations rather than variability in their isolation and identification.

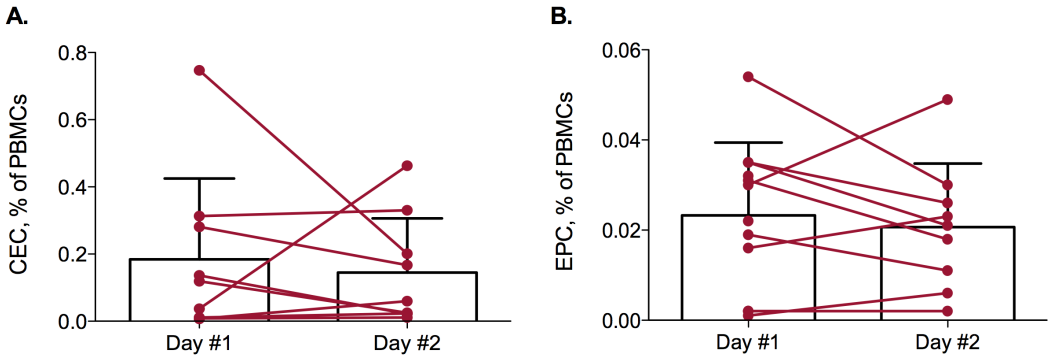

Figure 2.4 *Reproducibility of CEC and EPC assessments.* CECs (A) and EPCs (B) assessed on day #1 and day #2 demonstrated poor and substantial reproducibility, respectively. Data are presented as group mean and standard deviations for each day (bars), with an overlay of paired individual samples.

Although CEC and EPC assessments proved to be repeatable and reliable in statistical tests, Bartlett and Frost emphasized that the acceptable limits of reliability and agreement must also be based on acceptable limits in a clinical context (Bartlett and Frost 2008). Table 2.1 presents the mean standard deviation of duplicates, and percentage of samples from repeatability, reliability, and reproducibility assessments that met the criteria for valid CEC and EPC enumeration in duplicate samples, as outlined by Duda et al. It is important to note that although the

variability (SD) in CEC and EPC measurements is similar, the recommended acceptable variability for EPCs is significantly lower than that of CECs (0.01% vs. 0.2%). This might be expected given the relatively narrower range for normal values of EPCs reported in adult populations (Duda et al. 2007). Thus, these data support the findings of repeatable and reliable CEC and EPC assessments, and further suggest that the low naturally occurring levels of EPCs may make their assessment less stable than that of CECs.

Table 2.1 *Mean values for the standard deviation between duplicate samples (SD), and percentage of duplicates (%) that meet the clinical recommendation of a standard deviation of ≤0.2% for CECs, and ≤0.01% for EPCs (Duda et al. 2007).*

	CECs		EPCs	
	SD	**%**	**SD**	**%**
Repeatability	0.009	100	0.019	77.8
Reliability	0.013	100	0.011	70.0
Reproducibility	0.108	77.9	0.008	88.9

2.3.4 Gating Strategies

The gating strategies employed in this thesis were developed to maximize objective selection of positive, negative, dim, and bright populations of interest, and were based on the available published literature (Kinik et al. 2005, Duda et al. 2007, Zaldivar et al. 2007, Walther et al. 2008, 2009, Park et al. 2012). For CEC and EPC analyses based on the Duda et al. protocol (Chapters 3, 4, and 5), sequential gating consisted of identification of the lymphocytes and monocytes from the forward vs. side scatter plots, followed by CECs defined as $CD31^{bright}CD45^{-}$

$CD34^{dim}CD133^{-}$ cells, and EPCs as $CD31^{+}CD45^{dim}CD34^{bright}CD133^{+}$ cells (Duda et al. 2007), as illustrated in Figures 3.1, and 4.1 in Chapters 3, and 4, respectively. Importantly, samples were analyzed consecutively so as to ensure a uniform and standardized approach to gating.

In Chapter 4, we examined the effect of eight different published EPC definitions on cell counts and proportions. Where possible, the gating strategies and calculation of EPC counts were performed as described in the literature. In the event that the authors did not provide sufficient detail to reproduce their gating strategy, a standard sequential strategy was developed and applied to all collected samples. Appendix B provides examples of the gating strategies and units of expression for each cell definition presented in Chapter 4.

For all analyses, a linear scale was used for forward scatter versus side scatter plots, while biexponential or hlog scales were used for all plots of monoclonal antibodies. In addition, negative gates were set based on the unstained control samples for each participant. Given the relatively dim expression of CD34 on CECs, fluorescence minus one samples (lacking the CD34 antibody) were used as an additional control to establish the limits of the $CD34^{-}$ gate. For both the unstained control as well as the fluorescence minus one samples, negative gates were consistently set at 99.5% of the collected PBMCs.

CHAPTER 3: CIRCULATING ENDOTHELIAL CELLS IN CHILDREN: ROLE OF FITNESS, ACTIVITY, AND ADIPOSITY

Originally published as:

Obeid J, Nguyen T, Walker RG, Gillis LJ, and Timmons BW (2014). Circulating endothelial cells in children: role of fitness, activity, and adiposity. *Medicine & Science in Sports & Exercise* 46: 1974-1980.

3.1 Abstract

Purpose: Circulating endothelial cells (CEC) are thought to be useful biomarkers of endothelial dysfunction and overall cardiovascular health. The extent to which CEC are influenced by fitness, physical activity and adiposity in youth remains unknown, as they have seldom been examined in the pediatric population. This study assessed resting levels of CEC in boys and girls of different chronological and biological age, and explored the relationship between these cells and aerobic fitness, physical activity, and adiposity. *Methods:* Seventy-five children (39 males, median [interquartile range], age: 14.4 [5.8]) completed two study visits. During the first visit, basic anthropometric data were collected and biological age was calculated. Peak mechanical power (W_{peak}) was determined using the McMaster All-Out Continuous cycling test. Participants then wore an accelerometer over a 7-day period to assess habitual levels of moderate-

to-vigorous physical activity (MVPA). During visit 2, percent body fat (%BF) was assessed by DXA. A fasted blood sample was also collected from which concentrations of CEC, identified as $CD31^{bright}CD34^{+}CD45^{-}CD133^{-}$, were quantified by flow cytometry. *Results:* No differences were seen in CEC by sex, chronological or biological age. The median (interquartile range) CEC concentration was 32.3×10^6 (63.0×10^6), representing 1.3% (2.7) of collected peripheral blood mononuclear cells. CEC concentration was associated with W_{peak} normalized to lean body mass ($r=0.36$, $p<0.01$), and time spent in MVPA ($r=-0.27$, $p=0.02$). No relationship was observed between CEC and %BF. *Conclusion:* Healthy children present with relatively low concentrations of CEC. Because CEC represent a population of mature endothelial cells shed from the intima following irreversible damage, they may be more reflective of recent physical activity levels rather than fitness or level of adiposity.

3.2 Introduction

Cardiovascular disease is the leading cause of mortality globally, representing approximately 30% of all deaths (38). Recent reports from the World Health Organization suggest that it will remain the single leading cause of mortality, with an estimated 23.3 million annual deaths by 2030 (25), likely because of high rates of obesity. The realization that

cardiovascular disease has its roots in childhood (4, 28) highlights the need for early identification of cardiovascular dysfunction and intervention to prevent the worsening of cardiovascular health into adulthood.

To date, several risk factors and biomarkers for the development of cardiovascular disease have been identified in youth. Although more traditional risk factors like family history, blood pressure, and adiposity have been examined extensively, emerging evidence suggests that nontraditional markers including C-reactive protein, endothelial progenitor cells, and circulating endothelial cells (CEC) may be more representative of early signs of cardiovascular damage (2, 6). In adults, elevated concentrations of CEC have been linked with well-established plasma-based markers of endothelial dysfunction, such as von Willebrand Factor, as well as physiological markers such as impaired flow-mediated dilation (21, 32). CEC, which represent a population of mature endothelial cells shed from the intima after irreversible structural damage and injury to the endothelial layer (5), have been identified in several disease states, including diabetes (26), atherosclerosis and hypertension (16), as well as in association with poor health behaviours, such as smoking (6, 31). Moreover, the CEC concentration in peripheral blood demonstrates a positive association with disease severity (5, 20), and is predictive of adverse cardiovascular events in those with acute coronary syndromes (7, 22). Taken together, these lines of evidence suggest that CEC in

peripheral blood may be clinically useful as a surrogate marker of early endothelial abnormalities in adulthood (5, 8). The extent to which these findings translate to a pediatric population remains unknown; however, the limited evidence suggests that CEC may be elevated in severely obese youth with elevated systolic blood pressure (19).

Both aerobic fitness and physical activity are also predictive of cardiovascular risk in children and adults (1, 23, 33, 34). Emerging evidence suggests a strong influence of fitness and physical activity on circulating progenitor cells (23, 30, 41). Unlike CEC, endothelial progenitor cells are thought to contribute to the neoangiogenic process or to the repair of the damaged endothelial cell layer. In this way, regular exercise is believed to mobilize these cells for the benefit of endothelial adaptation (23). However, very little is known about the relationships between fitness, physical activity, and CEC, which are more representative of endothelial abnormalities. We sought to further understand these relationships in children to provide an additional perspective on fitness, physical activity and cardiovascular risk.

Clearly, more work is needed to identify and characterize CEC in children as potentially sensitive and specific markers of endothelial damage. Given their predictive value in endothelial dysfunction, it is important to study CEC in the context of fitness, physical activity, and adiposity. Such information may reveal pathways through which regular

exercise affects vascular health and may ultimately help to optimize physical activity recommendations according to age and sex. Therefore, the objectives of this study were to 1) examine resting levels of CEC in boys and girls of different chronological and biological age, and 2) explore the relationship between these cells and aerobic fitness, physical activity, and adiposity. We hypothesized that 1) given the relatively low level of endothelial damage expected in youth, CEC would be similar in boys and girls, and increase with both chronological and biological age in accordance with increasing, albeit still low, levels of endothelial damage over time, and 2) youth demonstrating lower levels of aerobic fitness and physical activity, along with higher levels of adiposity, would present with the highest concentrations of CEC.

3.3 Methods

Participants. Boys and girls between the ages of 8-10 yr (N = 48) and 14-16 yr (N=46) participated in this study, which was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board. Participants were recruited from the general community, as well as from randomly selected elementary and secondary schools from a local school board. Overweight and obese participants (23% of study sample) were recruited, by design, from the Children's Exercise and Nutrition Centre's weight management program. None of the children reported any

medical conditions at the time of participation. A parent or guardian and each participant provided written informed consent and assent, respectively, before enrollment in the study. Participant characteristics are presented in Table 1.

Table 3.1 *Participant characteristics.*

	All	Males	Females
N	75	39	36
Age (yr)	14.4 (5.8)	10.7 (5.7)	14.7 (6.0)
Height (m)	1.6 (0.3)	1.5 (0.3)	1.6 (0.3)
Weight (kg)	46.2 (31.5)	40.0 (31.7)	50.8 (30.3)
BMI Percentiles[a]	57.1 (50.3)	55.6 (45.7)	65.3 (52.5)
%BF*	20.2 (8.4)	16.9 (6.0)	24.0 (10.5)
Overweight/Obese (%)	22.7	23.1	22.2
W_{peak} ($W \cdot kg_{Lean}^{-1}$)	4.6 (1.0)	4.6 (1.0)	4.7 (1.4)
MVPA ($min \cdot d^{-1}$)*	57.9 (27.7)	64.7 (20.5)	50.3 (24.8)
MVPA ($min \cdot h^{-1}$ monitoring time)*	4.5 (2.3)	5.0 (2.4)	3.9 (1.9)

Data are presented as median (IQR).
[a]BMI percentiles are based on the CDC charts for age (28).
**Significant difference between males and females, $P < 0.05$.*
%BF, percentage body fat from DXA; W_{peak} *,peak mechanical power relative to lean body mass; MVPA, moderate-to-vigorous physical activity.*

Study overview. Data collection occurred between September 2009 and July 2011. Participants were invited to our laboratory on two occasions separated by ~7 days. During the first visit, basic anthropometric data were collected and fitness testing was performed. Participants were then provided with an accelerometer to wear for a 7-d period to assess levels of habitual physical activity. At the end of the week participants returned to the laboratory for their second visit, at which time adiposity was assessed and a fasting blood sample was collected.

Biological age and Adiposity. Standing and seated height (to the nearest 0.1 cm) and nude weight (to the nearest 0.1 kg, BWB-800, Tanita Corp., Japan) were assessed. Body mass index (BMI) was calculated as weight / height^2 and used for descriptive purposes only, where overweight or obese participants were defined as having a BMI ≥85th percentile for age (29). Biological age was assessed as maturity offset, defined as the estimated number of years from the age of peak height velocity (YPHV) and calculated from anthropometric measures according to Mirwald et al. (27). The primary measure of adiposity was % body fat (%BF) assessed using dual energy x-ray absorptiometry (DXA). Whole body DXA scans were performed in a supine position on a Hologic QDR 4500A scanner (Hologic Inc., Waltham, MA, USA). Accompanying software (Version 12.3) was used to determine total body fat mass.

Aerobic fitness. Aerobic fitness was assessed using the McMaster All-Out Progressive Continuous cycling test on a mechanically braked cycle ergometer (Fleisch-Metabo, Geneva, Switzerland). Participants were instructed to cycle at a pace of 60 rpm as the workload was increased in constant increments (10-50 Watts, based on height and estimated fitness level) every 2 min until they were no longer able to maintain the prescribed cadence, despite strong verbal encouragement. Peak mechanical power in Watts (W_{peak}) was defined as the highest achieved power output, pro-rated to the time completed in the final stage. To account for differences in

body size, W_{peak} was normalized to DXA-derived lean body mass (i.e., $W \cdot kg_{Lean}^{-1}$).

Physical activity. At the end of their first visit, participants were outfitted with an ActiGraph GT1M accelerometer and instructed to wear the device over the right hip during all waking hours, with the exception of water activities. The accelerometer was set to record in 3-sec epochs over 7 consecutive days. Participants were also provided with a logbook to record the times the device was worn and removed during the week. Only participants who wore the accelerometer for ≥ 10 hours on ≥ 4 days (at least 1 weekend day) were included in the analyses (N = 82). Accelerometer data were downloaded and visually inspected to ensure that the time recorded in the logbook matched the accelerometer output. Any activity recorded during participant-reported nonwear time was excluded from analysis. A Microsoft Excel-based Visual Basic data reduction program was then used to determine moderate-to-vigorous physical activity (MVPA). Activity intensity was determined using cut points developed by Evenson et al. (13), which have since been identified as the most sensitive and specific activity intensity cut-points for youth between the ages of 5 and 15 yr (36). MVPA was defined as ≥ 115 counts per 3 s and used in analyses as the primary physical activity variable because it is the target of world-wide physical activity recommendations for children and youth (24).

Blood sampling and analysis. Participants were instructed to refrain from participating in any strenuous activity for a 24-hour period, as well as to fast for a 10-hour period before providing a blood sample, which was drawn by venipuncture from the median cubital vein. Testing schedules were organized for postpubertal females such that blood samples would be collected during the follicular phase (days 7-10) of their menstrual cycle. A complete blood count was performed on a portion of each sample by the McMaster Core Facility (McMaster University). The remainder of the sample was processed for flow cytometric analysis according to the protocol described by Duda et al. (11). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from 10 mL of whole blood by density gradient centrifugation according to manufacturer protocols (Lymphoprep™, Axis-Shield, Oslo, Norway). Once isolated, the sample was washed and incubated for 10 min with an Fc-receptor blocking reagent so as to minimize nonspecific binding. This was followed by a 20-min incubation with the following antibodies (with conjugated fluorochromes): CD31 (FITC), CD34 (APC), CD45 (PerCP), and CD133 (PE). The sample was then lysed, fixed, and stored at 2°C–8°C until further analysis. CEC were defined as $CD31^{bright}CD34^{+}CD45^{-}CD133^{-}$ (Fig. 1), and expressed as both concentrations as well as percent of collected PBMCs. CEC were enumerated within 24-hours of sample collection on a 15-color BD LSRII flow cytometer (McMaster Flow Cytometry Facility,

McMaster University), and subsequently analyzed with FlowJo (Version 8.7 for MacIntosh, Tree Star Inc.).

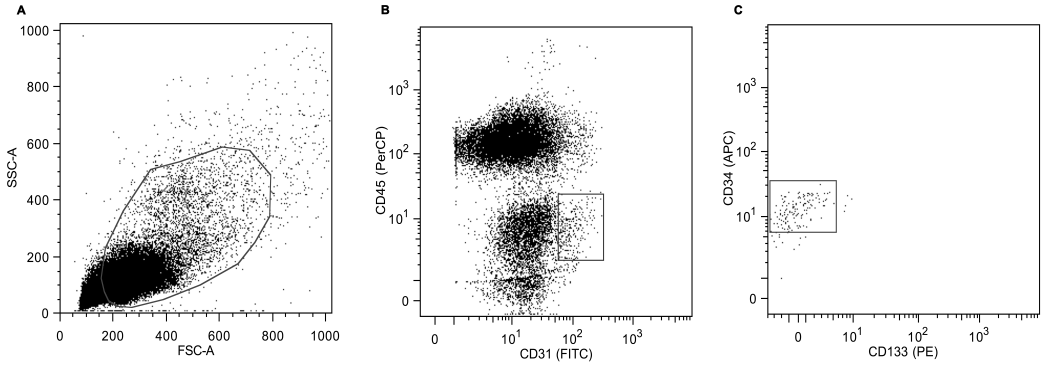

Figure 3.1 *A representative 4-colour analysis of circulating endothelial cells.* Peripheral blood mononuclear cells are stained for CD31 (FITC), CD34 (APC), CD45 (PerCP), and CD133 (PE) and first gated based on forward (size) and side (granularity) scatter characteristics (A). CECs were then identified as $CD31^{bright}CD45^{-}$ (B), and $CD133^{-}$ $CD34^{+}$ (C).

Statistical analyses. All variables were tested for normality using the Shapiro-Wilk test. To examine the influence of sex, chronological age and biological age, cell populations were compared between males and females, between different age groups as well as between participants who were ≥ +1 YPHV and ≤ -1 YPHV, respectively, using either independent sample t-tests or Mann-Whitney *U*-tests. Correlation coefficients (Pearson and Spearman) were calculated to assess the relationship between CEC and %BF, W_{peak}, and MVPA. If these variables demonstrated strong bivariate relationships (r≥0.5), they were entered into backward linear regression analyses to examine the degree to which these variables could predict measured CEC concentrations. Statistical significance was set at $P \leq 0.05$. All statistical analyses were performed in

Statistical Package for the Social Sciences (version 20.0, IBM, Chicago, IL).

3.4 Results

CEC in children. Complete data sets were available for 75 participants (39 males; 12 did not comply with physical activity assessments, 7 did not provide a fasted blood sample). CEC were not normally distributed; non-parametric statistics were employed and all data are presented as median (interquartile range [IQR]). Both the CEC concentration and the percentage of collected PBMC were similar in males and females (P > 0.2; Table 2). When compared across chronological age groups (8-10 vs. 14-16 yr) and by biological age (≥ +1 YPHV vs. ≤ -1 YPHV), there were no differences in the concentration or proportion of CEC (P > 0.2; Table 2), thus, all data were combined. The median (IQR) CEC concentration was 32.3 × 10^5 (63.0 × 10^5), representing 1.3% (2.7%) of collected PBMC.

Table 3.2 *Circulating endothelial cells by sex, chronological, and biological age.*

		Sex		Chronological Age (yr)		Biological Age (YPHV)	
	All	Males	Females	8-10	14-16	≤ -1	≥ +1
N	75	39	36	37	38	34	36
[CEC] (× 10^5 cells per liter)	32.3 (63.0)	32.6 (90.7)	32.1 (50.9)	26.4 (63.5)	46.8 (62.6)	26.4 (77.8)	38.2 (39.2)
%CEC (% of PBMC)	1.3 (2.7)	1.4 (2.8)	1.1 (2.2)	1.0 (2.0)	1.7 (2.8)	1.0 (2.6)	1.6 (2.1)

Data are presented as median (IQR).
[CEC], concentration of circulating endothelial cells; %CEC, circulating endothelial cells as a percentage of peripheral blood mononuclear cells (PBMC).

Relationships between CEC and fitness, physical activity, and adiposity. Spearman's correlations between CEC and W_{peak}, MVPA, and %BF are presented in Table 3. Both CEC concentration and CEC expressed as a percentage of PBMC were positively associated with W_{peak} normalized to lean body mass. Only the concentration of CEC was negatively associated with time spent in MVPA. No relationship was observed between CEC and %BF. Because none of the bivariate correlations achieved a correlation coefficient ≥0.5, backward linear regression analyses were not performed.

Table 3.3 *Spearman rank order correlations between CECs and fitness, physical activity and body composition.*

	[CEC]	%CEC
W_{peak} ($W \cdot kg_{Lean}^{-1}$)	0.36**	0.35**
MVPA ($min \cdot d^{-1}$)	-0.24*	-0.18
MVPA ($min \cdot hr^{-1}$ monitoring)	-0.27*	-0.22
%BF	-0.16	-0.19

*Significant correlation, $P < 0.05$.
**Significant correlation, $P < 0.01$.
W_{peak}, peak mechanical power; MVPA, moderate-to-vigorous physical activity; %BF, percentage body fat from DXA;
[CEC], concentration of circulating endothelial cells;
%CEC, CEC expressed as a percentage of collected PBMC.

3.5 Discussion

We simultaneously assessed the degree to which physical activity and fitness are correlated with CEC concentrations, while considering adiposity (%BF) in a group of apparently healthy youth. The main findings of this study were that aerobic fitness was positively associated with the concentration and proportion of CEC, whereas MVPA was negatively

associated with the concentration of CEC only. Conversely, %BF was not correlated with either concentrations or proportions of CEC. We also found that males and females as well as less biologically mature versus more biologically mature youths expressed similar concentrations and proportions of CEC.

There remains a great deal of controversy around the best methods and markers used for the identification of CEC (8, 11, 40). The literature to date has included CEC identified by immunobeads, density centrifugation, standard centrifugation, and flow cytometry, with each technique yielding a broad range values from as little as <3 to 18,896 cells per milliliter of healthy, adult whole blood (3, 6). Although flow cytometry, the technique used in the current study, may be more sensitive to the identification of these rare cells, marker selection may have an additional bearing on CEC counts (6). To date, the more commonly used phenotypic markers include expression of CD31, a marker of endothelial cells, together with the absence of CD45 and CD133, markers of leukocytes and immaturity, respectively (6, 11). In our study, we enumerated CEC as per the recommendations set out by Duda et al. (11) because this protocol has been utilized successfully in both healthy and clinical populations and represents a more thorough assessment of CEC than the more commonly utilized techniques that employ single markers for CEC identification. We found that CEC ranged from 0.1 to 6.9% of PBMC, which falls in line with

the 0.1 to 6.0% range reported by Duda et al. (11). Our median of 1.3% fell on the lower side of this range, which may be due to a limited degree of endothelial damage in a sample of relatively healthy youth.

To our knowledge, only two studies have examined CEC in a pediatric population; however, the focus of these papers was primarily on the association between these cells and adiposity (10, 19). As such, there are no data to describe the influence of normal growth and development on CEC. Such information is critical to our understanding of the potential role of CEC in cardiovascular health by distinguishing normal growth and development from factors such as physical activity, fitness, or disease. To begin to fill this gap in the literature, we examined CEC in youth of varying chronological and biological age, as defined by the maturity offset of estimated years from peak height velocity, and found that CEC concentrations are similar regardless of biological age. As previously mentioned, the extent of endothelial damage may be low in this relatively healthy sample and so it can be concluded that CEC are normally quite low in healthy children. In future studies, it will be important to evaluate CEC in children with chronic medical conditions, such as inflammatory diseases, which are associated with elevated cardiovascular risk (15, 17, 18).

In apparently healthy children, strong evidence links physical activity and fitness with favourable levels of traditional physiological risk

factors for cardiovascular disease and biomarkers of vascular structure and function (1, 14, 33-35). Unfortunately, we know virtually nothing about the mechanisms that potentially link being active and fit with improved cardiovascular health for children, or the extent to which these relationships may be modified by adiposity. In the current study, we found that aerobic fitness was positively associated with CEC, whereas the correlation between MVPA and CEC concentration was negative – the opposite direction than what we expected. Because fitness and physical activity represent different attributes, these discordant findings may be explained by the fact that fitness is a state of physiological function whereas MVPA represents a behavior. In our study, CEC were analyzed in a blood sample collected after 7 d of physical activity monitoring; thus, the MVPA data reflect recent physical activity behavior and CEC may be responsive to recent physical activity. It is also important to note that most of the studies examining CEC to date report significantly elevated concentrations in adults with chronic medical conditions compared with healthy controls (8, 9, 26). It is therefore possible that the relationship between fitness, physical activity and CEC is influenced by health status. Clearly, more work is needed to understand the potential role of CEC in cardiovascular health during growth and the extent to which recent physical activity behavior and/or fitness influences their status.

Given the commonly reported negative association between weight status and endothelial function (37), we hypothesized a positive relationship between adiposity and CEC. Nevertheless, we found no association between %BF or BMI percentile (not reported) and proportions or concentrations of CEC. Our finding are in line with those reported by Dias et al., wherein activated CEC demonstrated no association with BMI z-score, waist circumference, and %BF as measured by DXA (10), although we did not specifically measure activated CEC. Conversely, Kelly et al. reported moderately strong relationships between BMI, waist circumference, and CEC (19). These discrepant findings may be partially explained by the fact that Kelly et al. included a large proportion of severely obese participants (25% of sample had a BMI ≥99th percentile). Indeed, when examined by weight status groups (normal, overweight, obese, and severely obese), only participants in the severely obese group were found to have a higher number of CEC per mL of blood (19). Our sample was relatively lean (median %BF of 20.2%), with only 5 participants (7% of sample) classified as severely obese, despite the fact that we deliberately recruited ~25% of our sample from a weight management program. Moreover, Kelly et al. enumerated CEC via immunohistochemical examination of buffy-coat smears (19), making it difficult to directly compare our findings. Future work should seek to

include additional participants at the extremes of weight status before any clinical interventions.

To our knowledge, this is the first study to examine CEC and their association with fitness, physical activity and adiposity in youth; however, our results must be interpreted in light of some limitations. Although we attempted to recruit participants that were representative of the healthy pediatric population, our sample remains fairly homogeneous, making it difficult to extrapolate our findings to the general pediatric population. Second, our definition of CEC was based on 4 markers (CD31, CD34, CD45, and CD133) as described by Duda et al. (11). We did not include a CD146 marker, which represents one of the commonly used primary antibodies for CEC identification (12). We, therefore, cannot exclude the possibility of some small degree of endothelial progenitor cell contamination in our CEC sample. Finally, we enumerated CEC based on phenotypic markers, which is common in the literature; however, we did not examine their *in vitro* function, nor did we assess CEC activation state in the peripheral circulation. More specifically, it has been suggested that circulating CEC consist of live cells, as well as those that are apoptotic or necrotic (39), which may ultimately impact their relationship with fitness, physical activity, and adiposity. This notion is supported by the fact that Kelly et al. reported stronger associations between BMI, waist circumference, and the proportion of activated CEC compared with total

CEC counts (19). Future work should seek to differentiate between these populations in a more diverse pediatric population.

In conclusion, we found that CEC were similar in boys and girls as well as by chronological and biological age. CEC were positively associated with aerobic fitness but demonstrated no association with adiposity. Conversely, youth engaging in higher levels of physical activity presented with fewer CEC. Because CEC represent a population of mature endothelial cells shed from the intima following irreversible damage (5), they may be more reflective of recent physical activity levels as opposed to a child's state of fitness or level of adiposity.

3.6 Acknowledgements

The authors thank the participants for their time and effort. They also acknowledge the assistance of Hong Liang at the McMaster Flow Cytometry Facility as well as Colleen Cordukes and Megan Gao. This study was made possible by a grant-in-aid from the Heart & Stroke Foundation of Ontario (Grant no. NA 6445). JO was supported by a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship. TN was supported by an NSERC Postgraduate Scholarship. RGW was supported by a CIHR Undergraduate Studentship in Mobility, Musculoskeletal Health and Arthritis Across the Lifespan. BWT is supported by a CIHR New Investigator Salary Award. The authors have

no conflicts of interest to declare. The results of the present study do not constitute endorsement by ACSM.

3.7 References

1. Abbott RA, Harkness MA, Davies PS. Correlation of habitual physical activity levels with flow-mediated dilation of the brachial artery in 5-10 year old children. *Atherosclerosis*. 2002;160(1):233-9.

2. Balagopal PB, de Ferranti SD, Cook S et al. Nontraditional risk factors and biomarkers for cardiovascular disease: mechanistic, research, and clinical considerations for youth: a scientific statement from the American Heart Association. *Circulation*. 2011;123(23):2749-69.

3. Batycka-Baran A, Paprocka M, Krawczenko A, Dus D, Szepietowski JC. Increased number of circulating endothelial cells (CECs) in patients with psoriasis - preliminary report. *J Eur Acad Dermatol Venereol*. 2014;28(1):116-9.

4. Berenson GS, Srinivasan SR, Bao W, Newman WP, III, Tracy RE, Wattigney WA. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *N Engl J Med*. 1998;338(23):1650-6.

5. Blann AD, Pretorius A. Circulating endothelial cells and endothelial progenitor cells: two sides of the same coin, or two different coins? *Atherosclerosis*. 2006;188(1):12-8.

6. Blann AD, Woywodt A, Bertolini F et al. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost*. 2005;93(2):228-35.

7. Boos CJ, Soor SK, Kang D, Lip GY. Relationship between circulating endothelial cells and the predicted risk of cardiovascular events in acute coronary syndromes. *Eur Heart J*. 2007;28(9):1092-101.

8. Burger D, Touyz RM. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. *J Am Soc Hypertens*. 2012;6(2):85-99.

9. Chong AY, Lip GY, Freestone B, Blann AD. Increased circulating endothelial cells in acute heart failure: comparison with von Willebrand factor and soluble E-selectin. *Eur J Heart Fail*. 2006;8(2):167-72.

10. Dias IB, Panazzolo DG, Marques MF et al. Relationships between emerging cardiovascular risk factors, z-BMI, waist circumference and body adiposity index (BAI) on adolescents. *Clin Endocrinol (Oxf)*. 2013.

11. Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc*. 2007;2(4):805-10.

12. Erdbruegger U, Dhaygude A, Haubitz M, Woywodt A. Circulating endothelial cells: markers and mediators of vascular damage. *Curr Stem Cell Res Ther*. 2010;5(4):294-302.

13. Evenson KR, Catellier DJ, Gill K, Ondrak KS, McMurray RG. Calibration of two objective measures of physical activity for children. *J Sports Sci*. 2008;26(14):1557-65.

14. Froberg K, Andersen LB. Mini review: physical activity and fitness and its relations to cardiovascular disease risk factors in children. *Int J Obes (Lond)*. 2005;29 Suppl 2):S34-S39.

15. Glowinska-Olszewska B, Moniuszko M, Hryniewicz A et al. Relationship between circulating endothelial progenitor cells and endothelial dysfunction in children with type 1 diabetes: a novel paradigm of early atherosclerosis in high-risk young patients. *Eur J Endocrinol*. 2013;168(2):153-61.

16. Hladovec J, Prerovsky I. Endothelial lesion in hypertension. *Cor Vasa*. 1989;31(1):51-4.

17. Jednacz E, Rutkowska-Sak L. Atherosclerosis in juvenile idiopathic arthritis. *Mediators Inflamm*. 2012;2012):714732.

18. Kavey RE, Allada V, Daniels SR et al. Cardiovascular risk reduction in high-risk pediatric patients: a scientific statement from the American Heart Association Expert Panel on Population and Prevention Science; the Councils on Cardiovascular Disease in the Young, Epidemiology and Prevention, Nutrition, Physical Activity and Metabolism, High Blood Pressure Research, Cardiovascular Nursing, and the Kidney in Heart Disease; and the Interdisciplinary Working Group on Quality of Care and Outcomes Research. *J Cardiovasc Nurs*. 2007;22(3):218-53.

19. Kelly AS, Hebbel RP, Solovey AN et al. Circulating activated endothelial cells in pediatric obesity. *J Pediatr*. 2010;157(4):547-51.

20. Koc M, Richards HB, Bihorac A, Ross EA, Schold JD, Segal MS. Circulating endothelial cells are associated with future vascular events in hemodialysis patients. *Kidney Int*. 2005;67(3):1078-83.

21. Lee KW, Blann AD, Lip GY. Inter-relationships of indices of endothelial damage/dysfunction [circulating endothelial cells, von Willebrand factor and flow-mediated dilatation] to tissue factor and interleukin-6 in acute coronary syndromes. *Int J Cardiol*. 2006;111(2):302-8.

22. Lee KW, Lip GY, Tayebjee M, Foster W, Blann AD. Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. *Blood*. 2005;105(2):526-32.

23. Lenk K, Uhlemann M, Schuler G, Adams V. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. *J Appl Physiol*. 2011;111(1):321-8.

24. Lipnowski S, Leblanc CM. Healthy active living: Physical activity guidelines for children and adolescents. *Paediatr Child Health*. 2012;17(4):209-12.

25. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med*. 2006;3(11):e442.

26. McClung JA, Naseer N, Saleem M et al. Circulating endothelial cells are elevated in patients with type 2 diabetes mellitus independently of HbA(1)c. *Diabetologia*. 2005;48(2):345-50.

27. Mirwald RL, Baxter-Jones AD, Bailey DA, Beunen GP. An assessment of maturity from anthropometric measurements. *Med Sci Sports Exerc*. 2002;34(4):689-94.

28. Newman WP, III, Wattigney W, Berenson GS. Autopsy studies in United States children and adolescents. Relationship of risk factors to atherosclerotic lesions. *Ann N Y Acad Sci*. 1991;623):16-25.

29. Ogden CL, Kuczmarski RJ, Flegal KM et al. Centers for Disease Control and Prevention 2000 growth charts for the United States: improvements to the 1977 National Center for Health Statistics version. *Pediatrics*. 2002;109(1):45-60.

30. Park JH, Miyashita M, Kwon YC et al. A 12-week after-school physical activity programme improves endothelial cell function in overweight and obese children: a randomised controlled study. *BMC Pediatr*. 2012;12):111.

31. Prerovsky I, Hladovec J. Suppression of the desquamating effect of smoking on the human endothelium by hydroxyethylrutosides. *Blood Vessels*. 1979;16(5):239-40.

32. Rajagopalan S, Somers EC, Brook RD et al. Endothelial cell apoptosis in systemic lupus erythematosus: a common pathway for abnormal vascular function and thrombosis propensity. *Blood*. 2004;103(10):3677-83.

33. Reed KE, Warburton DE, Lewanczuk RZ et al. Arterial compliance in young children: the role of aerobic fitness. *Eur J Cardiovasc Prev Rehabil*. 2005;12(5):492-7.

34. Saakslahti A, Numminen P, Varstala V et al. Physical activity as a preventive measure for coronary heart disease risk factors in early childhood. *Scand J Med Sci Sports*. 2004;14(3):143-9.

35. Shea S, Basch CE, Gutin B et al. The rate of increase in blood pressure in children 5 years of age is related to changes in aerobic fitness and body mass index. *Pediatrics*. 1994;94(4 Pt 1):465-70.

36. Trost SG, Loprinzi PD, Moore R, Pfeiffer KA. Comparison of accelerometer cut points for predicting activity intensity in youth. *Med Sci Sports Exerc*. 2011;43(7):1360-8.

37. Woo KS, Chook P, Yu CW et al. Overweight in children is associated with arterial endothelial dysfunction and intima-media thickening. *Int J Obes Relat Metab Disord*. 2004;28(7):852-7.

38. World Health Organization. *Global status report on noncummunicable disease 2010*. Geneva, Switzerland: World Health Organization; 2011, p. 9-28.

39. Woywodt A, Bahlmann FH, De GK, Haller H, Haubitz M. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant*. 2002;17(10):1728-30.

40. Woywodt A, Kirsch T, Haubitz M. Immunomagnetic isolation and FACS--competing techniques for the enumeration of circulating endothelial cells. *Thromb Haemost*. 2006;96(1):1-2.

41. Zaldivar F, Eliakim A, Radom-Aizik S, Leu SY, Cooper DM. The effect of brief exercise on circulating CD34+ stem cells in early and late pubertal boys. *Pediatr Res*. 2007;61(4):491-5.

CHAPTER 4: CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN YOUTH: FITNESS, PHYSICAL ACTIVITY, AND ADIPOSITY

Submitted for publication as:

Obeid J, Nguyen T, and Timmons BW. Circulating endothelial progenitor cells in children and adolescents: impact of aerobic fitness, physical activity, and cell definition. *International Journal of Sports Medicine*.

4.1 Abstract

Background: Circulating endothelial progenitor cells (EPCs) are early markers of cardiovascular impairment. The role of EPCs in youth remains unclear, and is complicated by differences in how cells are identified. This study (1) described EPCs in pre- and late-pubertal males and females, (2) examined their association with fitness, activity and adiposity, and (3) compared EPCs by published cell definitions. *Methods:* Ninety-four participants completed two sessions. During the first session, aerobic fitness (W_{peak}) and moderate-to-vigorous physical activity (MVPA) were assessed. During the second session, percent body fat (%BF) was determined by DXA, and a fasted blood sample was collected to measure EPCs by flow cytometry. EPCs were identified as $CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}$. Samples were reanalyzed and cell counts were compared according to 8 previously published EPC definitions. *Results:* EPCs were similar in pre- and late-pubertal males and

females (p>0.05). Neither EPC concentrations nor proportions were correlated with W_{peak} (ρ=-0.04- -0.06), MVPA (ρ=-0.09- -0.07), or %BF (ρ=0.20–0.14). Agreement between cell data analyzed according the different cell definitions ranged from K=-0.06-0.82. *Conclusion:* EPCs were not associated with fitness, MVPA or adiposity in youth. The overall poor agreement across definitions may be indicative of distinct EPC subpopulations.

4.2 Introduction

Physical activity and aerobic fitness are among the most well established predictors of cardiovascular health in both children and adults [1,31,36]; however, the mechanisms underlying these relationships are only beginning to emerge. Identification and characterization of new, sensitive, and specific markers of cardiovascular dysfunction in youth could help to optimize clinical physical activity recommendations, which are important now more than ever in light of persistently high childhood obesity rates [32]. To this end, emerging evidence supports a critical role for bone marrow-derived circulating endothelial progenitor cells (EPCs) in supporting post-natal vasculogenesis and vascular repair [5,10,38], and suggests that their concentration in peripheral blood may be clinically useful as an early marker of cardiovascular risk, at least among adults [19,42].

A potent stimulus to increase EPCs is physical exercise [6,25,34]. Research involving healthy adults and those with diagnosed cardiovascular disease has demonstrated that acute bouts of exercise can lead to 66-309% increases in EPCs [35,39]. Consistent with an apparent benefit of specific episodes of exercise, adults with higher levels of habitual physical activity [26] and physical fitness [6] experience higher EPCs concentrations. A small number of studies suggest that acute exercise, physical activity and fitness can also increase EPCs in youth, albeit to a lesser degree [3,30,40,41,43]. Specifically, Zaldivar et al. reported 83% and 170% increases in EPCs following acute exercise in pre- and post-pubertal males, respectively [43]. Moreover, the addition of 45 min of daily physical activity in a school setting over a 1-year period led to an 18% increase in EPCs, with a concomitant 29% increase in aerobic fitness [40]. Taken together, these data indicate that EPC mobilization may represent one mechanism translating physical activity and aerobic fitness into improved cardiovascular health.

Despite the promising links between exercise and EPCs, our understanding of the naturally occurring concentrations of these cells in youth is limited by the equivocal findings of the available literature. In a comparison of EPCs across the lifespan, children and adolescents (1-17 years of age) demonstrated three times higher EPC counts compared with adult age groups (20-60, and 60-81 years)[22]. Conversely, Jung et al.

found lower levels of EPCs in adolescents (13-17 years) compared with adults (>34 years)[23]. Only one study from Zaldivar et al. examined differences in EPCs by maturation and reported higher resting concentrations in pre-pubertal compared with post-pubertal males, suggesting that the biology of these cells and/or their precursors may be influenced by puberty or chronological age [43]. Conflicting findings on the effect of weight status on EPCs have also been reported, with data to support a drastic reduction [7], no change [24], and an increase [23] in EPCs in overweight or obese youth compared with their healthy weight peers. These findings highlight the need for a comprehensive assessment of EPCs, as well as the factors influencing their resting concentrations, so as to explore their potential as markers of cardiovascular health in youth.

The discrepancies between studies in the pediatric population are likely related to the different protocols and cell markers (i.e., phenotype) used to identify EPCs. In fact, a number of adult-based studies have revealed little to no agreement in EPC concentrations when cells were quantified using different isolation protocols (i.e., whole blood vs. peripheral blood mononuclear cells), cell markers (i.e., CD34, CD45, CD133, CD309), and enumeration techniques (i.e., flow cytometry vs. cell culture) [8,9,14,15,17]. Importantly, marker selection continues to be a matter of debate since most available monocloncal antibodies are not specific to EPCs, and as such, often fail to distinguish between

hematopoietic and endothelial progenitor cell lineages [15,37]. A better understanding of the impact of cell marker selection on EPC identification is necessary to allow for comparison and interpretation of the previously reported findings in the pediatric literature, as well as for the design of future studies of EPCs in youth.

To begin addressing these gaps in the literature, the primary objectives of this study were to 1) assess and compare resting levels of EPCs in typically developing, pre- and post-pubertal males and females, and 2) examine the relationship between these cells and aerobic fitness, physical activity, and body composition. Given the wide range of markers used to identify EPCs in youth (Table 1), the secondary objective of this study was to compare EPC concentrations in the same sample of participants according to previously reported protocols in the pediatric population.

Table 4.1 *Characteristics of EPC populations assessed by flow cytometry in association with fitness, physical activity and/or body composition in the pediatric population.*

Study (Reference)	Cells of Interest	Cell Definition	Units of Expression	Group‡	N (males)	Age (yrs)	Resting Concentrations
Kinik et al. (2005)	CD34+ Hematopoietic stem cells	**CD34$^+$**	% of mononuclear cells	Obese	31 (15)	4.7 – 7.4	0.0 – 2.4
				Control	30 (17)	5.0 – 8.7	0.0 – 2.4
Zaldivar et al. (2007)	Peripheral blood hematopoietic stem cells	**CD45^{dim}CD34$^+$**	Cells per µL of blood	Pre-Pubertal	14 (14)	10.3 ± 0.3	112 ± 21
				Post-Pubertal	13 (13)	16.5 ± 0.42	63 ± 8
Walther et al. (2008)	Circulating endothelial progenitor cells	(1) CD3$^-$CD34$^+$KDR$^+$; **(2) CD34$^+$;** **(3) CD133$^+$;** **(4) CD45$^+$;** (5) CD133$^+$KDR$^+$	Cells per mL blood	Intervention Class	50 (24)	11.1 ± 0.1	(1) 600 ± 34 (2) 2,759 ± 190 (3) 12,347 ± 1,074 (4) 2.05 x 10^6 ± 10,6001 (5) 301 ± 27
				Control Class	42 (18)	11.3 ± 0.1	(1) 466 ± 28 (2) 2,388 ± 155 (3) 11,104 ± 1,498 (4) 2.01 x 10^6 ± 88,663 (5) 189 ± 10
				Sports Gymnasium	19 (12)	11.4 ± 0.1	(1) 768 ± 42 (2) 3,458 ± 371 (3) 17,110 ± 4032 (4) 2.05 x 10^6 ± 11,515 (5) 529 ± 83
Walther et al. (2009)	Circulating endothelial progenitor cells	**(1) CD34$^+$;** (2) CD34$^+$KDR$^+$; (3) CD45^{dim}KDR$^+$CD34$^+$	Cells per mL blood	Intervention Class	84 – 101 (NS)	NS	(1) 1,269 ± 1,297 (2) 224 ± 191 (3) 305 ± 132
				Control Class	57 – 61 (NS)	NS	(1) 1,485 ± 867 (2) 312 ± 204 (3) 308 ± 103
				Sport Gymnasium	29 (17)	11.4 ± 0.5	(1) NS (2) NS (3) 524 ± 134
Jung et al. (2009)	Circulating endothelial progenitor cells	(1) CD34$^+$CD133$^+$KDR$^+$ (2) CD34$^-$CD133$^+$KDR$^+$	Cells per mL blood	Overweight	37 (37)	15.0 ± 1.4	(1) 30.0 ± 16.1 (2) 94.6 ± 76.8
				Control	42 (42)	15.4 ± 0.9	(1) 24.5 ± 14.4 (2) 59.1 ± 54.9
Arnold et al. (2010)	Circulating endothelial progenitor cells	(1) CD34$^+$; (2) CD34$^+$KDR$^+$; (3) CD34$^+$CD133$^+$; (4) CD34$^+$CD117$^+$	Cells per 10^6 leucocytes	N/A	24 (9)	8.2 – 16.3	(1) 70 – 390 (2) 0 – 114 (3) 30.1 – 299.3 (4) 30.1 – 277.4
Park et al. (2012)*	Circulating endothelial progenitor cells	**(1) CD34$^+$;** **(2) CD133$^+$;** **(3) CD34$^+$CD133$^+$**	% of lymphocytes	Exercise Group	15 (7)	12.1 ± 0.1	(1) 0.03 ± 0.01 (2) NS (3) NS
				Control Group	14 (7)	12.2 ± 0.1	(1) 0.03 ± 0.01 (2) NS (3) NS
Bruyndonckx et al. (2014)†	Circulating endothelial progenitor cells	CD34$^+$KDR$^+$CD45$^{dim/-}$	Cells per 10^6 mononuclear cells	Obese	57 (15)	15.2 ± 1.4	16.8 (12.3, 24.2)
				Control	30 (9)	15.4 ± 1.5	27.1 (22.0, 42.9)
D'Ascenzi et al. (2014)	Hematopoietic stem cells and Endothelial Progenitor Cells	**(1) CD34$^+$CD45^{dim};** (2) KDR$^+$; (3) KDR$^+$CD133$^+$CD34$^+$; (4) KDR$^+$CD133$^+$CD34$^-$; (5) KDR$^+$CD133$^-$CD34$^+$;	Cells per 10^6 events and **Cells per mL blood**	Athletes	20 (20)	18.4 ± 0.5	(1) NS (2) NS (3) NS (4) NS (5) NS
				Sedentary	9 (9)	18.7 ± 0.4	(1) NS (2) NS (3) NS (4) NS (5) NS

Values are presented as range or mean ± SD where available. *Values presented are Mean ± SEM. † Values presented are median (IQR). ‡ Groups were defined by the authors of each respective paper. Underlined and bolded cell definitions represent those analyzed in the current study. NS = not specified.

4.3 Materials and Methods

Participants. A total of 94 participants (52 males) were recruited from the general community, local recreation centres, as well as a random selection of elementary and secondary schools from the Hamilton region. A subset of children (N = 21, 22.7% of total) was also recruited from the weight management program at the Children's Exercise & Nutrition Centre so as to ensure that our sample was more representative of the general Canadian pediatric population from a body composition perspective [32]. By design, an equal number of participants were selected for the pre-pubertal (8-10 years, N=48) and late or post-pubertal groups (14-16 years, N=46). None of the children reported any medical conditions at the time of participation. A parent or guardian and each participant provided written informed consent and assent, respectively, prior to their enrollment in this study, which was approved by the Hamilton Health Science/Faculty of Health Sciences Research Ethics Board.

Study Overview. Participants were invited to visit the Child Health & Exercise Medicine research laboratory on two occasions separated by approximately 1 week. During the first visit, basic anthropometric measures were collected including weight, standing and sitting height. Pubertal status was defined as maturity offset or the estimated number of years from peak height velocity (YPHV) calculated from anthropometric measures according to Mirwald et al [27]. This was followed by an

assessment of aerobic fitness, which was defined as the highest power output (W_{peak}) achieved during the McMaster All-Out Progressive Continuous cycling test. Finally, participants were asked to wear an ActiGraph GT1M accelerometer during all waking hours over a 7-day period so as to quantify of habitual levels of moderate-to-vigorous physical activity (MVPA) [13]. At the end of the week, participants returned to the laboratory for their second visit where body composition (% body fat) was assessed using dual energy X-ray absorptiometry, and a fasted blood sample was collected for the enumeration of EPCs. A detailed description of body composition, maturation status, aerobic fitness, and physical activity assessments is available elsewhere [29].

Blood sampling and analysis. A 12-mL blood sample was drawn from each participant by venipuncture from the median cubital vein for determination of complete blood count (2 mL, McMaster Core Facility) as well as EPC levels (10 mL). Participants were instructed to refrain from eating for at least 10-hours prior to sample collection, and the timing of the sample for post-pubertal females was arranged such that it coincided with the follicular phase (days 7 to 10) of their menstrual cycle. All blood samples were processed within 2 hours of collection in accordance with the protocol described by Duda et al [12]. More specifically, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation according to manufacturer protocols

(Lymphoprep, Axis-Shield, Oslo, Norway). The sample was then incubated with an Fc-receptor blocking reagent for 10-min so as to minimize non-specific binding. This was followed by a 20-min incubation with fluorochrome-conjugated monoclonal antibodies, including: CD31-FITC, CD34-APC, CD45-PerCP, and CD133-PE. Finally, the sample was lysed, fixed, and stored at 2-8°C until analysis on a 15-color BD LSRII flow cytometer (McMaster Flow Cytometry Facility, McMaster University). All analyses were performed within 24 hours of sample collection. EPCs, defined according to Duda et al. as $CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}$ (Figure 1, [12], were enumerated from 8.0×10^{5} to 1.0×10^{6} events collected in the PBMC gate, and analyzed offline using FlowJo (Version 8.7 for MacIntosh, Tree Star Inc.). In a separate experiment using 10 samples not from the participants in the current study, intraclass correlation coefficients for repeatability and test-retest reliability assessments of EPCs were 0.920 (95% lower, upper confidence intervals: 0.708, 0.981, $p<0.001$), and 0.975 (0.907, 0.994, $p<0.001$), respectively.

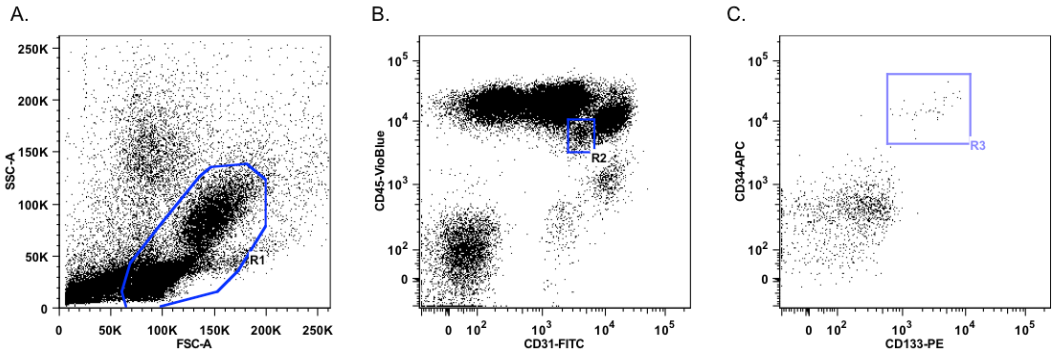

Figure 4.1 *Circulating endothelial progenitor cells, as defined by Duda et al. (2007).* Peripheral blood mononuclear cells (lymphocytes and monocytes) were isolated and gated (A, R1), from which $CD31^{+}CD45^{dim}$ cells were identified (B, R2), and further classified into $CD133^{+}CD34^{br}$ cells (C, R3).

To address the secondary objective of this study, each sample was examined an additional 8 times according to the EPC definitions set out by Kinik et al. [24], Zaldivar et al. [43], Walther et al. [40], Arnold et al. [3], and Park et al [30]. Since a 4-marker protocol was employed in the current study, only EPC definitions that included one or more of these markers were included in the analysis (See Table 1, all bolded and underlined EPC definitions), and expressed in the units described in the original study from which the definition was retrieved.

Statistical analyses. Statistical analyses were performed in SPSS (version 20.0, IBM, Chicago, IL). All variables were tested for normality using the Shapiro Wilk test; however, the ability of this statistical tool to detect non-normal distributions may be affected by a moderate (~50+) sample size [2,33]. As such, each variable was also visually examined to confirm sample distribution, and subsequent statistical analyses were

performed using a bootstrap technique, where possible, so as to reduce the impact of potentially skewed distributions and maximize robustness of the reported findings [18]. A total of 1,000 stratified resamples based on pubertal group and sex were employed for all bootstrap analyses, and bias-corrected and accelerated 95% confidence intervals were calculated. To examine the influence of sex and pubertal status, two-way ANOVAs were performed using sex (males vs. females) and pubertal status (pre-pubertal: ≥ +1 YPHV vs. late/post-pubertal: ≤ -1 YPHV) as factors. Tukey's HSD post hoc tests were used to examine any significant interaction. Spearman correlation coefficients were calculated to assess the relationship between EPCs, as either a concentration or percentage of PBMCs, and aerobic fitness (W_{peak}), physical activity (MVPA), and body composition (% body fat). If these variables demonstrated strong bivariate relationships ($r \geq 0.5$), they were entered into backward linear regression analyses to examine the degree to which these variables could predict measured EPC levels.

Given that the units of expression for the EPC definitions outlined in Table 1 varied by study (i.e., % of PBMCs, cells/mL blood, % lymphocytes, etc.), it was impossible to directly compare counts. Rather, we sought to examine the degree to which these EPC definitions were assessing the same attribute, whereby those participants presenting with the highest or lowest EPCs should retain their relative rank, regardless of the markers

used for identification. First, cell counts within each of the cell definitions were converted into percentile ranks. Second, Kendall's coefficient of concordance (Kendall's W) was used to examine whether participant ranks were maintained across each of the 10 cell definitions. Kendall's W values range from 0, indicating no agreement, to 1, suggesting complete agreement [16]. Finally, participants were split into tertiles based on EPCs (high, mid, or low) within each cell definition, and pairwise Kappa statistics were performed to assess the level of agreement in overall ranking across the 10 cell definitions (i.e., were participants in the high $CD34^+$ group also in the high $CD45^{dim}CD34^+$ group?). Kappa statistics were interpreted based on Munoz et al., where <0 are considered poor, 0-0.19 are slight, 0.2-0.4 are fair, 0.4-0.59 are moderate, 0.6-0.79 are substantial, and 0.8-<1.0 are almost perfect strengths of agreement [28]. All data are presented as median and bootstrap bias-corrected and accelerated 95% confidence intervals (BC_a 95% CI), unless otherwise indicated.

4.4 Results

EPCs by sex and pubertal status. Of the 94 participants enrolled in this study, complete data sets were available for 75 (39 male) participants (12 did not comply with physical activity assessments, 7 did not provide a fasted blood sample). When examined as a whole group, all variables, with the exception of physical activity outcomes, were not normally

distributed. When split by sex and pubertal group, both proportion and concentrations of EPCs demonstrated normal distributions. Two-way ANOVAs of EPC concentrations revealed no main effects for sex ($F(1, 78) = 0.59$; $p = 0.44$), pubertal group ($F(1, 78) = 0.11$, $p = 0.74$), and no significant interaction ($F(1, 78) = 0.85$, $p = 0.34$). Similarly, there were no significant differences in the proportion of EPCs by sex $F(1, 78) = 0.08$; $p = 0.78$), pubertal group ($F(1, 78) = 0.19$, $p = 0.66$), nor any interactions ($F(1, 78) = 1.0$, $p = 0.32$). Table 2 provides participant characteristic as well as a breakdown of EPCs by group. All data were combined for subsequent analyses.

Table 4.2 *Participant characteristics.*

	N	All	≤ -1 YPHV		≥ +1 YPHV	
			Females	Males	Females	Males
Age (yrs)	94	13.2 (10.4, 14.8)	9.4 (9.3, 10.0)	9.8 (9.8, 9.8)	15.3 (15.1, 15.8)	15.2 (15.0, 15.7)
Height (m)	94	1.5 (1.4, 1.6)	1.4 (1.4, 1.4)	1.4 (1.4, 1.4)	1.7 (1.7, 1.7)	1.7 (1.7, 1.8)
Weight (kg)	94	47.4 (36.4, 56.8)	31.3 (29.5, 34.5)	32.2 (30.7, 34.4)	61.7 (57.7, 66.7)	68.4 (63.5, 78.7)
BMI Percentiles	94	61.0 (53.2, 72.4)	55.2 (33.4, 79.4)	52.0 (39.0, 57.5)	76.4 (62.6, 82.4)	85.5 (57.1, 89.4)
%BF	89	20.3 (19.2, 22.3)	23.5 (21.1, 29.6)	17.8 (17.0, 18.2)	24.2 (20.7, 25.2)	16.5 (14.3, 17.3)
Overweight/Obese (N (%))	94	21 (22.7)	4 (21.0)	2 (6.9)	5 (21.7)	7 (30.4)
W_{peak} (W/kg_{Lean})	89	4.6 (4.4, 4.9)	4.3 (3.8, 4.7)	4.5 (4.2, 4.8)	4.9 (4.2, 5.0)	4.6 (4.4, 5.1)
MVPA (min/d)	82	58.0 (54.3, 65.0)	58.1 (50.3, 59.4)	70.1 (66.3, 74.6)	47.2 (40.5, 50.5)	54.3 (47.6, 63.1)
MVPA (min/hr monitoring time)	82	4.5 (4.0, 5.0)	4.4 (3.7, 5.0)	5.6 (5.1, 6.0)	3.6 (3.1, 4.0)	3.8 (3.3, 4.6)
[EPC] ($\times 10^5$ cells/L)	82	3.3 (2.4, 4.0)	2.5 (1.8, 7.4)	2.9 (1.7, 4.0)	3.9 (2.6, 4.8)	3.8 (1.9, 5.7)
% EPC (% PBMCs)	82	0.01 (0.0, 0.02)	0.01 (0.01, 0.02)	0.01 (0.01, 0.02)	0.01 (0.01, 0.02)	0.02 (0.01, 0.02)

Data are presented as median (lower, upper BC_a 95% CI). YPHV = years from peak height velocity (Mirwald et al. 2002), %BF = percentage body fat from DXA, W_{peak} = peak mechanical power relative to lean body mass, MVPA = moderate-to-vigorous physical activity. BMI percentiles and overweight/obesity % calculated according to CDC criteria for age (Ogden et al. 2002).

Relationship between fitness, physical activity, body composition and EPCs. Spearman's correlations between EPCs and W_{peak}, MVPA, and %BF are presented in Table 3. Neither concentrations of EPCs nor cells expressed as a % of PBMCs were associated with W_{peak} normalized to lean body mass, MVPA or % BF. Because none of the bivariate

correlations achieved a correlation coefficient ≥0.5, backward linear regression analyses were not performed.

Table 4.3 *Spearman rank order correlations between EPCs, and fitness, physical activity and body composition.*

	[EPC]	%EPC
W_{peak} (W/kg_{Lean})	-0.06 (-0.29, 0.18)	-0.04 (-0.26, 0.18)
MVPA (min/d)	-0.09 (-0.29, 0.15)	-0.07 (-0.28, 0.15)
MVPA (min/hr monitoring)	-0.07 (-0.26, 0.16)	-0.06 (-0.24, 0.17)
%BF	0.20 (-0.02, 0.40)	0.14 (-0.08, 0.37)

Data are presented as Spearman's rho and lower, upper BC_a 95% CI. W_{peak} = peak mechanical power, MVPA = moderate-to-vigorous physical activity, %BF = percentage body fat from DXA, [EPC] = concentration of circulating endothelial progenitor cells, %EPC = EPCs expressed as a percentage of collected PBMCs.

Assessment of Agreement by EPC definitions. Median and BC_a 95% CI are presented for each of the cell definitions assessed in the current study in Table 4. Kendall's W statistic suggested very little agreement in percentile ranks across cell definitions (Kendall's W = 0.02, p = 0.212). Kappa statistics revealed a broad range of agreements by cell definition, from poor to almost perfect (Table 5).

Table 4.4 *EPC levels by cell definition.*

Cell Definition	Reference	Min.	Max.	Median	BC_a 95% CI	
					Lower	Upper
(1) $CD34^{br}CD133^{+}CD45^{dim}CD31^{+}$ (% of PBMCs)	Duda et al. 2007	0.0	0.1	0.01	0.0	0.02
(2) $CD34^{br}CD133^{+}CD45^{dim}CD31^{+}$ (× 10^5 cells/L)	Duda et al. 2007	0.02	21.3	3.3	2.4	4.0
(3) $CD34^{+}$ (% of mononuclear cells)	Kinik et al. 2005	0.01	1.0	0.1	0.1	0.1
(4) $CD34^{+}$ (% of lymphocytes)	Park et al. 2012	0.02	1.0	0.1	0.1	0.1
(5) $CD34^{+}$ (cells/mL)	Walther et al. 2008, 2009	477.2	26,970.5	1,935.2	1,574.7	2,698.5
(6) $CD45^{+}$ (cells/mL)	Walther et al. 2008	0.55 × 10^6	3.78 × 10^6	1.87 × 10^6	1.71 × 10^6	1.97 × 10^6
(7) $CD133^{+}$ (% of lymphocytes)	Park et al. 2012	0.02	9.6	0.3	0.3	0.4
(8) $CD133^{+}$ (cells/mL)	Walther et al. 2008	504.5	297,794.9	7,233.1	5,925.5	8,932.6
(9) $CD34^{+}CD133^{+}$ (% of lymphocytes)	Park et al. 2012	0.00	0.4	0.03	0.02	0.04
(10) $CD45^{dim}CD34^{+}$ (cells/μL)	Zaldivar et al. 2007, D'Ascenzi et al. 2014*	0.04	38.8	2.8	2.2	3.6

*D'Ascenzi et al. reported data using the same definition as Zaldivar et al., but in different units (cells/mL).

Table 4.5 *Kappa statistics for pairwise comparisons of percentile ranks by cell definition.*

	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1)	0.82† (0.71, 0.91)	0.21† (0.06, 0.36)	0.16* (-0.01, 0.32)	0.07 (-0.09. 0.22)	0.25† (0.07, 0.42)	-0.06 (-0.20, 0.09)	-0.03 (-0.17, 0.14)	0.12 (-0.02, 0.27)	0.03 (-0.13, 0.19)
(2)	-	0.27† (0.12, 0.40)	0.14 (-0.02, 0.32)	0.12 (-0.05, 0.31)	0.36† (0.18, 0.54)	-0.06 (-0.21, 0.10)	0.05 (-0.11, 0.22)	0.14 (0.0, 0.29)	0.10 (-0.06, 0.26)
(3)	-	-	0.58† (0.44, 0.71)	0.52† (0.37, 0.67)	0.03 (-0.14, 0.19)	0.16* (0.0, 0.32)	0.19* (0.02, 0.37)	0.42† (0.27, 0.56)	0.27† (0.12, 0.43)
(4)	-	-	-	0.67† (0.53, 0.78)	-0.04 (-0.19, 0.11)	0.16* (0.0, 0.29)	0.25† (0.09, 0.40)	0.41† (0.27, 0.54)	0.29† (0.15, 0.43)
(5)	-	-	-	-	0.10 (-0.06, 0.27)	0.25† (0.09, 0.41)	0.29† (0.11, 0.46)	0.40† (0.23, 0.54)	0.42† (0.26, 0.56)
(6)	-	-	-	-	-	0.07 (-0.08, 0.22)	0.10 (-0.06, 0.27)	0.05 (-0.10, 0.19)	0.16* (-0.0, 0.31)
(7)	-	-	-	-	-	-	0.74† (0.62, 0.89)	0.18* (0.02, 0.32)	0.14 (-0.01, 0.31)
(8)	-	-	-	-	-	-	-	0.20* (0.04, 0.35)	0.14 (-0.02, 0.29)
(9)	-	-	-	-	-	-	-	-	0.29 (0.13, 0.44)

Values are presented as Kappa statistic (lower, upper BC_a 95% CI). (1) $CD34^{br}CD133^{+}CD45^{dim}CD31^{+}$ (% of PBMCs), (2) $CD34^{br}CD133^{+}CD45^{dim}CD31^{+}$ ($\times 10^6$ cells/L), (3) $CD34^{+}$ (% of mononuclear cells), (4) CD34+ (% of lymphocytes), (5) CD34+ (cells/mL), (6) $CD45^{+}$ (cells/mL), (7) $CD133^{+}$ (% of lymphocytes), (8) $CD133^{+}$ (cells/mL), (9) $CD34^{+}CD133^{+}$ (% of lymphocytes), (10) $CD45^{dim}CD34^{+}$ (cells/μL). * $p \leq 0.05$; † $p \leq 0.001$.

4.5 Discussion

There is limited evidence in the pediatric population for links between fitness, physical activity and EPCs, which are emerging as potentially important effectors of cardiovascular health. For this reason, we simultaneously assessed the degree to which physical activity and fitness are associated with EPC concentrations, while considering body composition in children and adolescents. The main findings of this study are that neither proportions nor concentrations of EPCs were associated with markers of aerobic fitness, physical activity, or body composition. We also found that these cells were expressed similarly in males and females

in early and late phases of maturation, as defined by estimated years from peak height velocity.

In a series of reviews of mainly the adult literature, convincing links between EPCs and measures of vascular function, fitness and physical activity were provided [26,35,39]. These findings are in line with some of the pediatric literature where EPCs were weakly, but positively associated with exercise capacity [41], and elevated by 25-54% in youth presenting with higher baseline aerobic fitness, assessed as maximal oxygen uptake, compared with their peers [40]. We found no association between fitness, physical activity and EPCs, which is consistent with a recent study demonstrating similar levels of EPCs in competitive athletes and sedentary controls [11]. Our findings may be attributable to the homogeneous nature of our participants. Indeed, the incidence of overt acquired cardiovascular disease in children is extremely low, and the reported BC_a 95% CI values reported in Tables 2 and 4 highlight the small degree of variability in our sample. Since D'Ascenzi et al. did not directly measure aerobic fitness or physical activity levels in their participants, the degree of difference between their active and sedentary groups remains unclear. Both studies from Walther et al. required full classroom participation and actively recruited students from a specialized school for competitive sport, which increased the probability that both active and fit, as well as sedentary and unfit students were included in their sample.

Aside from fitness and physical activity, we also reported no association between percentage body fat and EPC proportion or concentration. This finding is consistent with that of Kinik et al. and is not all together surprising in light of the work of Arnold et al. suggesting that physical fitness, and not body fatness or body mass index, is associated with EPC number in obese youth [3,24]. More recently, Bruyndonckx et al. reported a 38% reduction in EPCs in obese youth (≥97th sex- and age-specific percentile for BMI) compared with healthy weight controls [7]. In the current study, only 7 of 21 participants met the criteria used by Bruyndonckx et al. for obese classification; of these, all 7 participants were enrolled in a lifestyle modification program that included both diet and activity counseling and as such, may not have been representative of overweight and obese youth in the general population. Although we aimed to recruit a sample that was representative of the general Canadian pediatric population, it may be important for future work to examine youth at the extremes of the population, specifically focusing on those presenting with increased risk of cardiovascular disease.

Pubertal development is associated with a number of physiological and behavioral changes that might also impact EPC concentrations; however, we did not observe any differences in EPC levels between pre- and late/post-pubertal youths. Conversely, Zaldivar et al. reported that $CD45^{dim}CD34^{+}$ cells were more abundant early pubertal compared to late

pubertal males, based on the development of secondary sex characteristics (i.e., Tanner stages). This difference is likely related to the cell populations assessed in each of the respective studies. Specifically, Zaldivar et al. examined a broader population of $CD34^+$ peripheral blood stem cells (PBSC) that may play a role in key tissue repair mechanisms, which include, but are not limited to, vascular repair [43]. Therefore, it may be that the cells examined in the current study represent a subset of the $CD34^+$ PBSC population.

Given the wide range of markers and gating strategies in previously reported exercise- and body composition-related pediatric studies of EPCs, our secondary objective was to compare EPC enumeration techniques in the same sample of youth. Our results suggest that there is little to no agreement in the overall ranking of participants according to the 10 examined phenotypic definitions of EPCs. This finding is consistent with previous studies in the adult population examining various protocols and phenotypic definitions of EPCs [8,17]. When participants were grouped into tertiles and examined in pairs of cell definitions, the $CD34^+$ definition employed by Kinik et al. demonstrated slight to moderate with all but one cell definition, $CD45^+$. This may explained by the fact that the Kinik et al. definition was relatively broad and included all $CD34^+$ events from both the lymphocyte and monocyte gates. Both the $CD45^+$ definition from Walther et al. and the Duda et al. definition employed in the current study were in

least agreement with the remainder of the cell definitions. This may be attributable to the more stringent criteria used in the cell gating of these definitions. Specifically, Walther et al. gated only on lymphocytes, while the Duda et al. definition employed included 4 markers for EPC characterization, which may have led to exclusion of some EPC subpopulations. Taken together, these data lend additional support to the finding that EPCs do not represent a single type of cell, but rather a group of cells at various stages of maturation [4,15,37]. To date, at least 3 EPC subtypes have been identified according to characteristics observed in vitro: early EPCS or circulating angiogenic cells, colony forming units, and late EPCs or endothelial colony forming cells [20]. Further, there is evidence to suggest that the early EPC subtype, which appears to be consistent with the phenotypes examined in the current study, represents a heterogeneous cell population in and of itself, and includes a subset of cells that may develop into late EPCs [21]. Therefore, it is likely that the large variability in EPC marker selection among the pediatric studies has led to an inadvertent assessment of distinct subpopulations of early and late EPCs. Given the reported differences in the contributions of these subpopulations to vasculogenesis and vascular repair [21], it is plausible that some subpopulations may also be more responsive and tightly linked with fitness, physical activity, and body composition than others. It is important that future studies clearly define their EPC of interest and,

ideally, isolate and assess the individual contributions of these cells to vascular maintenance and repair so as to better understand their potential as early markers of cardiovascular impairments in youth.

The findings of this study must be interpreted with some limitations in mind. The EPC enumeration protocol we employed was developed and verified by Duda et al.; however, we did not isolate the cells of interest to verify that their morphology was consistent with that of EPCs. Our protocol used peripheral blood mononuclear cells, whereas the majority of studies in listed Table 1 used whole blood. This limited our ability to directly compare our findings to previously published studies since PBMC and whole blood EPC enumeration protocols are known to demonstrate relatively low levels of agreement [8]. However, it is important to note that PBMC isolation may yield more valid estimates of EPCs than whole blood, which may be more susceptible to false positives [8,9]. Given that the Duda et al. protocol required 4 markers for EPC identification, and that most of the available literature employs a 2- or 3-marker approach, it is possible that we excluded some cells that might fall under the EPC umbrella. Moreover, our 4-marker protocol did not include KDR (or CD309/VEGFR2), a commonly used EPC marker, nor did we examine definitions including CD3 or CD117, thus we were unable to compare our results to all of the previously reported cell populations in exercise- and body composition-related studies youth. Finally, our study sample

represented a relatively healthy and homogeneous group children and adolescents. It may be that differences in EPCs are only seen at the extremes of the population; therefore, future work should seek to include children with chronic medical conditions that may predispose them to increased risk of cardiovascular disease.

To our knowledge, this was the first study to examine resting EPC concentrations and their associations with fitness, physical activity and body composition in a sample of healthy children and adolescents. We found that EPCs were similar in pre- and post-pubertal males and females, and were not associated with aerobic fitness, physical activity or body composition. We also reported overall poor agreement with regard to EPC concentrations identified using previously reported phenotypic definitions, which may be indicative of various subsets of EPCs involved in the complex process of vascular repair and remodeling. Future work should seek to isolate and examine the quantity, mobilization and function of these cells in relation to an exercise stimulus, so as to better understand the role of EPCs in exercise-induced improvements in cardiovascular health during the pediatric years.

4.6 Acknowledgements

The authors extend their sincere thanks to participants and their families for their time and effort. We also acknowledge the assistance of

Hong Liang at the McMaster Flow Cytometry Facility, as well as Colleen Cordukes, Megan Gao, and Rachel Walker. This study was made possible by a grant-in-aid from the Heart & Stroke Foundation of Canada (#NA 6445). JO is supported by a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship. TN is supported by an NSERC Postgraduate Scholarship. BWT is supported by a CIHR New Investigator Salary Award.

4.7 References

1 *Abbott RA, Harkness MA, Davies PSW.* Correlation of habitual physical activity levels with flow-mediated dilation of the brachial artery in 5-10 year old children. Atherosclerosis 2002; 160: 233–239

2 *Altman DG.* Practical Statistics for Medical Research. CRC Press, 1990

3 *Arnold C, Wenta D, Müller-Ehmsen J, Sreeram N, Graf C.* Progenitor cell number is correlated to physical performance in obese children and young adolescents. Cardiol Young 2010; 20: 381–386

4 *Asahara T, Kawamoto A.* Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 2004; 287: C572–C579

5 *Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM.* Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964–967

6 *Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U, Hogg JC.* Circulating hematopoietic progenitor cells in runners. J Appl Physiol Bethesda Md 1985 2002; 93: 1691–1697

7 *Bruyndonckx L, Hoymans VY, Frederix G, De Guchtenaere A, Franckx H, Vissers DK, Vrints CJ, Ramet J, Conraads VM.* Endothelial progenitor cells and endothelial microparticles are independent predictors of endothelial function. J Pediatr 2014; 165: 300–305

8 *Van Craenenbroeck EMF, Conraads VMA, Van Bockstaele DR, Haine SE, Vermeulen K, Van Tendeloo VF, Vrints CJ, Hoymans VY.* Quantification of circulating endothelial progenitor cells: a methodological comparison of six flow cytometric approaches. J Immunol Methods 2008; 332: 31–40

9 *Van Craenenbroeck EM, Van Craenenbroeck AH, van Ierssel S, Bruyndonckx L, Hoymans VY, Vrints CJ, Conraads VM.* Quantification of circulating CD34+/KDR+/CD45dim endothelial progenitor cells: analytical considerations. Int J Cardiol 2013; 167: 1688–1695

10 *Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF.* Endothelial cells of hematopoietic origin

make a significant contribution to adult blood vessel formation. Circ Res 2000; 87: 728–730

[11] *D'Ascenzi F, Zacà V, Maiorca S, Neri A, Aldinucci C, Sorrentino V, Causarano A, Bonifazi M, Mondillo S.* A quantitative assessment of circulating progenitor cells in competitive athletes and in sedentary subjects. J Sports Med Phys Fitness 2014;

[12] *Duda DG, Cohen KS, Scadden DT, Jain RK.* A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2007; 2: 805–810

[13] *Evenson KR, Catellier DJ, Gill K, Ondrak KS, McMurray RG.* Calibration of two objective measures of physical activity for children. J Sports Sci 2008; 26: 1557–1565

[14] *Fadini GP, Avogaro A, Agostini C.* Critical assessment of putative endothelial progenitor phenotypes. Exp Hematol 2007; 35: 1479–1480; author reply 1481–1482

[15] *Fadini GP, Losordo D, Dimmeler S.* Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. Circ Res 2012; 110: 624–637

[16] *Field A.* Discovering Statistics Using SPSS. SAGE, 2007

[17] *George J, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A.* Comparative analysis of methods for assessment of circulating endothelial progenitor cells. Tissue Eng 2006; 12: 331–335

[18] *Haukoos JS, Lewis RJ.* Advanced statistics: bootstrapping confidence intervals for statistics with "difficult" distributions. Acad Emerg Med Off J Soc Acad Emerg Med 2005; 12: 360–365

[19] *Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T.* Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348: 593–600

[20] *Hirschi KK, Ingram DA, Yoder MC.* Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008; 28: 1584–1595

[21] *Hur J, Yoon C-H, Kim H-S, Choi J-H, Kang H-J, Hwang K-K, Oh B-H, Lee M-M, Park Y-B.* Characterization of two types of endothelial

progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004; 24: 288–293

22 *Jie KE, Goossens MHJ, van Oostrom O, Lilien MR, Verhaar MC.* Circulating endothelial progenitor cell levels are higher during childhood than in adult life. Atherosclerosis 2009; 202: 345–347

23 *Jung C, Fischer N, Fritzenwanger M, Thude H, Ferrari M, Fabris M, Brehm BR, Barz D, Figulla HR.* Endothelial progenitor cells in adolescents: impact of overweight, age, smoking, sport and cytokines in younger age. Clin Res Cardiol Off J Ger Card Soc 2009; 98: 179–188

24 *Kinik ST, Ozbek N, Yücel M, Haberal A, Cetintas S.* Correlations among serum leptin levels, complete blood count parameters and peripheral CD34(+) cell count in prepubertal obese children. Ann Hematol 2005; 84: 605–608

25 *Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Böhm M, Kindermann W, Nickenig G.* Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol 2005; 12: 407–414

26 *Lenk K, Uhlemann M, Schuler G, Adams V.* Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. J Appl Physiol Bethesda Md 1985 2011; 111: 321–328

27 *Mirwald RL, Baxter-Jones ADG, Bailey DA, Beunen GP.* An assessment of maturity from anthropometric measurements. Med Sci Sports Exerc 2002; 34: 689–694

28 *Munoz SR, Bangdiwala SI.* Interpretation of Kappa and B statistics measures of agreement. J Appl Stat 1997; 24: 105–112

29 *Obeid J, Nguyen T, Walker RG, Gillis LJ, Timmons BW.* Circulating endothelial cells in children: role of fitness, activity, and adiposity. Med Sci Sports Exerc 2014; 46: 1974–1980

30 *Park J-H, Miyashita M, Kwon Y-C, Park H-T, Kim E-H, Park J-K, Park K-B, Yoon S-R, Chung J-W, Nakamura Y, Park S-K.* A 12-week after-school physical activity programme improves endothelial cell function

in overweight and obese children: a randomised controlled study. BMC Pediatr 2012; 12: 111

31 *Reed KE, Warburton DER, Lewanczuk RZ, Haykowsky MJ, Scott JM, Whitney CL, McGavock JM, McKay HA.* Arterial compliance in young children: the role of aerobic fitness. Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol 2005; 12: 492–497

32 *Roberts KC, Shields M, de Groh M, Aziz A, Gilbert J-A.* Overweight and obesity in children and adolescents: results from the 2009 to 2011 Canadian Health Measures Survey. Health Rep 2012; 23: 37–41

33 *Rochon J, Gondan M, Kieser M.* To test or not to test: Preliminary assessment of normality when comparing two independent samples. BMC Med Res Methodol 2012; 12: 81

34 *Shaffer RG, Greene S, Arshi A, Supple G, Bantly A, Moore JS, Parmacek MS, Mohler ER.* Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease. Vasc Med Lond Engl 2006; 11: 219–226

35 *Silva JFR da, Rocha NG, Nóbrega ACL da.* Mobilization of endothelial progenitor cells with exercise in healthy individuals: a systematic review. Arq Bras Cardiol 2012; 98: 182–191

36 *Strath SJ, Kaminsky LA, Ainsworth BE, Ekelund U, Freedson PS, Gary RA, Richardson CR, Smith DT, Swartz AM, American Heart Association Physical Activity Committee of the Council on Lifestyle and Cardiometabolic Health and Cardiovascular, Exercise, Cardiac Rehabilitation and Prevention Committee of the Council on Clinical Cardiology, and Council.* Guide to the assessment of physical activity: Clinical and research applications: a scientific statement from the American Heart Association. Circulation 2013; 128: 2259–2279

37 *Timmermans F, Plum J, Yöder MC, Ingram DA, Vandekerckhove B, Case J.* Endothelial progenitor cells: identity defined? J Cell Mol Med 2009; 13: 87–102

38 *Tongers J, Roncalli JG, Losordo DW.* Role of endothelial progenitor cells during ischemia-induced vasculogenesis and collateral formation. Microvasc Res 2010; 79: 200–206

39 *Volaklis KA, Tokmakidis SP, Halle M.* Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and

diseased patients. Clin Res Cardiol Off J Ger Card Soc 2013; 102: 249–257

[40] *Walther C, Adams V, Bothur I, Drechsler K, Fikenzer S, Sonnabend M, Bublitz B, Körner A, Erbs S, Busse M, Schuler G.* Increasing physical education in high school students: effects on concentration of circulating endothelial progenitor cells. Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol 2008; 15: 416–422

[41] *Walther C, Gaede L, Adams V, Gelbrich G, Leichtle A, Erbs S, Sonnabend M, Fikenzer K, Körner A, Kiess W, Bruegel M, Thiery J, Schuler G.* Effect of increased exercise in school children on physical fitness and endothelial progenitor cells: a prospective randomized trial. Circulation 2009; 120: 2251–2259

[42] *Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M, Nickenig G.* Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005; 353: 999–1007

[43] *Zaldivar F, Eliakim A, Radom-Aizik S, Leu S-Y, Cooper DM.* The effect of brief exercise on circulating CD34+ stem cells in early and late pubertal boys. Pediatr Res 2007; 61: 491–495

CHAPTER 5: EFFECTS OF EXERCISE ON CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN CHILDREN AND ADOLESCENTS WITH JUVENILE IDIOPATHIC ARTHRITIS AND HEALTHY CONTROLS

Submitted for publication as:

Obeid J, Nguyen T, Cellucci T, Larché MJ, and Timmons BW. Effect of exercise on circulating endothelial and progenitor cells in children and adolescents with juvenile idiopathic arthritis and healthy controls. *Pediatric Rheumatology*.

5.1 Abstract

Background: Youth with juvenile idiopathic arthritis (JIA) may be at risk of poor cardiovascular health. Circulating endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) are markers of cardiovascular repair and damage, respectively, and respond to exercise. The objectives of this study were to compare resting levels of EPCs and CECs in JIA and controls, and to assess the effects of distinct types of exercise on EPCs and CECs in JIA and controls. *Methods:* Seven youth with JIA and six controls completed 3 visits. First, aerobic fitness was assessed. Participants then performed either moderate intensity, continuous exercise (MICE) or high intensity, intermittent exercise (HIIE) on separate days. Blood samples were collected at the beginning (REST), mid-point (MID) and end of exercise (POST) for determination of EPCs

($CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}$) and CECs ($CD31^{bright}CD34^{+}CD45^{-}CD133^{-}$) by flow cytometry. Between group differences in EPCs and CECs were examined using two-way ANOVA, followed by Tukey's HSD post hoc, where appropriate. Statistical significance set at $p \leq 0.05$. *Results:* Both EPCs and CECs were similar between groups at REST (p=0.18 – 0.94). During MICE, EPCs remained unchanged in JIA (p=0.95) but increased significantly at POST in controls (REST: 0.91 ± 0.55 × 10^6 cells/L vs. POST: 1.53 ± 0.36 × 10^6 cells/L, p=0.04). Compared with controls, lower levels of EPCs were observed in JIA at MID (0.48 ± 0.50 × 10^6 cells/L vs. 1.10 ± 0.39 × 10^6 cells/L, p=0.01) and POST (0.38 ± 0.34 × 10^6 cells/L vs. 1.53 ± 0.36 × 10^6 cells/L, p<0.001) during MICE. No changes were detected in CECs with MICE in JIA and controls (p=0.69). Neither EPCs nor CECs were modified with HIIE (p=0.28-0.69). *Conclusion:* Youth with JIA demonstrated a blunted EPC response to MICE when compared with controls. Future work should examine factors that may increase or normalize EPC mobilization in JIA.

Keywords: juvenile idiopathic arthritis, flow cytometry, exercise, cardiovascular risk factors, endothelial progenitor cells, circulating endothelial cells.

5.2 Background

Children and adolescents with juvenile idiopathic arthritis (JIA) may be at an increased risk of developing poor cardiovascular health, with early signs of atherosclerosis manifesting as young as 4 years of age [1, 2]. This finding might be expected given that cardiovascular disease (CVD) is the leading cause of death among adults with rheumatoid arthritis (RA), and a number of predisposing factors for CVD, including chronic inflammation and low levels of physical activity, are also present on a long-term basis in JIA [3–6]. In fact, recent evidence suggests that approximately 41% of patients with JIA maintain an active disease state, marked by increased levels of inflammation and medication usage, 30 years after disease onset [7]. Interestingly, the increased incidence of CVD in adults with RA cannot be solely explained by traditional risk factors, highlighting the need for sensitive and specific alternative markers of CV health in this population and in younger patients with JIA [3, 8].

Recent evidence from healthy controls and patients supports a critical role for two distinct cell populations, circulating endothelial progenitor cells and circulating endothelial cells, in CV health [9, 10]. Circulating endothelial progenitor cells (EPCs) are a heterogeneous population of cells derived from the bone marrow that contribute to vascular repair and post-natal vasculogenesis via paracrine secretion of angiogenic factors or differentiation into mature endothelial cells [11, 12].

Concentrations of EPCs are inversely associated with markers of endothelial dysfunction and are known to predict occurrence of cardiovascular events [9, 13]. Moreover, EPCs are reduced in numerous pathologic conditions, including RA [13–17]. Much less is known about circulating endothelial cells (CECs), which represent a population of mature endothelial cells shed from the intima following irreversible structural damage to the endothelium [18]. Elevated levels of CECs are consistently reported in individuals with known risk factors for cardiovascular disease, including RA, and may be predictive of cardiovascular events [10, 13, 19]. Only one study has assessed EPCs in children with JIA and reported similar concentrations to healthy controls [20]. Conversely, CECs have yet to be assessed in JIA; however, the available evidence in various clinical populations suggest that both EPCs and CECs cells may provide important, clinically relevant insight into CV repair and damage, respectively.

Given the altered EPC and CEC profiles observed in clinical populations at risk of CVD, identification of methods to enhance their mobilization may allow for the development of therapeutic agents to improve CV remodeling. Exercise may represent one such stimulus as it has been shown to increase peripheral blood EPCs in adults and children [21–25]. Specifically, acute bouts of both high and moderate intensity running in adults led to similar increases in EPCs of 34-120% and 20-

163%, respectively [22]. In healthy children, EPCs have only been examined in response to an acute bout of high intensity intermittent cycling, which increased EPCs by 83-170% [23]. Unlike EPCs, the effect of acute exercise on CECs has only been examined in two studies of older adults with CVD with mixed results; however, a negative association between CECs and habitual physical activity among healthy children was recently reported [26–28]. Thus, exercise-induced changes in EPCs and CECs require additional study as one potential mechanism to enhance CV repair and remodeling, and ultimately impact overall CV health.

To date, no study has examined both resting and exercise-related changes in EPCs and CECs in JIA or compared the effects of distinct types of exercise on these cells. As such, the objectives of this study were to: 1) compare resting levels of EPCs and CECs in youth with JIA and healthy, age-matched controls; 2) assess the effects of acute bouts of high intensity and moderate intensity exercise on EPCs and CECs in JIA; and 3) compare exercise-induced changes in EPCs and CECs in JIA and healthy controls. Given the high levels of inflammation and low levels of physical activity and fitness reported in JIA, it is plausible that they would demonstrate lower levels of EPCs and elevated CECs at rest compared with their healthy peers [6, 29]. Further, we hypothesized that specific episodes of exercise, regardless of intensity, would lead to a transient increase in EPCs in both JIA and controls. Since CECs are negatively

associated with CV health, and exercise is a potent stimulus to improve CV health, we hypothesized that acute exercise may transiently decrease CECs [18, 30, 31].

5.3 Methods

Children with JIA were recruited by a research coordinator during their regularly scheduled visit to the McMaster Children's Hospital Pediatric Rheumatology Clinic. All patients were 8 to 17 years of age, and were diagnosed with JIA in accordance with the International League of Associations for Rheumatology criteria [32]. Patients were only excluded from participation if they did not have a confirmed JIA diagnosis, were diagnosed with any other medical condition, or had contraindications for exercise, including joint pain or swelling that would prevent completion of the exercise tests. They were also excluded if they were currently on biologic therapy or had received corticosteroid injections within 3 months of study participation as these treatments may alter resting EPCs or their precursors [20, 33, 34]. Healthy controls were matched by age to participants with JIA (within 1 year) and were either friends of participants with JIA or were recruited from the general community. Participants were not matched by sex as our previous work revealed no sex differences in either EPCs or CECs [26]. Healthy participants were excluded if they had any known medical conditions or had a BMI ≥ 85th percentile for their age,

since these might impact EPCs and CECs. All participants and parents/guardians provided written informed consent and assent, respectively, prior to enrollment in this study, which was approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board (REB #08-276).

Study Overview. Demographic and disease-related characteristics were retrieved by retrospective review of each participant's health records. All participants were invited to the Child Health & Exercise Medicine Program research laboratory on 3 occasions. During their first visit, basic anthropometric data were collected, including standing height measured to the nearest 0.1 cm, and weight measured to the nearest 0.1 kg. This was followed by an assessment of aerobic fitness. During their second and third visits, participants performed either a moderate intensity, continuous cycling exercise (MICE) or a high intensity, intermittent cycling exercise (HIIE) in a randomized, counterbalanced order. Blood samples were collected to assess EPC and CEC levels at rest, mid- and immediately post-exercise. Participants were then provided with an accelerometer to wear over a 9-day period to assess levels of moderate-to-vigorous physical activity (MVPA).

Aerobic fitness assessment. Aerobic fitness was assessed using the McMaster All Out Continuous Progressive test on either a mechanically or electromagnetically braked cycle ergometer (Flesich-

Metabo, Geneva, Switzerland; Lode Corival, Lode, The Netherlands, respectively). Progression in this test was achieved by fixed increases in workload every 2-min, such that the participant's maximal workload was achieved by 8- to 12-min. The test was terminated when the participant was no longer able to maintain the prescribed pedaling cadence of 60-70 rpm, despite strong verbal encouragement. To assess aerobic fitness, defined here as the maximal volume of oxygen consumed over 30-sec ($\dot{V}O_{2peak}$), participants were asked to breathe into a mouthpiece connected to a calibrated metabolic cart (Care Fusion, Cardinal Health) for determination of breath-by-breath inspired O_2 and expired CO_2. Maximal workload, defined as the peak power (W_{peak}) achieved during the test prorated to the time completed in the last stage, was also determined so as to normalize the workload for the subsequent cycling tasks.

MICE and HIIE Protocols. During visits 2 and 3, participants performed either the MICE or HIIE protocols on the same cycle ergometer used in their aerobic fitness assessment. These sessions were performed at the same time of day, at least 4 days apart. The MICE protocol consisted of 2 × 30-min bouts of cycling at 50% of W_{peak}, with a 6-min rest between bouts. The continuous nature of this exercise was designed to mimic traditional adult-based exercise prescriptions that have commonly been utilized in the JIA population [35, 36]. The HIIE protocol consisted of 6 sets of 4 × 15-sec bouts of cycling at 100% of W_{peak} for a total of 6-min

of exercise; participants were given a 1-min rest between bouts, and 6-min rest between sets. The intermittent, but intense, nature of this exercise was selected to reflect the typical physical activity patterns of children [37].

Physical Activity Assessment. Each participant was outfitted with an ActiGraph GT1M accelerometer (The ActiGraph, Pensacola, FL), which is a small device that provides objective and valid measures of habitual physical activity in youth [38]. Accelerometers were initialized to sample data in 3-sec intervals, and participants were instructed to wear the device over the right hip during all waking hours, with the exception of water activities, for 9 consecutive days. Levels of MVPA were determined and reported in minutes per day and minutes per hour of monitoring time, as previously described [26, 38]. Participants were included in the analyses if they wore the device for ≥ 10 hours on ≥ 4 days, including 1 weekend day.

Blood Samples. Participants were instructed to avoid engaging in any strenuous activity for at least 24 hours, and to refrain from eating and drinking, with the exception of water, for 3 hours prior to arrival to the laboratory. Blood samples during both MICE and HIIE protocols were collected at rest approximately 15 min before exercise (REST), at the mid point of exercise (MID), and immediately at the end of exercise (POST) from an indwelling catheter placed in the ante-cubital region of the arm. For each blood sampling time point, 12 mL of whole blood was collected of which 2 mL were processed for a complete blood count by the McMaster

Core Facility, and the remainder was stained for determination of EPCs and CECs, as previously described [26, 39]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation according to manufacturer protocols (Histopaque 1077, Sigma-Aldrich). Samples were then incubated with an FcR blocking reagent so as to minimize non-specific, receptor-mediated antibody binding. This was followed by a 20-min incubation with antibodies and conjugated fluorochromes for CD31-FITC, CD34-APC, CD45-PerCP and CD133-PE. Cells were then lysed and fixed prior to analysis for EPCs, defined as $CD31^{+}CD45^{dim}CD34^{br}CD133^{+}$, and CECs, identified as $CD31^{br}CD45^{-}CD34^{dim}CD133^{-}$ [26, 39]. Samples were analyzed within 24 hours of collection on either a BD LSRII flow cytometer (2 JIA and 2 matched controls) or a Miltenyi Biotec MACSQuant flow cytometer. Pilot data from samples assessed on both units revealed no significant differences in EPC and CEC counts (Obeid et al., unpublished observation). All analyses were performed using FlowJo (Version 8.7 for MacIntosh, Treestar Inc.), and both EPC and CEC levels are reported as a concentration as well as a percentage of PBMCs.

Statistical Analyses. All variables were assessed for normality using the Shapiro-Wilk test. Independent sample t-tests were used to assess differences in anthropometric, fitness, and physical activity variables between groups. To examine differences in resting EPCs and CECs, two-

way ANOVAs were performed with group (JIA vs. control) and day (Visit 2 vs. Visit 3) as factors. The effects of exercise on EPCs and CECs were compared in JIA and healthy controls by two-way ANOVA, with time (REST vs. MID vs. POST) and group (JIA vs. controls) as factors, for MICE and HIIE separately. Given the previously reported associations between EPCs, CECs, and physical activity, ANOVAs were repeated with levels of MVPA included as a covariate. Two-way ANCOVAs were also performed using resting EPC and CEC concentrations as a covariate. Significant main effects and interactions were further examined using Tukey's HSD post hoc, with statistical significance set at $p \leq 0.05$. Cohen's *d* (ES) was calculated as a measure of effect size, standardized to the variance in the control group, where small, medium, and large effects were defined as 0.2 – 0.49, 0.5 – 0.79, and >0.79, respectively [40]. All statistical analyses were performed in Statistica (version 10.0, Statsoft, Inc., Tulsa, OK). Data are presented as mean ± SD and 95% confidence intervals, unless otherwise specified.

5.4 Results

Participants Characteristics. Seven patients with JIA and 6 healthy controls completed this study. Disease-related participant characteristics are presented in Table 1. Participant characteristics, fitness, and physical activity are compared in children with JIA and healthy controls in Table 2.

$\dot{V}O_{2peak}$, a measure of aerobic fitness, tended to be lower in JIA vs. controls, but W_{peak} was similar. There were also no differences between groups in average time spent in MVPA per day or per hour.

Table 5.1 *Disease-related characteristics of children with JIA.*

	Age (Sex)	JIA Type	Disease Duration (years)	CRP	ESR	Active Joints	Medications
I	12.8 (F)	Psoriatic	3.8	0.34	6.0	Wrists	NSAID
II	10.8 (M)	Systemic	6.0	0.05[a]	n/a	None	None
III	17.4 (M)	Oligoarthritis (ANA-, RF+)	14.2	0.75[a]	6.0	None	None
IV	13.8 (M)	Polyarthritis (ANA-, RF-)	3.6	2.80	11.0	None	Methotrexate
V	11.1 (F)	Polyarthritis (ANA-, RF+)	3.6	0.90	15.0	Ankles	Methotrexate Corticosteroid
VI	11.5 (F)	Polyarthritis (ANA-, RF-)	7.3	<0.20	9.0	None	Methotrexate
VII	16.5 (F)	Oligoarthritis (ANA+, RF-)	15.2	n/a	n/a	None	NSAID

ANA: Antinuclear Antibody; CRP: C-reactive protein; ESR: Erythrocyte Sedimentation Rate; n/a: not available; NSAID: Non-Steroidal Anti-Inflammatory Drugs; RF: Rheumatoid Factor. Disease duration was calculated as the date of confirmed diagnosis of arthritis to date of enrollment in the study. CRP values were based on a blood sample taken at the clinic visit closest to study participation ([a] CRP was measured from a study visit blood sample since participants did not have any recent clinic visits). Active Joints refer to joints assessed as tender or swollen by the participant's rheumatologist at the clinic visit closest to study participation. Medications refer to those taken regularly by the participant at the time of study completion (Off medication duration: II = 5.0 years, and III = 0.9 years).

Table 5.2 Participant characteristics by group.

	JIA	Control	Mean Difference (Lower, Upper 95% CI)	P	ES
N (Males)	7 (3)	5 (1)	n/a	n/a	n/a
Age (years)	13.4 ± 2.6	14.0 ± 2.3	-0.6 (-3.8, 2.6)	0.67	0.26
Height (cm)	157.5 ± 15.0	162.1 ± 5.2	-4.6 (-20.4, 11.1)	0.53	0.88
Weight (kg)	56.0 ± 16.7	50.1 ± 7.1	5.9 (-11.9, 23.8)	0.48	0.83
$\dot{V}O_{2peak}$ (ml/kg/min)	45.6 ± 11.4	57.9 ± 8.3	-12.4 (-25.7, 1.0)	0.07	1.48
$\dot{W}_{peak}$ (W/kg)	3.0 ± 1.0	3.5 ± 0.5	-0.4 (-1.5, 0.7)	0.43	1.00
MVPA (min/d)	35.7 ± 13.0	26.1 ± 9.7	9.6 (-6.3, 25.5)	0.21	0.99
MVPA (min/hr wear time)	4.4 ± 2.0	3.8 ± 1.9	0.6 (-2.0, 3.3)	0.61	0.32

ES: effect size; $\dot{V}O_{2peak}$: maximal volume of oxygen consumed over 30-sec during the aerobic fitness test; W_{peak}: peak workload achieved during the aerobic fitness test; MVPA: moderate-to-vigorous physical activity. Mean difference calculated as JIA – control. Statistical significance set at $p \leq 0.05$.

Resting EPCs and CECs. No differences were observed in resting levels of EPCs or CECs in youth with JIA compared with healthy controls. This finding was consistent when cells were expressed as either a proportion of PBMCs or as a concentration (p = 0.18 – 0.94). Mean resting EPC and CEC values by group are presented in Table 3.

Exercise responses in JIA and Healthy Controls. While the MICE protocol led to a significant post-exercise increase in the concentration of EPCs in healthy controls, these cells remained unaltered in JIA (group × time F(2, 20) = 5.48, p = 0.01). Both MID (1.11 ± 0.39 × 10^6 cells/L) and POST (1.68 ± 0.33 × 10^6 cells/L) EPCs were significantly higher in healthy controls compared with REST (ES = 1.44, 2.71), MID (0.48 ± 0.50 × 10^6 cells/L, p = 0.02-0.04; ES = 1.60, 2.89) and POST (0.43 ± 0.33 × 10^6 cells/L, p < 0.01; ES = 1.86, 3.17) EPCs in JIA. A similar interaction was

observed when EPCs were expressed as a proportion of PBMCs ($F(2,20) = 5.11$, $p = 0.01$; ES = 0.60-2.41; Figure 1). No changes were detected in proportions or concentrations of EPCs in response to the HIIE protocol in either JIA or healthy controls ($p = 0.33$-0.38; ES = 0.22-1.13). Moreover, neither the MICE nor HIIE protocol had an effect on proportions or concentrations of CECs in JIA and healthy controls, as presented in Figure 1 ($p = 0.28$-0.69; ES = 0.08-4.1). These findings were unchanged when resting concentrations and/or MVPA were included as covariates in the analyses.

Table 5.3 *Resting EPC and CEC concentrations in JIA and controls.*

	JIA		Controls		Effect Size
	MICE	**HIIE**	**MICE**	**HIIE**	
EPC					
% of PBMCs	0.02 ± 0.02 (0.004, 0.03)	0.02 ± 0.02 (0.003, 0.04)	0.03 ± 0.02 (0.01, 0.05)	0.02 ± 0.01 (-0.003, 0.05)	0.25
× 10^6 cells/L	0.55 ± 0.48 (0.10, 0.99)	0.55 ± 0.50 (0.03, 1.08)	0.91 ± 0.55 (0.22, 1.60)	0.79 ± 0.42 (-0.24, 1.83)	0.55
CEC					
% of PBMCs	0.23 ± 0.26 (-0.01, 0.46)	0.11 ± 0.13 (-0.03, 0.24)	0.20 ± 0.23 (-0.09, 0.48)	0.22 ± 0.22 (-0.33, 0.77)	0.17
× 10^6 cells/L	6.36 ± 9.61 (-2.52, 15.24)	2.85 ± 3.19 (-0.49, 6.20)	6.28 ± 7.24 (-2.71, 15.28)	8.26 ± 8.88 (-13.81, 30.3)	0.37

Data are presented as mean ± SD (lower, upper 95% CI). Effect size calculated in JIA vs. controls, standardized to the pooled SD of the control group.

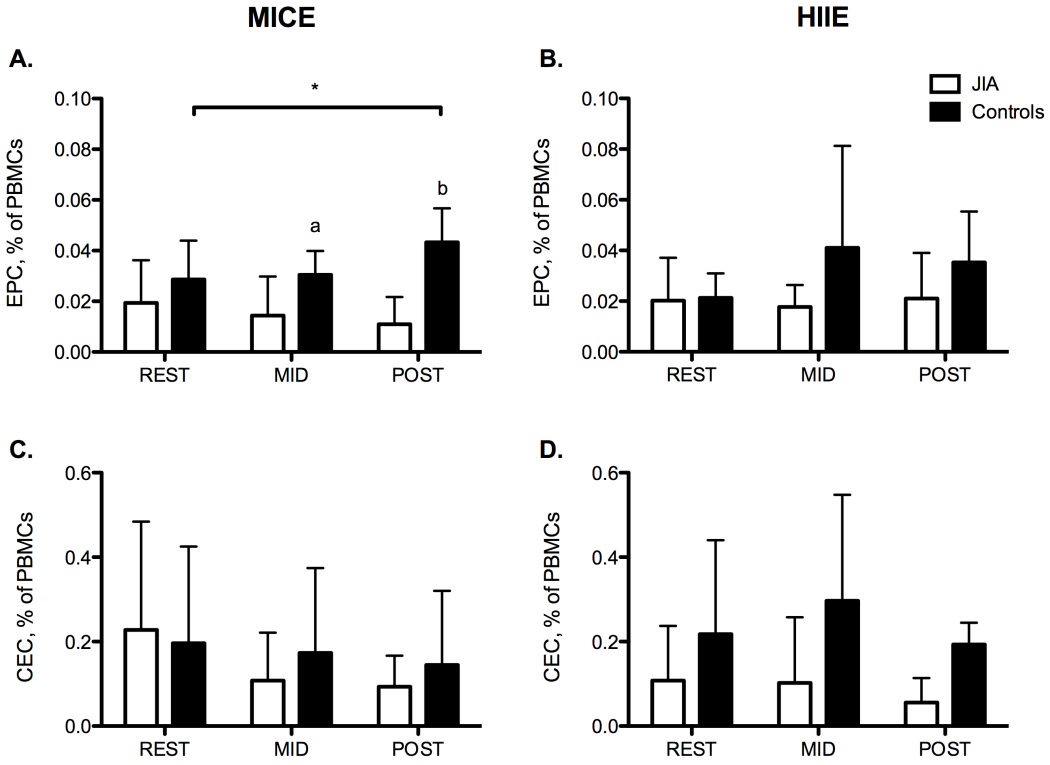

Figure 5.1 *Resting, mid- and post-exercise proportions EPCs and CECs in youth with JIA and healthy controls*. A significant interaction was seen for EPCs as a proportion of PBMCs during MICE (A), whereby healthy controls demonstrated an increase in EPCs while EPCs in JIA remained unchanged. Conversely, HIIE (B) did not alter EPCs in healthy controls or JIA. Similarly, neither MICE (C) nor HIIE (D) had any effect on CECs in JIA and healthy controls. * significant difference from rest, $p = 0.05$; [a] significant difference between JIA and healthy controls, $p = 0.05$; [b] significant difference between JIA and controls, $p < 0.01$.

5.5 Discussion

This is the first study to compare exercise-induced changes in EPCs and CECs in children with JIA and healthy controls. Our findings suggest that similar resting concentrations and proportions of EPCs and CECs are present in both groups. During MICE, healthy controls demonstrated a steady increase in EPCs, which was not observed in

participants with JIA. Conversely, CECs were unaltered in both groups during MICE, and HIIE had no effect on either CECs or EPCs.

Resting EPC and CEC concentrations were similar in JIA and controls, which is similar to the recently reported findings of Rusak et al. in JIA, but differs from previous studies that showed altered EPC and CEC levels in children and adults with chronic medical conditions compared with controls [14–17, 20, 41–45]. This may be related to the fact that our participants with JIA had a small number of active joints and relatively low C-reactive protein (CRP) and erythrocyte sedimentation rates (ESR), which may suggest they also had lower levels of disease activity at the time of participation. Data from adults with RA lend additional support to this finding. More specifically, a comparison of adults with active RA (disease activity score of ≥ 3.2), low or inactive RA and healthy controls revealed that only those with active disease demonstrated significantly impaired EPCs (active: 0.026 ± 0.002% vs. no/low active: 0.052 ± 0.006% vs. controls: 0.045 ± 0.008%) [14]. The authors also reported a significant, negative relationship between EPCs and disease activity score ($r= -0.38$, $p<0.01$) [14]. Similarly, Egan et al. reported significant negative associations between EPCs and ESR as well as rheumatoid factor level, as indicators of inflammation [15]. While the only study to assess CECs in adults with RA reported higher concentrations of these cells compared with controls, the 2-fold difference between groups was less than expected

when compared with other disease groups that demonstrate 6- to 20-fold higher levels of CECs relative to controls [10]. The authors reasoned that this was likely related to low disease activity and drug management in the RA group [10].

The exact underlying mechanism linking EPCs, CECs, and disease activity remains unclear but is likely mediated, at least in part, by systemic levels of inflammation and medication use. Grisar et al. reported that patients with high levels of tumor necrosis factor alpha (TNF-α) presented with low EPCs, and when examined according to medication use, those on anti-TNF therapy demonstrated similar levels of EPCs to healthy controls [14]. More recently, Rusak et al. found that patients with JIA treated with a combination of glucocorticoids, methotrexate, and anti-TNF therapy presented with reduced EPCs compared with those treated with either methotrexate alone, a combination of methotrexate and glucocorticoids, and healthy controls [20]. The pilot nature of this study did not allow us to examine these factors; however, it may be important to consider both factors in future study designs.

Exercise is a potent stimulus to transiently increase EPCs in adults, whereby a single bout can lead to a 66-309% increase in peripheral blood concentrations of these cells [25]. In children, only two studies have examined the effect of an acute bout of exercise on EPCs [23, 46]. Zaldivar et al. assessed healthy, pre- and post-pubertal males and

reported an 83-170% increase in EPCs [23]. Conversely, Lau et al. examined youth with chronic kidney disease and reported no change in EPCs [46]. In the present study, a single bout of moderate intensity, continuous exercise led to a ~100% increase in EPCs in healthy children, but did not elicit any change in EPCs in participants with JIA. The fact that we were unable to detect a change in EPCs in JIA may suggest an impaired mobilization of EPCs from the bone marrow. In healthy individuals, a number of angiogenic factors are associated with increased EPC proliferation and mobilization into the circulation; chief among these is Vascular Endothelial Growth Factor (VEGF) [47]. Interestingly, elevated VEGF levels are consistently reported in RA, but are not matched by an increase in EPCs [14, 41, 43, 47]. The mechanisms inhibiting the commonly reported effects of VEGF on EPCs in RA remain unknown. If our participants presented with chronically elevated VEGF, it is plausible that the exercise stimulus may not have been sufficient to stimulate additional VEGF production to promote EPC mobilization; however, this cannot be ascertained since VEGF levels were not assessed. Alternatively, we may have failed to detect a change in peripheral blood EPCs if they were rapidly taken up by another tissue. In fact, there is some evidence to suggest that EPCs may be recruited from the peripheral blood into the synovium in RA [48]. It has been hypothesized that EPCs may be trapped in the synovium leading to increased synovial blood vessel formation,

recruitment of inflammatory markers to the affected joints, and a reduced ability for EPCs to respond to CV damage [43, 48, 49]. Since EPCs were similar in JIA and healthy controls at rest, it seems unlikely that an acute bout of exercise would lead to a substantial increase in EPC recruitment to the synovium. However, we cannot rule out the possibility that the timing of our blood samples may have limited our ability to detect changes in EPCs in JIA. Taken together, our data suggest that exercise affects EPCs levels differently in youth with JIA and healthy controls.

Given that short bouts of intense exercise led to significant increases in EPCs in the work of Zaldivar et al., we anticipated similar increases with our HIIE protocol [23]. However, we did not detect any changes in EPCs in either JIA or healthy controls during HIIE. This finding may be related to the timing of our blood collection (too early, or too late), which may have missed a peak in EPCs. It is more likely that the exercise duration of our HIIE protocol was not sufficient to alter levels of these cells. In fact, when EPC responses in adults were compared following 30-min of high intensity, 30-min of moderate intensity, or 10-min of moderate intensity running, only the 30-min bouts led to similar increases in EPCs [22].

Neither HIIE nor MICE had any effect on CEC concentrations in participants with JIA or healthy controls. Given that CECs are a population mature endothelial cells detached from the vasculature following

irreparable damage, they are likely more representative of long-term dysfunction and cumulative injury to the endothelium [10]. It is plausible that a single, acute bout of exercise, regardless of intensity or duration, may not be sufficient to induce any changes in CEC concentrations. Whether repeated bouts of exercise can modify CEC concentrations remains to be determined.

A number of limitations should be considered in the interpretation of our findings. First, this study assessed a small group of patients with different types of JIA making it difficult to determine the generalizability of our results. However, the majority of the calculated effect sizes were medium or large, suggesting the reported results were meaningful, irrespective of sample size. Second, there is no consensus with respect to the best protocol and markers for EPC and CEC enumeration [18, 39, 50]. Because of similarities in cell surface markers, there is a chance that some mature endothelial cells and hematopoietic stem cells were gated with EPCs and vice versa. Despite this potential overlap, the high degree of repeatability and reliability of our EPC (intraclass correlation coefficient, ICC = 0.92 and 0.98, respectively) and CEC (ICC = 0.96 and 0.82) enumeration protocol lends support to the sensitivity of our measurements (Obeid, unpublished observation). Third, our EPCs and CECs response profile is limited to 2 time points at MID and POST, which may not reflect the peak response times of these cells. Finally, we assessed EPC and

CEC concentrations but did not assess their function in the circulation. Future work should seek to include functional assays given that there may be a disconnect in the quantity and function of EPCs in adults with chronic conditions [41, 47, 49].

5.6 Conclusion

To our knowledge, this was the first study to simultaneously compare EPCs and CECs at rest and in response to exercise in youth with JIA and healthy controls. Our results suggest that resting levels of both EPCs and CECs are similar in JIA and healthy controls. In addition, EPCs in healthy controls increased by an average of ~100% in response to an acute bout of moderate intensity, continuous cycling but remained unchanged in JIA. Conversely, high intensity, intermittent exercise had no effect on either EPCs or CECs in JIA and controls. The EPC response to exercise in JIA may be blunted as a result of disease-related inflammation and medication use, but may also reflect recruitment of EPCs from the circulation into the synovium. Future studies should explore factors that may be inhibiting exercise-induced changes in EPCs, as well as the impact of blood sample timing on EPC and CEC responses to exercise. Given the growing body of evidence supporting the role of these cells in maintaining and improving vascular health, a better understanding of the effects of exercise on their concentrations and function may allow for

development of an appropriate adjuvant therapy to maintain CV health in JIA.

5.7 Acknowledgements

The authors extend their sincere thanks to participants and their families for their time and effort. We also acknowledge the assistance of Dr. Peter Dent, Stephanie Sharpe, and Janice Falcone with patient recruitment, as well as Hong Liang at the McMaster Flow Cytometry Facility. This study was made possible by funding from the Natural Sciences and Engineering Research Council of Canada. JO is supported by a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship. TN is supported by an NSERC Postgraduate Scholarship. BWT is supported by a CIHR New Investigator Salary Award.

5.8 References

1. Argyropoulou MI, Kiortsis DN, Daskas N, Xydis V, Mavridis A, Efremidis SC, Siamopoulou A: Distensibility and pulse wave velocity of the thoracic aorta in patients with juvenile idiopathic arthritis: an MRI study. *Clin Exp Rheumatol* 2003, 21:794–797.

2. El-Sayed ZA, Mostafa GA, Aly GS, El-Shahed GS, El-Aziz MMA, El-Emam SM: Cardiovascular autonomic function assessed by autonomic function tests and serum autonomic neuropeptides in Egyptian children and adolescents with rheumatic diseases. *Rheumatol Oxf Engl* 2009, 48:843–848.

3. Peltomaa R, Paimela L, Kautiainen H, Leirisalo-Repo M: Mortality in patients with rheumatoid arthritis treated actively from the time of diagnosis. *Ann Rheum Dis* 2002, 61:889–894.

4. Metsios GS, Stavropoulos-Kalinoglou A, Panoulas VF, Wilson M, Nevill AM, Koutedakis Y, Kitas GD: Association of physical inactivity with increased cardiovascular risk in patients with rheumatoid arthritis. *Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol* 2009, 16:188–194.

5. Hulsegge G, Henschke N, McKay D, Chaitow J, West K, Broderick C, Singh-Grewal D: Fundamental movement skills, physical fitness and physical activity among Australian children with juvenile idiopathic arthritis. *J Paediatr Child Health* 2014.

6. Lelieveld OTHM, Armbrust W, van Leeuwen MA, Duppen N, Geertzen JHB, Sauer PJJ, van Weert E: Physical activity in adolescents with juvenile idiopathic arthritis. *Arthritis Rheum* 2008, 59:1379–1384.

7. Selvaag AM, Aulie HA, Lilleby V, Flatø B: Disease progression into adulthood and predictors of long-term active disease in juvenile idiopathic arthritis. *Ann Rheum Dis* 2014.

8. Del Rincón ID, Williams K, Stern MP, Freeman GL, Escalante A: High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. *Arthritis Rheum* 2001, 44:2737–2745.

9. Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003, 348:593–600.

10. Foster W, Shantsila E, Carruthers D, Lip GYH, Blann AD: Circulating endothelial cells and rheumatoid arthritis: relationship with plasma markers of endothelial damage/dysfunction. *Rheumatol Oxf Engl* 2009, 48:285–288.

11. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997, 275:964–967.

12. Hur J, Yoon C-H, Kim H-S, Choi J-H, Kang H-J, Hwang K-K, Oh B-H, Lee M-M, Park Y-B: Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol* 2004, 24:288–293.

13. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M, Nickenig G: Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005, 353:999–1007.

14. Grisar J, Aletaha D, Steiner CW, Kapral T, Steiner S, Seidinger D, Weigel G, Schwarzinger I, Wolozcszuk W, Steiner G, Smolen JS: Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis. *Circulation* 2005, 111:204–211.

15. Egan CG, Caporali F, Garcia-Gonzalez E, Galeazzi M, Sorrentino V: Endothelial progenitor cells and colony-forming units in rheumatoid arthritis: association with clinical characteristics. *Rheumatol Oxf Engl* 2008, 47:1484–1488.

16. Hörtenhuber T, Rami-Mehar B, Satler M, Nagl K, Höbaus C, Höllerl F, Koppensteiner R, Schernthaner G, Schober E, Schernthaner G-H: Endothelial progenitor cells are related to glycemic control in children with type 1 diabetes over time. *Diabetes Care* 2013, 36:1647–1653.

17. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C, Avogaro A: Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* 2005, 45:1449–1457.

18. Burger D, Touyz RM: Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. *J Am Soc Hypertens JASH* 2012, 6:85–99.

19. Boos CJ, Soor SK, Kang D, Lip GYH: Relationship between circulating endothelial cells and the predicted risk of cardiovascular events in acute coronary syndromes. *Eur Heart J* 2007, 28:1092–1101.

20. Rusak M, Radzikowska U, Glowinska-Olszewska B, Dobrenko E, Piotrowska-Jastrzebska J, Dabrowska M, Bodzenta-Lukaszyk A, Bossowski A, Moniuszko M: Endothelial progenitor cell levels in juvenile idiopathic arthritis patients; effects of anti-inflammatory therapies. *Pediatr Rheumatol Online J* 2015, 13:6.

21. Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U, Hogg JC: Circulating hematopoietic progenitor cells in runners. *J Appl Physiol Bethesda Md 1985* 2002, 93:1691–1697.

22. Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Böhm M, Kindermann W, Nickenig G: Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. *Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol* 2005, 12:407–414.

23. Zaldivar F, Eliakim A, Radom-Aizik S, Leu S-Y, Cooper DM: The effect of brief exercise on circulating CD34+ stem cells in early and late pubertal boys. *Pediatr Res* 2007, 61:491–495.

24. Walther C, Adams V, Bothur I, Drechsler K, Fikenzer S, Sonnabend M, Bublitz B, Körner A, Erbs S, Busse M, Schuler G: Increasing physical education in high school students: effects on concentration of circulating endothelial progenitor cells. *Eur J Cardiovasc Prev Rehabil Off J Eur Soc Cardiol Work Groups Epidemiol Prev Card Rehabil Exerc Physiol* 2008, 15:416–422.

25. Volaklis KA, Tokmakidis SP, Halle M: Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and diseased patients. *Clin Res Cardiol Off J Ger Card Soc* 2013, 102:249–257.

26. Obeid J, Nguyen T, Walker RG, Gillis LJ, Timmons BW: Circulating endothelial cells in children: role of fitness, activity, and adiposity. *Med Sci Sports Exerc* 2014, 46:1974–1980.

27. Boos CJ, Balakrishnan B, Lip GYH: The effects of exercise stress testing on soluble E-selectin, von Willebrand factor, and circulating

endothelial cells as indices of endothelial damage/dysfunction. *Ann Med* 2008, 40:66–73.

28. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F: Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999, 93:2951–2958.

29. Klepper S: Making the case for exercise in children with juvenile idiopathic arthritis: what we know and where we go from here. *Arthritis Rheum* 2007, 57:887–890.

30. Abbott RA, Harkness MA, Davies PSW: Correlation of habitual physical activity levels with flow-mediated dilation of the brachial artery in 5-10 year old children. *Atherosclerosis* 2002, 160:233–239.

31. Strath SJ, Kaminsky LA, Ainsworth BE, Ekelund U, Freedson PS, Gary RA, Richardson CR, Smith DT, Swartz AM, American Heart Association Physical Activity Committee of the Council on Lifestyle and Cardiometabolic Health and Cardiovascular, Exercise, Cardiac Rehabilitation and Prevention Committee of the Council on Clinical Cardiology, and Council: Guide to the assessment of physical activity: Clinical and research applications: a scientific statement from the American Heart Association. *Circulation* 2013, 128:2259–2279.

32. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, He X, Maldonado-Cocco J, Orozco-Alcala J, Prieur A-M, Suarez-Almazor ME, Woo P, International League of Associations for Rheumatology: International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol* 2004, 31:390–392.

33. Ablin JN, Boguslavski V, Aloush V, Elkayam O, Paran D, Caspi D, George J: Effect of anti-TNFalpha treatment on circulating endothelial progenitor cells (EPCs) in rheumatoid arthritis. *Life Sci* 2006, 79:2364–2369.

34. Lill-Elghanian D, Schwartz K, King L, Fraker P: Glucocorticoid-induced apoptosis in early B cells from human bone marrow. *Exp Biol Med Maywood NJ* 2002, 227:763–770.

35. Klepper SE: Effects of an eight-week physical conditioning program on disease signs and symptoms in children with chronic arthritis. *Arthritis Care Res Off J Arthritis Health Prof Assoc* 1999, 12:52–60.

36. Takken T, van Brussel M, Engelbert RHH, Van der Net J, Kuis W, Helders PJM: Exercise therapy in juvenile idiopathic arthritis. *Cochrane Database Syst Rev* 2008:CD005954.

37. Baquet G, Stratton G, Van Praagh E, Berthoin S: Improving physical activity assessment in prepubertal children with high-frequency accelerometry monitoring: a methodological issue. *Prev Med* 2007, 44:143–147.

38. Evenson KR, Catellier DJ, Gill K, Ondrak KS, McMurray RG: Calibration of two objective measures of physical activity for children. *J Sports Sci* 2008, 26:1557–1565.

39. Duda DG, Cohen KS, Scadden DT, Jain RK: A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* 2007, 2:805–810.

40. Olejnik null, Algina null: Measures of Effect Size for Comparative Studies: Applications, Interpretations, and Limitations. *Contemp Educ Psychol* 2000, 25:241–286.

41. Herbrig K, Haensel S, Oelschlaegel U, Pistrosch F, Foerster S, Passauer J: Endothelial dysfunction in patients with rheumatoid arthritis is associated with a reduced number and impaired function of endothelial progenitor cells. *Ann Rheum Dis* 2006, 65:157–163.

42. Lee KW, Lip GYH, Tayebjee M, Foster W, Blann AD: Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. *Blood* 2005, 105:526–532.

43. Paleolog E: It's all in the blood: circulating endothelial progenitor cells link synovial vascularity with cardiovascular mortality in rheumatoid arthritis?. *Arthritis Res Ther* 2005, 7:270–272.

44. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC: Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002, 106:2781–2786.

45. Głowińska-Olszewska B, Moniuszko M, Hryniewicz A, Jeznach M, Rusak M, Dąbrowska M, Łuczyński W, Bodzenta-Łukaszyk A, Bossowski A: Relationship between circulating endothelial progenitor cells and endothelial dysfunction in children with type 1 diabetes: a

novel paradigm of early atherosclerosis in high-risk young patients. *Eur J Endocrinol Eur Fed Endocr Soc* 2013, 168:153–161.

46. Lau KK, Obeid J, Breithaupt P, Belostotsky V, Arora S, Nguyen T, Timmons BW: Effects of acute exercise on markers of inflammation in pediatric chronic kidney disease: a pilot study. *Pediatr Nephrol Berl Ger* 2014.

47. Van Zonneveld AJ, de Boer HC, van der Veer EP, Rabelink TJ: Inflammation, vascular injury and repair in rheumatoid arthritis. *Ann Rheum Dis* 2010, 69 Suppl 1:i57–60.

48. Rüger B, Giurea A, Wanivenhaus AH, Zehetgruber H, Hollemann D, Yanagida G, Groger M, Petzelbauer P, Smolen JS, Hoecker P, Fischer MB: Endothelial precursor cells in the synovial tissue of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum* 2004, 50:2157–2166.

49. Avouac J, Uzan G, Kahan A, Boileau C, Allanore Y: Endothelial progenitor cells and rheumatic disorders. *Jt Bone Spine Rev Rhum* 2008, 75:131–137.

50. Fadini GP, Losordo D, Dimmeler S: Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circ Res* 2012, 110:624–637.

CHAPTER 6: SUMMARY OF MAIN FINDINGS AND GENERAL DISCUSSSION

The general aim of this thesis was to enhance our understanding of naturally occurring levels of CECs and EPCs in the pediatric years. To do this, the three included investigations: 1) examined differences in CECs and EPCs by sex, chronological, and biological age (Chapters 3 & 4); 2) assessed the relationships between CECs, EPCs and fitness, physical activity, and adiposity (Chapters 3 & 4); and 3) determined the effects of acute exercise on CECs and EPCs in healthy children and those with JIA (Chapter 5). The main findings of the conducted studies suggest that children and adolescents demonstrate a relatively broad range of CECs and EPCs, with most clustering at the lower range of reported values in adults. There were no differences in the levels of either cell population between males and females, chronological or biological age groups (Chapters 3 & 4). In addition, CECs, but not EPCs, demonstrated a significant positive association with fitness, and negative relationship with levels of habitual physical activity (Chapters 3 & 4). Neither CECs nor EPCs were related to level of adiposity (Chapters 3 & 4). Finally, although children with JIA and healthy controls presented with similar resting levels of CECs and EPCs, only healthy children demonstrated a significant, transient increase in EPCs with a single bout of continuous, moderate intensity cycling (Chapter 5). The following discussion will address the

main results and novel findings in this thesis, as well as future research directions. Selected data from Chapters 3, 4, and 5 are combined, and supplemental data are presented where appropriate to synthesize and support the interpretation of these findings.

6.1 The Interaction of CECs and EPCs in Youth

We examined CECs and EPCs separately throughout this thesis; however, there is some evidence to suggest that concurrent assessment of these cells may provide better insight into the balance between damage and repair of the vascular endothelium (Blann and Pretorius 2006). To examine the potential link between CECs and EPCs in youth, supplementary analyses were performed using pooled data from chapters 3, 4, and 5. A trend toward a weak, negative relationship between CECs and EPCs was observed (Appendix C, C-1), but this did not reach statistical significance. Since CECs have been linked with both stimulation and inhibition of EPC mobilization (Holmén et al. 2005, Goon et al. 2006), it may be important to consider CEC status (i.e., activated, necrotic, or apoptotic) in future assessments of the potential interaction between CECs and EPCs. There may also be differences in the factors regulating CECs and EPCs in youth. This appears to be in line with the findings of this thesis, which suggest that while fitness and habitual physical activity

do not have any lasting effects on the resting concentrations of EPCs, they may be significant mediators of naturally occurring levels of CECs.

6.2 The Influence of Sex, Chronological, and Biological Age

As expected, our data indicate that there were no differences in CECs between males and females, regardless of chronological or biological age. One important consideration for post-menarcheal females is the cyclical fluctuations in hormones associated with the menstrual cycle (Schisterman et al. 2014). Only one study has examined changes in CECs based on phase of menstrual cycle in young, healthy women (ages: 23-51 years), and reported consistent CEC concentrations across the menstrual, follicular, periovulatory, early luteal, middle luteal, and late luteal phases (Tanaka et al. 2012). Thus, based on these findings, and the fact that male and female participants in our study were similar with respect to fitness and physical activity levels, as well as prevalence of overweight or obesity, there is no obvious physiological rationale that would support a difference in resting CECs between sexes.

Unlike CECs, there is some evidence to suggest that EPCs are sensitive to the fluctuations in reproductive hormones (Hoetzer et al. 2007, Fadini et al. 2008, Lemieux et al. 2009, Tanaka et al. 2012). Indeed, the cyclical increase in EPC number and function in females of reproductive age is believed to represent one mechanism underlying the protective

effects of estrogens on cardiovascular health (Mendelsohn and Karas 1999, Fadini et al. 2008). More specifically, EPCs peak during the ovulatory and middle luteal phases, both of which correspond to changes in estradiol, luteinizing hormone, and G-CSF (Tanaka et al. 2012). Not only do EPC concentrations increase roughly 2-fold during ovulation, but they also demonstrate improved colony forming capacity, and adhesion to a mature endothelial layer when compared with cells from age-matched males (Fadini et al. 2008). Interestingly, exposure of EPCs from male participants to estradiol led to similar increases in colony formation and adhesive properties, thereby highlighting the key role of estrogens in modulating EPC number and function (Fadini et al. 2008). In this thesis, both pre/early-pubertal and late/post-pubertal males and females presented with similar levels of EPCs. This finding is likely related to the timing of our blood sample, wherein all post-menarcheal females were tested during the follicular stage of their menstrual cycles, which precedes the peaks in progesterone and estrogen, and most closely matches hormonal profiles in males.

Resting levels of CECs and EPCs were similar in the two chronological and biological age groups assessed in Chapters 3 and 4. In adults, EPCs consistently decrease in number and/or function with age (Rauscher et al. 2003, Heiss et al. 2005, Jie et al. 2009), while the CEC literature suggests no change or an increase in CECs with age (Shaffer et

al. 2006, Strijbos et al. 2008, Fabbri-Arrigoni et al. 2012). It is important to note that these observations in adults were made in vastly different age groups, with at least a 10-year difference between the “young” and “old” participants. Therefore, it is plausible that the extent of endothelial damage in childhood, and more specifically across a narrow 2-year age range, is not sufficient to detect a notable change in either resting CECs or EPCs at the group level. In both chapters 3 and 4 analyses were performed with participants grouped dichotomously by chronological (8-10 vs. 14-16 years) and biological age (≤ -1 YPHV vs. ≥ + 1 YPHV), making it difficult to comment on the distinct effects of age and puberty. To further assess the effect of puberty on CECs and EPCs, supplementary exploratory analyses were performed in 1-year increments of estimated YPHV (range: -3 to +3 years). There was no main effect of YPHV on either concentrations or proportions of CECs and EPCs, but a trend toward reduced CECs and increased EPCs at -1 YPHV was observed (Appendix C, Figure C-2), which may be indicative of a pubertal effect. This finding must be interpreted with some caution since we excluded participants in the mid-pubertal group. It is interesting to note that even when participants were grouped in smaller, and likely more homogenous, bins according to YPHV there remained a large degree of inter-individual variability in both CECs and EPCs. As such, it may be more informative to assess individual trajectories of CECs or EPCs over time rather than group level differences.

6.3 The Influence of Fitness

Aerobic fitness, defined in our study as peak mechanical power normalized to lean body mass, demonstrated a moderate, positive association with CECs in healthy children and adolescents. O'Sullivan et al. reported a similar, but slightly stronger, association between CECs and aerobic fitness, defined as maximal volume of oxygen uptake, in healthy high-fit and low-fit adults (O'Sullivan 2003). The authors proposed that individuals with higher fitness levels likely engage in more exercise and therefore experience more vascular wall shear stress, which in turn may stimulate increased endothelial cell turnover (O'Sullivan 2003). While it is plausible that an increase in shear stress would lead to greater endothelial cell detachment from the vessel wall, our findings do not appear to support the hypothesis of increased endothelial cell turnover, as we did not observe any relationship between fitness and EPCs in our participants.

The first study to examine the relationship between aerobic fitness and EPCs was performed in a sample of 11- to 12-year-old boys and girls upon completion of a 1-year school-based physical education intervention. When both the control and intervention classes were pooled, a weak, but significant positive relationship was observed between $\dot{V}O_{2max}$ and $CD34^{+}CD309^{+}$ EPCs ($r = 0.33$, $p < 0.001$) (Walther et al. 2009). The discrepancy between our findings may be related to the differences in indicators of aerobic fitness. Although we recognize that $\dot{V}O_{2max}$ is a more

direct measure of aerobic fitness, the use of peak mechanical power in this thesis was due to technical difficulties with our oxygen analyzer. The only other study to assess this relationship in youth was performed in a smaller sample of obese participants, and used both $\dot{V}O_{2max}$ and peak mechanical power, as well as 4 separate EPC definitions (Arnold et al. 2010). Unlike Walther et al., the authors reported that $\dot{V}O_{2max}$ was not related to $CD34^+CD309^+$ EPCs or $CD34^+CD117^+$ EPCs, but a moderate relationship was observed between $\dot{V}O_{2max}$ and EPCs defined as $CD34^+$, and $CD34^+CD133^+$ (Arnold et al. 2010). A positive association of similar magnitude was reported for peak mechanical power normalized to body weight and $CD34^+$ cells, as well as for $CD34^+CD117^+$ EPCs (Arnold et al. 2010). Taken together, these findings suggest that the association between aerobic fitness and EPCs may differ by EPC subtype; however, our knowledge of the exact number, or relationship between the different EPC subtypes and their respective roles in the maintenance of cardiovascular health remains limited (Timmermans et al. 2009).

6.4 The Influence of Physical Activity

The studies in this thesis are the first to directly assess the relationship between CECs, EPCs, and objectively measured physical activity. In fact, fitness (usually aerobic fitness defined as maximal oxygen uptake) is assessed in every pediatric study examining EPCs, while

habitual physical activity levels are not considered or are assumed based on level sport participation. For example, Walther et al. reported lower levels of resting EPCs ($CD45^{dim}CD34^{+}CD309^{+}$) in children receiving standard 2 × 45-min of weekly physical education classes compared with those enrolled in a specialized sport school who received 12 × 45-min of weekly physical education classes in addition to their involvement in competitive sporting events (Walther et al. 2009). Whether this difference is a result of increased fitness (measured), physical activity (not measured), or an interaction of the two is unknown. The same can be said for the differences in EPCs reported between high- and low-fit groups (Arnold et al. 2010, D'Ascenzi et al. 2014), as well as pre- and post-exercise or lifestyle interventions (Walther et al. 2008, Park et al. 2012, Bruyndonckx et al. 2015).

Our findings suggest that average daily minutes of MVPA were inversely associated with CECs (Chapter 3), but were not related to EPCs (Chapter 4) in children and adolescents. The decision to examine CECs and EPCs in relation to MVPA was based on the fact that this intensity is the focus of activity recommendations in Canada and abroad (Tremblay et al. 2014). Supplementary analysis was performed to examine potential differences in CECs and EPCs among participants who were meeting the current Canadian physical activity guidelines of at least 60-min daily MVPA compared with those not meeting this recommendation (Tremblay

et al. 2011). Although there were no differences in CECs or EPCs between activity groups (Appendix C, Figure C-3), it must be noted that the analysis was complicated by the fact that only 3 (4%) of the 82 participants with valid physical activity data were meeting the MVPA guideline. Conversely, 30 (37%) participants did not meet the guideline on more than 1 monitoring day. This distribution may help to explain the observed relationships or lack thereof between CECs, EPCs, and MVPA. Moreover, we only examined total daily volume of MVPA, which includes bouts of MVPA that might be as short as 3 sec. Based on the findings in Chapter 5, it may be important to consider both intensity and the duration of activity bouts in future assessments of the relationship between MVPA and EPCs.

6.5 The Effects of Acute Exercise

6.5.1 Healthy Controls

We reported that neither moderate intensity, continuous exercise nor high intensity, intermittent exercise had an effect on peripheral blood concentrations or proportion of CECs in children. This finding is consistent with some but not all of the adult literature (Mutin et al. 1999, O'Sullivan 2003). Indeed, there is some evidence to suggest an increase in CECs immediately following exercise (Boos et al. 2008), while others have reported a delayed CEC response, peaking between 2- and 4-h into

recovery (Möbius-Winkler et al. 2009, Sandri et al. 2011). Thus, it is possible that our sampling time points (midpoint and end of exercise) may not have been appropriate to detect a change in CECs.

Aside from this potential methodological limitation, it is also important to consider the effects of acute exercise on immune cells and inflammatory markers, which are thought to be involved in cleavage of the integrins and adhesion molecules that anchor endothelial cells to the basement membrane (See Section 1.2.4). Previous work from our laboratory demonstrated that compared with adults, boys experienced a smaller change in natural killer cells and IL-6, as well as a faster recovery of lymphocyte subsets following an acute bout of exercise performed at the same relative intensity (Timmons et al. 2004). In a separate study, Timmons et al. reported consistently lower levels of neutrophils, and smaller exercise-induced perturbations to the immune system in younger boys and girls (12-years-old) compared with their older counterparts (14-years-old) (Timmons et al. 2006). Moreover, TNF-α only increased in the older boys and men, but remained consistent in young boys and girls, as well as in older girls (Timmons et al. 2004, 2006). The authors suggested that children might be relatively resistant to major inflammatory responses during and following exercise so as to maintain the anabolic pathways that support optimal growth and development (Timmons 2005, Timmons et al. 2006). Although we did not measure inflammatory markers in Chapter 5,

seven of our twelve participants were ≤12 years of age at the time of assessment, therefore, it is possible that neither HIIE nor MICE led to a sufficient change in the immune cells and cytokines involved in endothelial cell detachment.

The data indicate that EPCs increased ~100% with MICE but remained unchanged with HIIE in healthy children. Our HIIE protocol consisted of 15-sec bouts of cycling at 100% of peak mechanical power for a total of 6 minutes. The fact that EPCs were unchanged with HIIE is consistent with the work of Laufs et al., which suggest that exercise duration is more important that intensity for EPC mobilization (Laufs et al. 2005). The only other study to examine the effects of acute exercise on EPCs in healthy children also used an intermittent exercise protocol; however, each bout consisted of 2 min of cycling at ~75% of VO_{2max}, and was repeated 10 times for a total of 20 minutes (Zaldivar et al. 2007). The authors reported an 83-170% increase in EPCs, which is comparable to the change observed in this thesis with MICE (Zaldivar et al. 2007). The increase in $CD34^{+}CD45^{dim}$ cells reported by Zaldivar et al. was also accompanied by an increase in FLT-3, G-CSF and to a lesser extent, SDF-1α, each of which have been implicated in EPC mobilization in the human adult and animal literature (Asahara et al. 1997, Aicher et al. 2005, Zaldivar et al. 2007, Tilling et al. 2009). Interestingly, the EPC response to exercise in children and adolescents appears to be smaller than that in

adults, which is reported to increase up to 300% from baseline values (Volaklis et al. 2013). This difference may be related to differences in the exercise protocols used, with most adult studies examining changes in EPCs following a maximal exercise test (Koutroumpi et al. 2012). Given that both immune cells and inflammatory mediators are involved in the mobilization and homing of EPCs (Aicher et al. 2005), this observation may be linked to the child-adult differences in immune responses to exercise described above (Timmons 2005). It also seems likely that the reduced EPC response in children may simply reflect the fact that they are less likely to demonstrate the same degree of cardiovascular damage as adults (Stary 1989).

In Chapter 4, we reported that there was no agreement between resting EPCs defined according to Duda et al. and Zaldivar et al. (κ = -0.13, 0.26, $p > 0.05$). It is interesting to note that despite this finding at rest, the effect of exercise and the magnitude of change in EPCs were similar in our study and that of Zaldivar et al. Moreover, the exercise-induced increase in EPCs following MICE was similar when we re-examined our cells according to the definition of Zaldivar et al. This observation is consistent with our previous work, where we reported that EPCs were unchanged following a 20-min bout of exercise in children with chronic kidney disease when cells were defined as $CD31^{+}CD34^{bright}CD45^{dim}CD133^{+}$ (Duda et al. 2007) or $CD34^{+}CD45^{dim}$

(Zaldivar et al. 2007, Lau et al. 2014). Taken together, these findings suggest that the effects of exercise are relatively consistent across more than one of the identified heterogeneous subpopulations of EPCs, which may have important implications for cardiovascular health.

6.5.2 Juvenile Idiopathic Arthritis

Unlike healthy controls, children with JIA did not exhibit any change in EPCs with MICE. Since both heart rate and ratings of perceived exertion were similar in the JIA and healthy controls throughout MICE (Appendix C, C-4), it seems unlikely that the difference in EPC response is related to the exercise stimulus. Although interpretation of this difference is somewhat limited by the lack of functional assays, there are a number of potential explanations for the blunted EPC response in JIA, including between group differences in endothelial dysfunction, exercise-induced inflammation, and autonomic activity.

Vlahos et al. recently reported that children with JIA between the ages of 7 and 18 years present with ~30% reduction in brachial FMD compared with healthy controls, regardless of JIA subtype (Vlahos et al. 2011). Endothelial dysfunction, as measured by FMD, is generally indicative of reduced NO bioavailability (Celermajer et al. 1992), a key regulator in the MMP-9-mediated pathway of EPC mobilization (described in Section 1.3.4) (Tilling et al. 2009). The authors found that endothelial dysfunction was largely attributable to inflammation in JIA, since between

group differences disappeared after adjustment for ICAM-1 and ESR (Vlahos et al. 2011). Given that the available resting ESR and CRP data from our JIA participants is similar to that of the healthy controls assessed by Vlahos et al. (ESR: 9 ± 4 vs. 8 ± 4 mm/h, CRP: 0.8 ± 1.0 vs. 1.7 ± 0.7 mg/dl, respectively) (Vlahos et al. 2011), it seems unlikely that our findings are related to endothelial dysfunction in JIA.

Although the data suggest low levels of inflammation in our participants with JIA at rest, it is possible that exercise-induced changes in inflammatory markers were more pronounced in JIA, which may have in turn inhibited EPC mobilization in these participants. However, two studies from our laboratory reported that the IL-6 and TNF-α response to MICE was similar in healthy controls and children with chronic inflammatory conditions, including cystic fibrosis (Nguyen et al. 2012), and inflammatory bowel disease (Ploeger et al. 2012). It is impossible to definitively conclude that youth with JIA demonstrated a similar response to these earlier studies since inflammatory markers were not measured in our participants.

In adults, an impaired EPC response to exercise was observed in individuals diagnosed cardiovascular disease including peripheral arterial disease (Shaffer et al. 2006, Sandri et al. 2011), microvascular angina (Scalone et al. 2013), and chronic heart failure (Van Craenenbroeck et al. 2011). Much like the results of the current study, Van Craenenbroeck et al.

reported similar pre-exercise EPCs in the patient group (chronic heart failure) and control groups, but a blunted EPC response immediately after exercise in the chronic heart failure group, which was maintained up to 24-h post-exercise (Van Craenenbroeck et al. 2011). The authors reasoned that the damaged endothelium in chronic disease states may lead to a compensatory increase in EPC mobilization from the bone marrow at rest such that the exercise stimulus is no longer sufficient to induce an additional increase in EPC mobilization from the "overdemanded" bone marrow (Van Craenenbroeck et al. 2011). It is important to note that the aforementioned studies in adults involve participants with a more prolonged history of chronic disease and exposure to cardiovascular risk factors than the young participants in our study; however, the extent of bone marrow exhaustion in JIA may be an important area for future investigations. In a separate study, the same authors suggested that since the bone marrow is innervated by the sympathetic nervous system, EPC proliferation and release might also be modulated by autonomic balance (Van Craenenbroeck et al. 2009). Interestingly, there is some evidence of autonomic nervous system dysregulation in youth with active and inactive JIA (Kuis et al. 1996, Kavelaars et al. 1998, El-Sayed et al. 2009). While it is plausible that this imbalance might affect on EPCs in JIA, it is not clear why this effect is only observed with exercise and not at rest. Indeed, the fact that EPCs were similar in JIA and healthy controls at rest may indicate

that the impairment in JIA may be related to one or more of the factors unique to exercise-induced EPC mobilization. Additional work is needed to more closely examine the mechanisms underlying the blunted EPC response in youth with JIA, and to determine whether this response is common among children with other chronic inflammatory conditions.

6.6 Novelty of Findings

This thesis represents the first efforts towards a more comprehensive understanding resting and acute exercise-induced changes in CEC and EPC levels in children and adolescents. The key novel findings and the contributions of this thesis to advancing the state of knowledge in this field are summarized as follows:

1. Chapter 3: This is the first study to assess CECs, and factors that might influence their resting concentrations, in a sample of healthy children and adolescents. In addition, it is the first study in both the pediatric and adult literature to assess objectively measured physical activity in relation to CECs. The findings suggest that CECs are similar in pre/early-pubertal and late/post-pubertal males and females. Children with the highest MVPA present with the lowest CEC levels; conversely, those with the highest power output have the highest CECs.

2. Chapter 4: This is the first study to concurrently examine the effects of sex, age, fitness, objectively measured physical activity, and adiposity on EPCs in children and adolescents. EPCs are similar in pre/early-pubertal and late/post-pubertal males and females. Fitness, physical activity, and adiposity are not related to resting EPCs in youth. This is also the first study to collate and compare EPCs based on the different cell surface marker definitions used in the pediatric exercise literature to date. There is very little agreement among these EPC cell definitions, which may have important implications for comparisons of the available studies examining EPCs in the context of health in children and adolescents.
3. Chapter 5: This is the first study to compare the effects of acute exercise on CECs and EPCs in children with JIA and healthy controls. This also represents the first study to assess the effects of two distinct types of exercise on CECs and EPCs in the pediatric population. CECs do not change with MICE or HIIE in either JIA or healthy controls. Conversely, healthy children demonstrate a robust increase in EPCs with MICE; this response is blunted in JIA.

6.7 Future Research Directions

Although the findings of this thesis begin to address some of the gaps in our understanding of CECs and EPCs in the pediatric population, they have also served to highlight a number of areas that require further investigation. Perhaps the most important issue to address, and one that is discussed repeatedly throughout this thesis, is the lack of consensus with respect to identification and isolation of CECs and EPCs. Given the large discrepancies in EPCs between cell definitions reported in Chapter 4, it is clear that this methodological issue has severely limited the growth CEC and EPC biology, and the potential application of these cells in the clinical setting. However, beyond this limitation, it is important that future studies in youth examine CEC and EPC number and function concurrently, along with assessment of the host of factors involved in their mobilization, differentiation (for EPCs), and eventual fate. In addition, although the adult literature has consistently demonstrated that resting CEC and EPC level and function are sensitive markers of cardiovascular health (Hill et al. 2003, Blann et al. 2005, Werner and Nickenig 2006, Erdbruegger et al. 2010), comparable literature in children is lacking. To better establish their importance in the pediatric population, further investigations that also include assessment of well-established measures of endothelial or general cardiovascular health, including ultrasound-based measures of endothelial structure and function (i.e., intima media thickness, FMD) are needed.

Given the large range of EPCs and CECs reported in this thesis, it may be critical to assess changes in these cells at the individual level over time. More specifically, a longitudinal analysis of the change in CEC and EPC number, function, and link to other markers of endothelial and cardiovascular health will provide us with a more complete picture of the role of these cells in the pediatric population.

The effect of a single bout of acute exercise differed between EPCs and CECs, exercise type, and between healthy children and those with JIA. These findings raise a number of potential questions for future research. First, is there an optimal exercise intensity or duration to elicit favourable changes in EPCs and CECs? The answer to this question may be particularly important for children with chronic medical conditions associated with an increased risk of poor cardiovascular health. Second, what factors are involved or responsible for the blunted EPC response in JIA? Understanding the mechanisms underlying this finding, which may be linked to an interaction between inflammation and EPC mobilization, may provide us with unique insights into the observations of increased cardiovascular risk factors in JIA and other chronic inflammatory conditions of childhood. Third, how do the effects of a single, acute bout of exercise change with chronic or repeated bouts of exercise? The limited training studies in youth suggest that chronic exercise can increase EPC levels (Walther et al. 2008, 2009, Park et al. 2012, Bruyndonckx et al.

2015), but the specific exercise protocols in these studies are unclear, and the effects on CECs have not been examined. Finally, do changes in CECs and EPCs with chronic exercise exposure lead to long-term improvements in cardiovascular outcomes? Examining these questions in the context of both CEC and EPC number and function will provide critical evidence to support the mechanisms translating exercise into improved cardiovascular health beginning in childhood.

.

CHAPTER 7: REFERENCES

Abbott, R.A., Harkness, M.A., and Davies, P.S.W. 2002. Correlation of habitual physical activity levels with flow-mediated dilation of the brachial artery in 5-10 year old children. Atherosclerosis 160(1): 233–239.

Ablin, J.N., Boguslavski, V., Aloush, V., Elkayam, O., Paran, D., Caspi, D., and George, J. 2006. Effect of anti-TNFalpha treatment on circulating endothelial progenitor cells (EPCs) in rheumatoid arthritis. Life Sci. 79(25): 2364–2369. doi: 10.1016/j.lfs.2006.07.035.

Adams, V., Lenk, K., Linke, A., Lenz, D., Erbs, S., Sandri, M., Tarnok, A., Gielen, S., Emmrich, F., Schuler, G., and Hambrecht, R. 2004. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. Arterioscler. Thromb. Vasc. Biol. 24(4): 684–690. doi: 10.1161/01.ATV.0000124104.23702.a0.

Adams, V., Linke, A., Breuckmann, F., Leineweber, K., Erbs, S., Kränkel, N., Bröcker-Preuss, M., Woitek, F., Erbel, R., Heusch, G., Hambrecht, R., Schuler, G., and Möhlenkamp, S. 2008. Circulating progenitor cells decrease immediately after marathon race in advanced-age marathon runners. Eur. J. Cardiovasc. Prev. Rehabil. Off. J. Eur. Soc. Cardiol. Work. Groups Epidemiol. Prev. Card. Rehabil. Exerc. Physiol. 15(5): 602–607. doi: 10.1097/HJR.0b013e328309c756.

Aicher, A., Heeschen, C., Mildner-Rihm, C., Urbich, C., Ihling, C., Technau-Ihling, K., Zeiher, A.M., and Dimmeler, S. 2003. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat. Med. 9(11): 1370–1376. doi: 10.1038/nm948.

Aicher, A., Zeiher, A.M., and Dimmeler, S. 2005. Mobilizing endothelial progenitor cells. Hypertension 45(3): 321–325. doi: 10.1161/01.HYP.0000154789.28695.ea.

Ameln, H., Gustafsson, T., Sundberg, C.J., Okamoto, K., Jansson, E., Poellinger, L., and Makino, Y. 2005. Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 19(8): 1009–1011. doi: 10.1096/fj.04-2304fje.

Argyropoulou, M.I., Kiortsis, D.N., Daskas, N., Xydis, V., Mavridis, A., Efremidis, S.C., and Siamopoulou, A. 2003. Distensibility and pulse wave velocity of the thoracic aorta in patients with juvenile idiopathic arthritis: an MRI study. Clin. Exp. Rheumatol. 21(6): 794–797. [accessed 19 January 2011].

Armstrong, N., Welsman, J.R., and Kirby, B.J. 1999a. Submaximal exercise and maturation in 12-year-olds. J. Sports Sci. 17(2): 107–114. doi: 10.1080/026404199366208.

Armstrong, N., Welsman, J.R., Nevill, A.M., and Kirby, B.J. 1999b. Modeling growth and maturation changes in peak oxygen uptake in 11-13 yr olds. J. Appl. Physiol. Bethesda Md 1985 87(6): 2230–2236.

Arnold, C., Wenta, D., Müller-Ehmsen, J., Sreeram, N., and Graf, C. 2010. Progenitor cell number is correlated to physical performance in obese children and young adolescents. Cardiol. Young 20(4): 381–386. doi: 10.1017/S1047951109990278.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275(5302): 964–967.

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M., and Isner, J.M. 1999. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J. 18(14): 3964–3972. doi: 10.1093/emboj/18.14.3964.

Asicioglu, E., Gogas Yavuz, D., Koc, M., Ozben, B., Yazici, D., Deyneli, O., and Akalin, S. 2010. Circulating endothelial cells are elevated in patients with type 1 diabetes mellitus. Eur. J. Endocrinol. Eur. Fed. Endocr. Soc. 162(4): 711–717. doi: 10.1530/EJE-09-0795.

Avouac, J., Uzan, G., Kahan, A., Boileau, C., and Allanore, Y. 2008. Endothelial progenitor cells and rheumatic disorders. Jt. Bone Spine Rev. Rhum. 75(2): 131–137. doi: 10.1016/j.jbspin.2007.09.006.

Balagopal, P.B., de Ferranti, S.D., Cook, S., Daniels, S.R., Gidding, S.S., Hayman, L.L., McCrindle, B.W., Mietus-Snyder, M.L., Steinberger, J., American Heart Association Committee on Atherosclerosis Hypertension and Obesity in Youth of the Council on Cardiovascular

Disease in the Young, Council on Nutrition, Physical Activity and Metabolism, and Council on Epidemiology and Prevention. 2011. Nontraditional risk factors and biomarkers for cardiovascular disease: mechanistic, research, and clinical considerations for youth: a scientific statement from the American Heart Association. Circulation 123(23): 2749–2769. doi: 10.1161/CIR.0b013e31821c7c64.

Bar-Or, O., and Rowland, T.W. 2004. Pediatric Exercise Medicine: From Physiologic Principles to Health Care Application. Human Kinetics.

Bartlett, J.W., and Frost, C. 2008. Reliability, repeatability and reproducibility: analysis of measurement errors in continuous variables. Ultrasound Obstet. Gynecol. Off. J. Int. Soc. Ultrasound Obstet. Gynecol. 31(4): 466–475. doi: 10.1002/uog.5256.

Bland, J.M., and Altman, D.G. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1(8476): 307–310.

Bland, J.M., and Altman, D.G. 2007. Agreement between methods of measurement with multiple observations per individual. J. Biopharm. Stat. 17(4): 571–582. doi: 10.1080/10543400701329422.

Blann, A.D. 2000. Endothelial cell activation, injury, damage and dysfunction: separate entities or mutual terms? Blood Coagul. Fibrinolysis Int. J. Haemost. Thromb. 11(7): 623–630.

Blann, A.D., and Pretorius, A. 2006. Circulating endothelial cells and endothelial progenitor cells: two sides of the same coin, or two different coins? Atherosclerosis 188(1): 12–18. doi: 10.1016/j.atherosclerosis.2005.12.024.

Blann, A.D., Woywodt, A., Bertolini, F., Bull, T.M., Buyon, J.P., Clancy, R.M., Haubitz, M., Hebbel, R.P., Lip, G.Y.H., Mancuso, P., Sampol, J., Solovey, A., and Dignat-George, F. 2005. Circulating endothelial cells. Biomarker of vascular disease. Thromb. Haemost. 93(2): 228–235. doi: 10.1267/THRO05020228.

Bonsignore, M.R., Morici, G., Santoro, A., Pagano, M., Cascio, L., Bonanno, A., Abate, P., Mirabella, F., Profita, M., Insalaco, G., Gioia, M., Vignola, A.M., Majolino, I., Testa, U., and Hogg, J.C. 2002. Circulating hematopoietic progenitor cells in runners. J. Appl. Physiol.

Bethesda Md 1985 93(5): 1691–1697. doi: 10.1152/japplphysiol.00376.2002.

Boos, C.J., Balakrishnan, B., Blann, A.D., and Lip, G.Y.H. 2008a. The relationship of circulating endothelial cells to plasma indices of endothelial damage/dysfunction and apoptosis in acute coronary syndromes: implications for prognosis. J. Thromb. Haemost. JTH 6(11): 1841–1850. doi: 10.1111/j.1538-7836.2008.03148.x.

Boos, C.J., Balakrishnan, B., and Lip, G.Y.H. 2008b. The effects of exercise stress testing on soluble E-selectin, von Willebrand factor, and circulating endothelial cells as indices of endothelial damage/dysfunction. Ann. Med. 40(1): 66–73. doi: 10.1080/07853890701652833.

Boos, C.J., Lane, D.A., Kang, D., Goon, P.K.Y., Blann, A.D., and Lip, G.Y.H. 2006a. Temporal and venepuncture-related decline in circulating endothelial cell capture from mixed venous blood. J. Thromb. Thrombolysis 22(2): 125–131. doi: 10.1007/s11239-006-8422-z.

Boos, C.J., Lip, G.Y.H., and Blann, A.D. 2006b. Circulating endothelial cells in cardiovascular disease. J. Am. Coll. Cardiol. 48(8): 1538–1547. doi: 10.1016/j.jacc.2006.02.078.

Boos, C.J., Soor, S.K., Kang, D., and Lip, G.Y.H. 2007. Relationship between circulating endothelial cells and the predicted risk of cardiovascular events in acute coronary syndromes. Eur. Heart J. 28(9): 1092–1101. doi: 10.1093/eurheartj/ehm070.

Bouvier, C.A., Gaynor, E., Cintron, J.R., Bernhardt, B., and Spaet, T.H. 1970. Circulating endothelium as an indicator of vascular injury. Thromb. Diath. Haemorrh. 40 (Supplement): 163–168.

Brogan, P.A., Shah, V., Brachet, C., Harnden, A., Mant, D., Klein, N., and Dillon, M.J. 2004. Endothelial and platelet microparticles in vasculitis of the young. Arthritis Rheum. 50(3): 927–936. doi: 10.1002/art.20199.

Bruyndonckx, L., Hoymans, V.Y., Van Craenenbroeck, A.H., Vissers, D.K., Vrints, C.J., Ramet, J., and Conraads, V.M. 2013. Assessment of endothelial dysfunction in childhood obesity and clinical use. Oxid. Med. Cell. Longev. 2013: 174782. doi: 10.1155/2013/174782.

Bruyndonckx, L., Hoymans, V.Y., Frederix, G., De Guchtenaere, A., Franckx, H., Vissers, D.K., Vrints, C.J., Ramet, J., and Conraads, V.M. 2014. Endothelial progenitor cells and endothelial microparticles are independent predictors of endothelial function. J. Pediatr. 165(2): 300–305. doi: 10.1016/j.jpeds.2014.04.015.

Bruyndonckx, L., Hoymans, V.Y., De Guchtenaere, A., Van Helvoirt, M., Van Craenenbroeck, E.M., Frederix, G., Lemmens, K., Vissers, D.K., Vrints, C.J., Ramet, J., and Conraads, V.M. 2015. Diet, exercise, and endothelial function in obese adolescents. Pediatrics 135(3): e653–661. doi: 10.1542/peds.2014-1577.

Burger, D., and Touyz, R.M. 2012. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J. Am. Soc. Hypertens. JASH 6(2): 85–99. doi: 10.1016/j.jash.2011.11.003.

Campuzano, R., Moya, J.L., García-Lledó, A., Tomas, J.P., Ruiz, S., Megías, A., Balaguer, J., and Asín, E. 2006. Endothelial dysfunction, intima-media thickness and coronary reserve in relation to risk factors and Framingham score in patients without clinical atherosclerosis. J. Hypertens. 24(8): 1581–1588. doi: 10.1097/01.hjh.0000239294.17636.27.

Case, J., Mead, L.E., Bessler, W.K., Prater, D., White, H.A., Saadatzadeh, M.R., Bhavsar, J.R., Yoder, M.C., Haneline, L.S., and Ingram, D.A. 2007. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. Exp. Hematol. 35(7): 1109–1118. doi: 10.1016/j.exphem.2007.04.002.

Celermajer, D.S., Sorensen, K.E., Gooch, V.M., Spiegelhalter, D.J., Miller, O.I., Sullivan, I.D., Lloyd, J.K., and Deanfield, J.E. 1992. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 340(8828): 1111–1115.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat. Med. 10(8): 858–864. doi: 10.1038/nm1075.

Cesari, F., Marcucci, R., Gori, A.M., Burgisser, C., Francini, S., Sofi, F., Gensini, G.F., Abbate, R., and Fattirolli, F. 2013. Impact of a cardiac

rehabilitation program and inflammatory state on endothelial progenitor cells in acute coronary syndrome patients. Int. J. Cardiol. 167(5): 1854–1859. doi: 10.1016/j.ijcard.2012.04.157.

Cesari, F., Sofi, F., Gori, A.M., Corsani, I., Capalbo, A., Caporale, R., Abbate, R., Gensini, G.F., and Casini, A. 2012. Physical activity and circulating endothelial progenitor cells: an intervention study. Eur. J. Clin. Invest. 42(9): 927–932. doi: 10.1111/j.1365-2362.2012.02670.x.

Chang, E., Paterno, J., Duscher, D., Maan, Z.N., Chen, J.S., Januszyk, M., Rodrigues, M., Rennert, R.C., Bishop, S., Whitmore, A.J., Whittam, A.J., Longaker, M.T., and Gurtner, G.C. 2015. Exercise induces stromal cell-derived factor-1α-mediated release of endothelial progenitor cells with increased vasculogenic function. Plast. Reconstr. Surg. 135(2): 340e–50e. doi: 10.1097/PRS.0000000000000917.

Charakida, M., Deanfield, J.E., and Halcox, J.P.J. 2007. Childhood origins of arterial disease. Curr. Opin. Pediatr. 19(5): 538–545. doi: 10.1097/MOP.0b013e3282eff585.

Charakida, M., Donald, A.E., Terese, M., Leary, S., Halcox, J.P., Ness, A., Davey Smith, G., Golding, J., Friberg, P., Klein, N.J., Deanfield, J.E., and ALSPAC (Avon Longitudinal Study of Parents and Children) Study Team. 2005. Endothelial dysfunction in childhood infection. Circulation 111(13): 1660–1665. doi: 10.1161/01.CIR.0000160365.18879.1C.

Chavakis, E., Aicher, A., Heeschen, C., Sasaki, K., Kaiser, R., El Makhfi, N., Urbich, C., Peters, T., Scharffetter-Kochanek, K., Zeiher, A.M., Chavakis, T., and Dimmeler, S. 2005. Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells. J. Exp. Med. 201(1): 63–72. doi: 10.1084/jem.20041402.

Chen, Y.-H., Lin, S.-J., Lin, F.-Y., Wu, T.-C., Tsao, C.-R., Huang, P.-H., Liu, P.-L., Chen, Y.-L., and Chen, J.-W. 2007. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. Diabetes 56(6): 1559–1568. doi: 10.2337/db06-1103.

Choi, J.K., Moon, K.M., Jung, S.Y., Kim, J.Y., Choi, S.H., Kim, D.Y., Kang, S., Chu, C.W., and Kwon, S.M. 2014. Regular exercise training increases the number of endothelial progenitor cells and decreases homocysteine levels in healthy peripheral blood. Korean J. Physiol.

Pharmacol. Off. J. Korean Physiol. Soc. Korean Soc. Pharmacol. 18(2): 163–168. doi: 10.4196/kjpp.2014.18.2.163.

Chong, A.Y., Blann, A.D., Patel, J., Freestone, B., Hughes, E., and Lip, G.Y.H. 2004. Endothelial dysfunction and damage in congestive heart failure: relation of flow-mediated dilation to circulating endothelial cells, plasma indexes of endothelial damage, and brain natriuretic peptide. Circulation 110(13): 1794–1798. doi: 10.1161/01.CIR.0000143073.60937.50.

Clarke, L.A., Hong, Y., Eleftheriou, D., Shah, V., Arrigoni, F., Klein, N.J., and Brogan, P.A. 2010. Endothelial injury and repair in systemic vasculitis of the young. Arthritis Rheum. 62(6): 1770–1780. doi: 10.1002/art.27418.

Conraads, V.M., Spruit, M.A., Braunschweig, F., Cowie, M.R., Tavazzi, L., Borggrefe, M., Hill, M.R.S., Jacobs, S., Gerritse, B., and van Veldhuisen, D.J. 2014. Physical activity measured with implanted devices predicts patient outcome in chronic heart failure. Circ. Heart Fail. 7(2): 279–287. doi: 10.1161/CIRCHEARTFAILURE.113.000883.

Croce, G., Passacquale, G., Necozione, S., Ferri, C., and Desideri, G. 2006. Nonpharmacological treatment of hypercholesterolemia increases circulating endothelial progenitor cell population in adults. Arterioscler. Thromb. Vasc. Biol. 26(5): e38–39. doi: 10.1161/01.ATV.0000218504.71680.b5.

Crosby, J.R., Kaminski, W.E., Schatteman, G., Martin, P.J., Raines, E.W., Seifert, R.A., and Bowen-Pope, D.F. 2000. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ. Res. 87(9): 728–730.

Cubbon, R.M., Murgatroyd, S.R., Ferguson, C., Bowen, T.S., Rakobowchuk, M., Baliga, V., Cannon, D., Rajwani, A., Abbas, A., Kahn, M., Birch, K.M., Porter, K.E., Wheatcroft, S.B., Rossiter, H.B., and Kearney, M.T. 2010. Human exercise-induced circulating progenitor cell mobilization is nitric oxide-dependent and is blunted in South Asian men. Arterioscler. Thromb. Vasc. Biol. 30(4): 878–884. doi: 10.1161/ATVBAHA.109.201012.

Custodis, F., and Laufs, U. 2007. Physical exercise and endothelial progenitor cells. J. Cardiopulm. Rehabil. Prev. 27(2): 74–75. doi: 10.1097/01.HCR.0000265032.04307.34.

D'Ascenzi, F., Zacà, V., Maiorca, S., Neri, A., Aldinucci, C., Sorrentino, V., Causarano, A., Bonifazi, M., and Mondillo, S. 2014. A quantitative assessment of circulating progenitor cells in competitive athletes and in sedentary subjects. J. Sports Med. Phys. Fitness.

Davis, G.E., and Senger, D.R. 2005. Endothelial Extracellular Matrix Biosynthesis, Remodeling, and Functions During Vascular Morphogenesis and Neovessel Stabilization. Circ. Res. 97(11): 1093–1107. doi: 10.1161/01.RES.0000191547.64391.e3.

Deanfield, J.E., Halcox, J.P., and Rabelink, T.J. 2007. Endothelial function and dysfunction: testing and clinical relevance. Circulation 115(10): 1285–1295. doi: 10.1161/CIRCULATIONAHA.106.652859.

Del Papa, N., Colombo, G., Fracchiolla, N., Moronetti, L.M., Ingegnoli, F., Maglione, W., Comina, D.P., Vitali, C., Fantini, F., and Cortelezzi, A. 2004. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. Arthritis Rheum. 50(4): 1296–1304. doi: 10.1002/art.20116.

Del Papa, N., Cortiana, M., Vitali, C., Silvestris, I., Maglione, W., Comina, D.P., Lucchi, T., and Cortelezzi, A. 2008. Simvastatin reduces endothelial activation and damage but is partially ineffective in inducing endothelial repair in systemic sclerosis. J. Rheumatol. 35(7): 1323–1328.

Dias, I.B.F., Panazzolo, D.G., Marques, M.F., Paredes, B.D., Souza, M.G.C., Manhanini, D.P., Morandi, V., Farinatti, P.T.V., Bouskela, E., and Kraemer-Aguiar, L.G. 2013. Relationships between emerging cardiovascular risk factors, z-BMI, waist circumference and body adiposity index (BAI) on adolescents. Clin. Endocrinol. (Oxf.) 79(5): 667–674. doi: 10.1111/cen.12195.

Dignat-George, F., and Sampol, J. 2000. Circulating endothelial cells in vascular disorders: new insights into an old concept. Eur. J. Haematol. 65(4): 215–220.

Dimmeler, S., and Zeiher, A.M. 2004. Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? J. Mol. Med. Berl. Ger. 82(10): 671–677. doi: 10.1007/s00109-004-0580-x.

Donald, A.E., Charakida, M., Cole, T.J., Friberg, P., Chowienczyk, P.J., Millasseau, S.C., Deanfield, J.E., and Halcox, J.P. 2006. Non-invasive

assessment of endothelial function: which technique? J. Am. Coll. Cardiol. 48(9): 1846–1850. doi: 10.1016/j.jacc.2006.07.039.

Duda, D.G., Cohen, K.S., Scadden, D.T., and Jain, R.K. 2007. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat. Protoc. 2(4): 805–810. doi: 10.1038/nprot.2007.111.

Duda, D.G., Cohen, K.S., di Tomaso, E., Au, P., Klein, R.J., Scadden, D.T., Willett, C.G., and Jain, R.K. 2006. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 24(9): 1449–1453. doi: 10.1200/JCO.2005.04.2861.

Eckel, R.H., Jakicic, J.M., Ard, J.D., Jesus, J.M. de, Miller, N.H., Hubbard, V.S., Lee, I.-M., Lichtenstein, A.H., Loria, C.M., Millen, B.E., Nonas, C.A., Sacks, F.M., Smith, S.C., Svetkey, L.P., Wadden, T.A., and Yanovski, S.Z. 2014. 2013 AHA/ACC Guideline on Lifestyle Management to Reduce Cardiovascular Risk A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation 129(25 suppl 2): S76–S99. doi: 10.1161/01.cir.0000437740.48606.d1.

Edelberg, J.M., Tang, L., Hattori, K., Lyden, D., and Rafii, S. 2002. Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. Circ. Res. 90(10): E89–93.

Egan, C.G., Caporali, F., Garcia-Gonzalez, E., Galeazzi, M., and Sorrentino, V. 2008. Endothelial progenitor cells and colony-forming units in rheumatoid arthritis: association with clinical characteristics. Rheumatol. Oxf. Engl. 47(10): 1484–1488. doi: 10.1093/rheumatology/ken299.

El Amine, M., Sohawon, S., Lagneau, L., Langneau, L., Gaham, N., and Noordally, S. 2010. Plasma levels of ICAM-1 and circulating endothelial cells are elevated in unstable types 1 and 2 diabetes. Endocr. Regul. 44(1): 17–24.

El-Sayed, Z.A., Mostafa, G.A., Aly, G.S., El-Shahed, G.S., El-Aziz, M.M.A., and El-Emam, S.M. 2009. Cardiovascular autonomic function assessed by autonomic function tests and serum autonomic neuropeptides in Egyptian children and adolescents with rheumatic

diseases. Rheumatol. Oxf. Engl. 48(7): 843–848. doi: 10.1093/rheumatology/kep134.

Eltayeb, A.A., Ahmad, F.-A., Sayed, D.M., and Osama, A.M. 2014. Subclinical vascular endothelial dysfunctions and myocardial changes with type 1 diabetes mellitus in children and adolescents. Pediatr. Cardiol. 35(6): 965–974. doi: 10.1007/s00246-014-0883-9.

Endemann, D.H., and Schiffrin, E.L. 2004. Endothelial Dysfunction. J. Am. Soc. Nephrol. 15(8): 1983–1992. doi: 10.1097/01.ASN.0000132474.50966.DA.

Erbs, S., Höllriegel, R., Linke, A., Beck, E.B., Adams, V., Gielen, S., Möbius-Winkler, S., Sandri, M., Kränkel, N., Hambrecht, R., and Schuler, G. 2010. Exercise training in patients with advanced chronic heart failure (NYHA IIIb) promotes restoration of peripheral vasomotor function, induction of endogenous regeneration, and improvement of left ventricular function. Circ. Heart Fail. 3(4): 486–494. doi: 10.1161/CIRCHEARTFAILURE.109.868992.

Erdbruegger, U., Dhaygude, A., Haubitz, M., and Woywodt, A. 2010. Circulating endothelial cells: markers and mediators of vascular damage. Curr. Stem Cell Res. Ther. 5(4): 294–302.

Erdbruegger, U., Haubitz, M., and Woywodt, A. 2006. Circulating endothelial cells: a novel marker of endothelial damage. Clin. Chim. Acta Int. J. Clin. Chem. 373(1-2): 17–26. doi: 10.1016/j.cca.2006.05.016.

Everaert, B.R., Van Craenenbroeck, E.M., Hoymans, V.Y., Haine, S.E., Van Nassauw, L., Conraads, V.M., Timmermans, J.-P., and Vrints, C.J. 2010. Current perspective of pathophysiological and interventional effects on endothelial progenitor cell biology: focus on PI3K/AKT/eNOS pathway. Int. J. Cardiol. 144(3): 350–366. doi: 10.1016/j.ijcard.2010.04.018.

Fabbri-Arrigoni, F.I., Clarke, L., Wang, G., Charakida, M., Ellins, E., Halliday, N., Brogan, P.A., Deanfield, J.E., Halcox, J.P., and Klein, N. 2012. Levels of circulating endothelial cells and colony-forming units are influenced by age and dyslipidemia. Pediatr. Res. 72(3): 299–304. doi: 10.1038/pr.2012.76.

Fadini, G.P., Agostini, C., Sartore, S., and Avogaro, A. 2007. Endothelial progenitor cells in the natural history of atherosclerosis. Atherosclerosis 194(1): 46–54. doi: 10.1016/j.atherosclerosis.2007.03.046.

Fadini, G.P., de Kreutzenberg, S., Albiero, M., Coracina, A., Pagnin, E., Baesso, I., Cignarella, A., Bolego, C., Plebani, M., Nardelli, G.B., Sartore, S., Agostini, C., and Avogaro, A. 2008. Gender differences in endothelial progenitor cells and cardiovascular risk profile: the role of female estrogens. Arterioscler. Thromb. Vasc. Biol. 28(5): 997–1004. doi: 10.1161/ATVBAHA.107.159558.

Fadini, G.P., Losordo, D., and Dimmeler, S. 2012. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. Circ. Res. 110(4): 624–637. doi: 10.1161/CIRCRESAHA.111.243386.

Fernandes, T., Nakamuta, J.S., Magalhães, F.C., Roque, F.R., Lavini-Ramos, C., Schettert, I.T., Coelho, V., Krieger, J.E., and Oliveira, E.M. 2012. Exercise training restores the endothelial progenitor cells number and function in hypertension: implications for angiogenesis. J. Hypertens. 30(11): 2133–2143. doi: 10.1097/HJH.0b013e3283588d46.

Flamme, I., Frölich, T., and Risau, W. 1997. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. J. Cell. Physiol. 173(2): 206–210. doi: 10.1002/(SICI)1097-4652(199711)173:2<206::AID-JCP22>3.0.CO;2-C.

Fletcher, G.F., Blair, S.N., Blumenthal, J., Caspersen, C., Chaitman, B., Epstein, S., Falls, H., Froelicher, E.S., Froelicher, V.F., and Pina, I.L. 1992. Statement on exercise. Benefits and recommendations for physical activity programs for all Americans. A statement for health professionals by the Committee on Exercise and Cardiac Rehabilitation of the Council on Clinical Cardiology, American Heart association. Circulation 86(1): 340–344.

Folkman, J., and Shing, Y. 1992. Angiogenesis. J. Biol. Chem. 267(16): 10931–10934.

Foster, W., Lip, G.Y.H., Raza, K., Carruthers, D., and Blann, A.D. 2012. An observational study of endothelial function in early arthritis. Eur. J. Clin. Invest. 42(5): 510–516. doi: 10.1111/j.1365-2362.2011.02607.x.

Foster, W., Shantsila, E., Carruthers, D., Lip, G.Y.H., and Blann, A.D. 2009. Circulating endothelial cells and rheumatoid arthritis: relationship with plasma markers of endothelial damage/dysfunction. Rheumatol. Oxf. Engl. 48(3): 285–288. doi: 10.1093/rheumatology/ken486.

Gatta, L., Armani, A., Iellamo, F., Consoli, C., Molinari, F., Caminiti, G., Volterrani, M., and Rosano, G.M.C. 2012. Effects of a short-term exercise training on serum factors involved in ventricular remodelling in chronic heart failure patients. Int. J. Cardiol. 155(3): 409–413. doi: 10.1016/j.ijcard.2010.10.045.

Gaynor, E., Bouvier, C.A., and Spaet, T.H. 1968. Circulating endothelial cells in endotoxin treated rabbits. Clin. Res. 16: 535.

Gehling, U.M., Ergün, S., Schumacher, U., Wagener, C., Pantel, K., Otte, M., Schuch, G., Schafhausen, P., Mende, T., Kilic, N., Kluge, K., Schäfer, B., Hossfeld, D.K., and Fiedler, W. 2000. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 95(10): 3106–3112.

George, F., P, B., Mc, B., M, M., M, D., C, B., D, R., and J, S. 1993. Demonstration of Rickettsia conorii-induced endothelial injury in vivo by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. Blood 82(7): 2109–2116. [accessed 7 April 2015].

George, J., Shmilovich, H., Deutsch, V., Miller, H., Keren, G., and Roth, A. 2006. Comparative analysis of methods for assessment of circulating endothelial progenitor cells. Tissue Eng. 12(2): 331–335. doi: 10.1089/ten.2006.12.331.

Glowinska, B., Urban, M., Peczynska, J., and Florys, B. 2005. Soluble adhesion molecules (sICAM-1, sVCAM-1) and selectins (sE selectin, sP selectin, sL selectin) levels in children and adolescents with obesity, hypertension, and diabetes. Metabolism. 54(8): 1020–1026. doi: 10.1016/j.metabol.2005.03.004.

Głowińska-Olszewska, B., Moniuszko, M., Hryniewicz, A., Jeznach, M., Rusak, M., Dąbrowska, M., Łuczyński, W., Bodzenta-Łukaszyk, A., and Bossowski, A. 2013. Relationship between circulating endothelial progenitor cells and endothelial dysfunction in children with type 1 diabetes: a novel paradigm of early atherosclerosis in high-risk young

patients. Eur. J. Endocrinol. Eur. Fed. Endocr. Soc. 168(2): 153–161. doi: 10.1530/EJE-12-0857.

Goon, P.K.Y., Boos, C.J., Stonelake, P.S., Blann, A.D., and Lip, G.Y.H. 2006a. Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. Thromb. Haemost. 96(1): 45–52. doi: 10.1160/TH06-04-0185.

Goon, P.K.Y., Lip, G.Y.H., Boos, C.J., Stonelake, P.S., and Blann, A.D. 2006b. Circulating Endothelial Cells, Endothelial Progenitor Cells, and Endothelial Microparticles in Cancer. Neoplasia 8(2): 79–88. doi: 10.1593/neo.05592.

Groner, J.A., Huang, H., Nagaraja, H., Kuck, J., and Bauer, J.A. 2015. Secondhand smoke exposure and endothelial stress in children and adolescents. Acad. Pediatr. 15(1): 54–60. doi: 10.1016/j.acap.2014.09.003.

Gustafsson, T., Puntschart, A., Kaijser, L., Jansson, E., and Sundberg, C.J. 1999. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am. J. Physiol. 276(2 Pt 2): H679–685.

Hagensen, M.K., Raarup, M.K., Mortensen, M.B., Thim, T., Nyengaard, J.R., Falk, E., and Bentzon, J.F. 2012. Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury. Cardiovasc. Res. 93(2): 223–231. doi: 10.1093/cvr/cvr278.

Hasmim, M., Vassalli, G., Alghisi, G.C., Bamat, J., Ponsonnet, L., Bieler, G., Bonnard, C., Paroz, C., Oguey, D., and Rüegg, C. 2005. Expressed isolated integrin beta1 subunit cytodomain induces endothelial cell death secondary to detachment. Thromb. Haemost. 94(5): 1060–1070.

Hattori, K., Heissig, B., Tashiro, K., Honjo, T., Tateno, M., Shieh, J.H., Hackett, N.R., Quitoriano, M.S., Crystal, R.G., Rafii, S., and Moore, M.A. 2001. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood 97(11): 3354–3360.

Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., Mildner-Rihm, C., Martin, H., Zeiher, A.M., and Dimmeler, S. 2003. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102(4): 1340–1346. doi: 10.1182/blood-2003-01-0223.

Heidenreich, P.A., Trogdon, J.G., Khavjou, O.A., Butler, J., Dracup, K., Ezekowitz, M.D., Finkelstein, E.A., Hong, Y., Johnston, S.C., Khera, A., Lloyd-Jones, D.M., Nelson, S.A., Nichol, G., Orenstein, D., Wilson, P.W.F., and Woo, Y.J. 2011. Forecasting the Future of Cardiovascular Disease in the United States A Policy Statement From the American Heart Association. Circulation 123(8): 933–944. doi: 10.1161/CIR.0b013e31820a55f5.

Heiss, C., Keymel, S., Niesler, U., Ziemann, J., Kelm, M., and Kalka, C. 2005. Impaired progenitor cell activity in age-related endothelial dysfunction. J. Am. Coll. Cardiol. 45(9): 1441–1448. doi: 10.1016/j.jacc.2004.12.074.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.S., Werb, Z., and Rafii, S. 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109(5): 625–637.

Herbeuval, H., and Fourot, M. 1964. [Comparative Study of the Vascular Endothelium and its Basal on a Section and in Leukoconcentration]. Comptes Rendus Séances Société Biol. Ses Fil. 158: 137–141.

Herbeuval, H., Fourot, M., Hettich, C., and Herbeuval, R. 1965. Endothelial cells and circulating-lood clumps: differential diagnosis. Acta Cytol. 9: 68–72.

Herbrig, K., Haensel, S., Oelschlaegel, U., Pistrosch, F., Foerster, S., and Passauer, J. 2006. Endothelial dysfunction in patients with rheumatoid arthritis is associated with a reduced number and impaired function of endothelial progenitor cells. Ann. Rheum. Dis. 65(2): 157–163. doi: 10.1136/ard.2005.035378.

Hill, J.M., Zalos, G., Halcox, J.P.J., Schenke, W.H., Waclawiw, M.A., Quyyumi, A.A., and Finkel, T. 2003. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N. Engl. J. Med. 348(7): 593–600. doi: 10.1056/NEJMoa022287.

Hirschi, K.K., Ingram, D.A., and Yoder, M.C. 2008. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler. Thromb. Vasc. Biol. 28(9): 1584–1595. doi: 10.1161/ATVBAHA.107.155960.

Hladovec, J. 1973. Circulating endothelial cells isolated together with platelets and the experimental modification of their counts in rats. Thromb. Res. 3(6): 665–674.

Hladovec, J. 1978. Circulating endothelial cells as a sign of vessel wall lesions. Physiol. Bohemoslov. 27(2): 140–144.

Hladovec, J. 1978. Endothelial injury by nicotine and its prevention. Experientia 34(12): 1585–1586.

Hoetzer, G.L., Van Guilder, G.P., Irmiger, H.M., Keith, R.S., Stauffer, B.L., and DeSouza, C.A. 2007a. Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. J. Appl. Physiol. Bethesda Md 1985 102(3): 847–852. doi: 10.1152/japplphysiol.01183.2006.

Hoetzer, G.L., MacEneaney, O.J., Irmiger, H.M., Keith, R., Van Guilder, G.P., Stauffer, B.L., and DeSouza, C.A. 2007b. Gender differences in circulating endothelial progenitor cell colony-forming capacity and migratory activity in middle-aged adults. Am. J. Cardiol. 99(1): 46–48. doi: 10.1016/j.amjcard.2006.07.061.

Holmén, C., Elsheikh, E., Stenvinkel, P., Qureshi, A.R., Pettersson, E., Jalkanen, S., and Sumitran-Holgersson, S. 2005. Circulating inflammatory endothelial cells contribute to endothelial progenitor cell dysfunction in patients with vasculitis and kidney involvement. J. Am. Soc. Nephrol. JASN 16(10): 3110–3120. doi: 10.1681/ASN.2005040347.

Holthöfer, H., Virtanen, I., Kariniemi, A.L., Hormia, M., Linder, E., and Miettinen, A. 1982. Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. Lab. Investig. J. Tech. Methods Pathol. 47(1): 60–66.

Hörtenhuber, T., Rami-Mehar, B., Satler, M., Nagl, K., Höbaus, C., Höllerl, F., Koppensteiner, R., Schernthaner, G., Schober, E., and Schernthaner, G.-H. 2013. Endothelial progenitor cells are related to

glycemic control in children with type 1 diabetes over time. Diabetes Care 36(6): 1647–1653. doi: 10.2337/dc12-1206.

Hristov, M., Erl, W., and Weber, P.C. 2003. Endothelial progenitor cells: isolation and characterization. Trends Cardiovasc. Med. 13(5): 201–206.

Hur, J., Yoon, C.-H., Kim, H.-S., Choi, J.-H., Kang, H.-J., Hwang, K.-K., Oh, B.-H., Lee, M.-M., and Park, Y.-B. 2004. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler. Thromb. Vasc. Biol. 24(2): 288–293. doi: 10.1161/01.ATV.0000114236.77009.06.

Hwang, S.J., Ballantyne, C.M., Sharrett, A.R., Smith, L.C., Davis, C.E., Gotto, A.M., and Boerwinkle, E. 1997. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 96(12): 4219–4225.

Jaffe, E.A. 1987. Cell biology of endothelial cells. Hum. Pathol. 18(3): 234–239.

Janssen, I., Shields, M., Craig, C.L., and Tremblay, M.S. 2012. Changes in the obesity phenotype within Canadian children and adults, 1981 to 2007-2009. Obes. Silver Spring Md 20(4): 916–919. doi: 10.1038/oby.2011.122.

Jiang, D.-M., Han, J., Zhu, J.-H., Fu, G.-S., and Zhou, B.-Q. 2013. Paracrine effects of bone marrow-derived endothelial progenitor cells: cyclooxygenase-2/prostacyclin pathway in pulmonary arterial hypertension. PloS One 8(11): e79215. doi: 10.1371/journal.pone.0079215.

Jie, K.E., Goossens, M.H.J., van Oostrom, O., Lilien, M.R., and Verhaar, M.C. 2009. Circulating endothelial progenitor cell levels are higher during childhood than in adult life. Atherosclerosis 202(2): 345–347. doi: 10.1016/j.atherosclerosis.2008.05.012.

Jung, C., Fischer, N., Fritzenwanger, M., Thude, H., Ferrari, M., Fabris, M., Brehm, B.R., Barz, D., and Figulla, H.R. 2009. Endothelial progenitor cells in adolescents: impact of overweight, age, smoking, sport and cytokines in younger age. Clin. Res. Cardiol. Off. J. Ger. Card. Soc. 98(3): 179–188. doi: 10.1007/s00392-008-0739-5.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M., and Asahara, T. 2000. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc. Natl. Acad. Sci. U. S. A. 97(7): 3422–3427. doi: 10.1073/pnas.070046397.

Kavelaars, A., de Jong-de Vos van Steenwijk, T., Kuis, W., and Heijnen, C.J. 1998. The reactivity of the cardiovascular system and immunomodulation by catecholamines in juvenile chronic arthritis. Ann. N. Y. Acad. Sci. 840: 698–704.

Kavey, R.-E.W., Allada, V., Daniels, S.R., Hayman, L.L., McCrindle, B.W., Newburger, J.W., Parekh, R.S., Steinberger, J., American Heart Association Expert Panel on Population and Prevention Science, American Heart Association Council on Cardiovascular Disease in the Young, American Heart Association Council on Epidemiology and Prevention, American Heart Association Council on Nutrition, Physical Activity and Metabolism, American Heart Association Council on High Blood Pressure Research, American Heart Association Council on Cardiovascular Nursing, American Heart Association Council on the Kidney in Heart Disease, and Interdisciplinary Working Group on Quality of Care and Outcomes Research. 2006. Cardiovascular risk reduction in high-risk pediatric patients: a scientific statement from the American Heart Association Expert Panel on Population and Prevention Science; the Councils on Cardiovascular Disease in the Young, Epidemiology and Prevention, Nutrition, Physical Activity and Metabolism, High Blood Pressure Research, Cardiovascular Nursing, and the Kidney in Heart Disease; and the Interdisciplinary Working Group on Quality of Care and Outcomes Research: endorsed by the American Academy of Pediatrics. Circulation 114(24): 2710–2738. doi: 10.1161/CIRCULATIONAHA.106.179568.

Kelly, A.S., Hebbel, R.P., Solovey, A.N., Schwarzenberg, S.J., Metzig, A.M., Moran, A., Sinaiko, A.R., Jacobs, D.R., and Steinberger, J. 2010. Circulating activated endothelial cells in pediatric obesity. J. Pediatr. 157(4): 547–551. doi: 10.1016/j.jpeds.2010.04.069.

Khan, S.S., Solomon, M.A., and McCoy, J.P. 2005. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin. Cytom. 64(1): 1–8. doi: 10.1002/cyto.b.20040.

Kim, J.H., Jung, J.-H., Phi, J.H., Kang, H.-S., Kim, J.E., Chae, J.H., Kim, S.-J., Kim, Y.-H., Kim, Y.Y., Cho, B.-K., Wang, K.-C., and Kim, S.-K. 2010. Decreased level and defective function of circulating endothelial progenitor cells in children with moyamoya disease. J. Neurosci. Res. 88(3): 510–518. doi: 10.1002/jnr.22228.

Kinik, S.T., Ozbek, N., Yücel, M., Haberal, A., and Cetintas, S. 2005. Correlations among serum leptin levels, complete blood count parameters and peripheral CD34(+) cell count in prepubertal obese children. Ann. Hematol. 84(9): 605–608. doi: 10.1007/s00277-005-1064-y.

Kinnaird, T., Stabile, E., Burnett, M.S., Shou, M., Lee, C.W., Barr, S., Fuchs, S., and Epstein, S.E. 2004. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109(12): 1543–1549. doi: 10.1161/01.CIR.0000124062.31102.57.

Kissel, C.K., Lehmann, R., Assmus, B., Aicher, A., Honold, J., Fischer-Rasokat, U., Heeschen, C., Spyridopoulos, I., Dimmeler, S., and Zeiher, A.M. 2007. Selective functional exhaustion of hematopoietic progenitor cells in the bone marrow of patients with postinfarction heart failure. J. Am. Coll. Cardiol. 49(24): 2341–2349. doi: 10.1016/j.jacc.2007.01.095.

Koc, M., Richards, H.B., Bihorac, A., Ross, E.A., Schold, J.D., and Segal, M.S. 2005. Circulating endothelial cells are associated with future vascular events in hemodialysis patients. Kidney Int. 67(3): 1078–1083. doi: 10.1111/j.1523-1755.2005.00173.x.

Korf-Klingebiel, M., Kempf, T., Sauer, T., Brinkmann, E., Fischer, P., Meyer, G.P., Ganser, A., Drexler, H., and Wollert, K.C. 2008. Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. Eur. Heart J. 29(23): 2851–2858. doi: 10.1093/eurheartj/ehn456.

Korkmaz, B., Horwitz, M.S., Jenne, D.E., and Gauthier, F. 2010. Neutrophil Elastase, Proteinase 3, and Cathepsin G as Therapeutic Targets in Human Diseases. Pharmacol. Rev. 62(4): 726–759. doi: 10.1124/pr.110.002733.

Koutroumpi, M., Dimopoulos, S., Psarra, K., Kyprianou, T., and Nanas, S. 2012. Circulating endothelial and progenitor cells: Evidence from

acute and long-term exercise effects. World J. Cardiol. 4(12): 312–326. doi: 10.4330/wjc.v4.i12.312.

Kränkel, N., Adams, V., Linke, A., Gielen, S., Erbs, S., Lenk, K., Schuler, G., and Hambrecht, R. 2005. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. Arterioscler. Thromb. Vasc. Biol. 25(4): 698–703. doi: 10.1161/01.ATV.0000156401.04325.8f.

Krüger, K., Klocke, R., Kloster, J., Nikol, S., Waltenberger, J., and Mooren, F.C. 2014a. Activity of daily living is associated with circulating CD34+/KDR+ cells and granulocyte colony-stimulating factor levels in patients after myocardial infarction. J. Appl. Physiol. Bethesda Md 1985 116(5): 532–537. doi: 10.1152/japplphysiol.01254.2013.

Krüger, K., Pilat, C., Schild, M., Lindner, N., Frech, T., Muders, K., and Mooren, F.C. 2014b. Progenitor cell mobilization after exercise is related to systemic levels of G-CSF and muscle damage. Scand. J. Med. Sci. Sports. doi: 10.1111/sms.12320.

Kucia, M., Dawn, B., Hunt, G., Guo, Y., Wysoczynski, M., Majka, M., Ratajczak, J., Rezzoug, F., Ildstad, S.T., Bolli, R., and Ratajczak, M.Z. 2004. Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. Circ. Res. 95(12): 1191–1199. doi: 10.1161/01.RES.0000150856.47324.5b.

Kuis, W., de Jong-de Vos van Steenwijk, C., Sinnema, G., Kavelaars, A., Prakken, B., Helders, P.J., and Heijnen, C.J. 1996. The autonomic nervous system and the immune system in juvenile rheumatoid arthritis. Brain. Behav. Immun. 10(4): 387–398.

Landis, J.R., and Koch, G.G. 1977. The measurement of observer agreement for categorical data. Biometrics 33(1): 159–174.

Laufs, U., Urhausen, A., Werner, N., Scharhag, J., Heitz, A., Kissner, G., Böhm, M., Kindermann, W., and Nickenig, G. 2005. Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. Eur. J. Cardiovasc. Prev. Rehabil. Off. J. Eur. Soc. Cardiol. Work. Groups Epidemiol. Prev. Card. Rehabil. Exerc. Physiol. 12(4): 407–414.

Lau, K.K., Obeid, J., Breithaupt, P., Belostotsky, V., Arora, S., Nguyen, T., and Timmons, B.W. 2014. Effects of acute exercise on markers of inflammation in pediatric chronic kidney disease: a pilot study. Pediatr. Nephrol. Berl. Ger. doi: 10.1007/s00467-014-2971-8.

Lee, K.W., Blann, A.D., and Lip, G.Y.H. 2006. Inter-relationships of indices of endothelial damage/dysfunction [circulating endothelial cells, von Willebrand factor and flow-mediated dilatation] to tissue factor and interleukin-6 in acute coronary syndromes. Int. J. Cardiol. 111(2): 302–308. doi: 10.1016/j.ijcard.2005.10.014.

Lee, K.W., Lip, G.Y.H., Tayebjee, M., Foster, W., and Blann, A.D. 2005. Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. Blood 105(2): 526–532. doi: 10.1182/blood-2004-03-1106.

Lee, P.S.S., and Poh, K.K. 2014. Endothelial progenitor cells in cardiovascular diseases. World J. Stem Cells 6(3): 355–366. doi: 10.4252/wjsc.v6.i3.355.

Lefevre, P., George, F., Durand, J.M., and Sampol, J. 1993. Detection of circulating endothelial cells in thrombotic thrombocytopenic purpura. Thromb. Haemost. 69(5): 522.

Lemieux, C., Cloutier, I., and Tanguay, J.-F. 2009. Menstrual cycle influences endothelial progenitor cell regulation: a link to gender differences in vascular protection? Int. J. Cardiol. 136(2): 200–210. doi: 10.1016/j.ijcard.2008.04.054.

Lenk, K., Uhlemann, M., Schuler, G., and Adams, V. 2011. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. J. Appl. Physiol. Bethesda Md 1985 111(1): 321–328. doi: 10.1152/japplphysiol.01464.2010.

Leone, A.M., Rutella, S., Bonanno, G., Abbate, A., Rebuzzi, A.G., Giovannini, S., Lombardi, M., Galiuto, L., Liuzzo, G., Andreotti, F., Lanza, G.A., Contemi, A.M., Leone, G., and Crea, F. 2005. Mobilization of bone marrow-derived stem cells after myocardial infarction and left ventricular function. Eur. Heart J. 26(12): 1196–1204. doi: 10.1093/eurheartj/ehi164.

Leone, A.M., Rutella, S., Bonanno, G., Contemi, A.M., de Ritis, D.G., Giannico, M.B., Rebuzzi, A.G., Leone, G., and Crea, F. 2006. Endogenous G-CSF and CD34+ cell mobilization after acute myocardial infarction. Int. J. Cardiol. 111(2): 202–208. doi: 10.1016/j.ijcard.2005.06.043.

Lévesque, J.-P., Hendy, J., Takamatsu, Y., Simmons, P.J., and Bendall, L.J. 2003. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J. Clin. Invest. 111(2): 187–196. doi: 10.1172/JCI15994.

Li, C., Wu, Q., Liu, B., Yao, Y., Chen, Y., Zhang, H., Wang, C., Cao, J., and Ge, S. 2013. Detection and validation of circulating endothelial cells, a blood-based diagnostic marker of acute myocardial infarction. PloS One 8(3): e58478. doi: 10.1371/journal.pone.0058478.

Lin, Y., Weisdorf, D.J., Solovey, A., and Hebbel, R.P. 2000. Origins of circulating endothelial cells and endothelial outgrowth from blood. J. Clin. Invest. 105(1): 71–77. doi: 10.1172/JCI8071.

Long, L., Roberts, S., Mcgrath, R., Mcgrew, E., and Cole, W.H. 1960. Cancer cells in the blood stream. Arch. Surg. Chic. Ill 1960 80: 639–645.

Loomans, C.J.M., de Koning, E.J.P., Staal, F.J.T., Rookmaaker, M.B., Verseyden, C., de Boer, H.C., Verhaar, M.C., Braam, B., Rabelink, T.J., and van Zonneveld, A.-J. 2004. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes 53(1): 195–199.

Lu, A., Wang, L., and Qian, L. 2014a. The role of eNOS in the migration and proliferation of bone-marrow derived endothelial progenitor cells and in vitro angiogenesis. Cell Biol. Int. doi: 10.1002/cbin.10405.

Lu, C., Zhang, J., Zhang, D., Uzan, G., and Li, M. 2014b. EPCs in vascular repair: how can we clear the hurdles between bench and bedside? Front. Biosci. Landmark Ed. 19: 34–48.

Luk, T.-H., Dai, Y.-L., Siu, C.-W., Yiu, K.-H., Chan, H.-T., Fong, D.Y.-T., Lee, S.W.L., Li, S.-W., Tam, S., Lau, C.-P., and Tse, H.-F. 2009. Habitual physical activity is associated with endothelial function and endothelial progenitor cells in patients with stable coronary artery

disease. Eur. J. Cardiovasc. Prev. Rehabil. Off. J. Eur. Soc. Cardiol. Work. Groups Epidemiol. Prev. Card. Rehabil. Exerc. Physiol. 16(4): 464–471. doi: 10.1097/HJR.0b013e32832b38be.

MacEneaney, O.J., Kushner, E.J., Van Guilder, G.P., Greiner, J.J., Stauffer, B.L., and DeSouza, C.A. 2009. Endothelial progenitor cell number and colony-forming capacity in overweight and obese adults. Int. J. Obes. 2005 33(2): 219–225. doi: 10.1038/ijo.2008.262.

Makin, A.J., Blann, A.D., Chung, N.A.Y., Silverman, S.H., and Lip, G.Y.H. 2004. Assessment of endothelial damage in atherosclerotic vascular disease by quantification of circulating endothelial cells. Relationship with von Willebrand factor and tissue factor. Eur. Heart J. 25(5): 371–376. doi: 10.1016/j.ehj.2003.04.001.

Mancuso, P., Burlini, A., Pruneri, G., Goldhirsch, A., Martinelli, G., and Bertolini, F. 2001. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 97(11): 3658–3661.

Manfredini, F., Rigolin, G.M., Malagoni, A.M., Catizone, L., Mandini, S., Sofritti, O., Mauro, E., Soffritti, S., Boari, B., Cuneo, A., Zamboni, P., and Manfredini, R. 2009. Exercise training and endothelial progenitor cells in haemodialysis patients. J. Int. Med. Res. 37(2): 534–540.

Manuel, D.G., Tuna, M., Hennessy, D., Bennett, C., Okhmatovskaia, A., Finès, P., Tanuseputro, P., Tu, J.V., Flanagan, W., and Team, for the S.T. for A.R.T. and the C.C.O.R. 2014. Projections of preventable risks for cardiovascular disease in Canada to 2021: a microsimulation modelling approach. Can. Med. Assoc. Open Access J. 2(2): E94–E101. doi: 10.9778/cmajo.2012-0015.

McMahan, C.A., Gidding, S.S., Malcom, G.T., Tracy, R.E., Strong, J.P., McGill, H.C., and Pathobiological Determinants of Atherosclerosis in Youth Research Group. 2006. Pathobiological determinants of atherosclerosis in youth risk scores are associated with early and advanced atherosclerosis. Pediatrics 118(4): 1447–1455. doi: 10.1542/peds.2006-0970.

Medina, R.J., O'Neill, C.L., Sweeney, M., Guduric-Fuchs, J., Gardiner, T.A., Simpson, D.A., and Stitt, A.W. 2010. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell

populations with different identities. BMC Med. Genomics 3: 18. doi: 10.1186/1755-8794-3-18.

Mendelsohn, M.E., and Karas, R.H. 1999. The protective effects of estrogen on the cardiovascular system. N. Engl. J. Med. 340(23): 1801–1811. doi: 10.1056/NEJM199906103402306.

Meyer, A.A., Kundt, G., Steiner, M., Schuff-Werner, P., and Kienast, W. 2006. Impaired flow-mediated vasodilation, carotid artery intima-media thickening, and elevated endothelial plasma markers in obese children: the impact of cardiovascular risk factors. Pediatrics 117(5): 1560–1567. doi: 10.1542/peds.2005-2140.

Mezzani, A., Grassi, B., Jones, A.M., Giordano, A., Corrà, U., Porcelli, S., Della Bella, S., Taddeo, A., and Giannuzzi, P. 2013. Speeding of pulmonary VO2 on-kinetics by light-to-moderate-intensity aerobic exercise training in chronic heart failure: clinical and pathophysiological correlates. Int. J. Cardiol. 167(5): 2189–2195. doi: 10.1016/j.ijcard.2012.05.124.

Mirwald, R.L., Baxter-Jones, A.D., Bailey, D.A., and Beunen, G.P. 2002. An assessment of maturity from anthropometric measurements. Med. Sci. Sports. Exerc. 34(4): 689–94.

Möbius-Winkler, S., Hilberg, T., Menzel, K., Golla, E., Burman, A., Schuler, G., and Adams, V. 2009. Time-dependent mobilization of circulating progenitor cells during strenuous exercise in healthy individuals. J. Appl. Physiol. Bethesda Md 1985 107(6): 1943–1950. doi: 10.1152/japplphysiol.00532.2009.

Müller-Ehmsen, J., Braun, D., Schneider, T., Pfister, R., Worm, N., Wielckens, K., Scheid, C., Frommolt, P., and Flesch, M. 2008. Decreased number of circulating progenitor cells in obesity: beneficial effects of weight reduction. Eur. Heart J. 29(12): 1560–1568. doi: 10.1093/eurheartj/ehn213.

Mutin, M., Canavy, I., Blann, A., Bory, M., Sampol, J., and Dignat-George, F. 1999. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. Blood 93(9): 2951–2958.

Mutunga, M., Fulton, B., Bullock, R., Batchelor, A., Gascoigne, A., Gillespie, J.I., and Baudouin, S.V. 2001. Circulating endothelial cells in

patients with septic shock. Am. J. Respir. Crit. Care Med. 163(1): 195–200. doi: 10.1164/ajrccm.163.1.9912036.

Myers, J., McAuley, P., Lavie, C.J., Despres, J.-P., Arena, R., and Kokkinos, P. 2015. Physical Activity and Cardiorespiratory Fitness as Major Markers of Cardiovascular Risk: Their Independent and Interwoven Importance to Health Status. Prog. Cardiovasc. Dis. 57(4): 306–314. doi: 10.1016/j.pcad.2014.09.011.

Nguyen, T., Obeid, J., Ploeger, H.E., Takken, T., Pedder, L., and Timmons, B.W. 2012. Inflammatory and growth factor response to continuous and intermittent exercise in youth with cystic fibrosis. J. Cyst. Fibros. Off. J. Eur. Cyst. Fibros. Soc. 11(2): 108–118. doi: 10.1016/j.jcf.2011.10.001.

Nissen, S.E., Gurley, J.C., Grines, C.L., Booth, D.C., McClure, R., Berk, M., Fischer, C., and DeMaria, A.N. 1991. Intravascular ultrasound assessment of lumen size and wall morphology in normal subjects and patients with coronary artery disease. Circulation 84(3): 1087–1099.

Ogden, C.L., Kuczmarski, R.J., Flegal, K.M., Mei, Z., Guo, S., Wei, R., Grummer-Strawn, L.M., Curtin, L.R., Roche, A.F., and Johnson, C.L. 2002. Centers for Disease Control and Prevention 2000 growth charts for the United States: improvements to the 1977 National Center for Health Statistics version. Pediatrics 109(1): 45–60.

Oguey, D., George, P.W., and Rüegg, C. 2000. Disruption of integrin-dependent adhesion and survival of endothelial cells by recombinant adenovirus expressing isolated beta integrin cytoplasmic domains. Gene Ther. 7(15): 1292–1303. doi: 10.1038/sj.gt.3301236.

O'Sullivan, S.E. 2003. The effects of exercise training on markers of endothelial function in young healthy men. Int. J. Sports Med. 24(6): 404–409. doi: 10.1055/s-2003-41183.

Pagidipati, N.J., and Gaziano, T.A. 2013. Estimating Deaths From Cardiovascular Disease: A Review of Global Methodologies of Mortality Measurement. Circulation 127(6): 749–756. doi: 10.1161/CIRCULATIONAHA.112.128413.

Paleolog, E. 2005. It's all in the blood: circulating endothelial progenitor cells link synovial vascularity with cardiovascular mortality in

rheumatoid arthritis? Arthritis Res. Ther. 7(6): 270–272. doi: 10.1186/ar1850.

Palmefors, H., DuttaRoy, S., Rundqvist, B., and Börjesson, M. 2014. The effect of physical activity or exercise on key biomarkers in atherosclerosis--a systematic review. Atherosclerosis 235(1): 150–161. doi: 10.1016/j.atherosclerosis.2014.04.026.

Papayannopoulou, T. 2004. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 103(5): 1580–1585. doi: 10.1182/blood-2003-05-1595.

Park, J.-H., Miyashita, M., Kwon, Y.-C., Park, H.-T., Kim, E.-H., Park, J.-K., Park, K.-B., Yoon, S.-R., Chung, J.-W., Nakamura, Y., and Park, S.-K. 2012. A 12-week after-school physical activity programme improves endothelial cell function in overweight and obese children: a randomised controlled study. BMC Pediatr. 12: 111. doi: 10.1186/1471-2431-12-111.

Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z., Lane, W.J., Williams, M., Oz, M.C., Hicklin, D.J., Witte, L., Moore, M.A., and Rafii, S. 2000. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 95(3): 952–958.

Van Pel, M., van Os, R., Velders, G.A., Hagoort, H., Heegaard, P.M.H., Lindley, I.J.D., Willemze, R., and Fibbe, W.E. 2006. Serpina1 is a potent inhibitor of IL-8-induced hematopoietic stem cell mobilization. Proc. Natl. Acad. Sci. U. S. A. 103(5): 1469–1474. doi: 10.1073/pnas.0510192103.

Pelosi, E., Valtieri, M., Coppola, S., Botta, R., Gabbianelli, M., Lulli, V., Marziali, G., Masella, B., Müller, R., Sgadari, C., Testa, U., Bonanno, G., and Peschle, C. 2002. Identification of the hemangioblast in postnatal life. Blood 100(9): 3203–3208. doi: 10.1182/blood-2002-05-1511.

Peña, A.S., Couper, J.J., Harrington, J., Gent, R., Fairchild, J., Tham, E., and Baghurst, P. 2012. Hypoglycemia, but not glucose variability, relates to vascular function in children with type 1 diabetes. Diabetes Technol. Ther. 14(6): 457–462. doi: 10.1089/dia.2011.0229.

Pitchford, S.C., Furze, R.C., Jones, C.P., Wengner, A.M., and Rankin, S.M. 2009. Differential mobilization of subsets of progenitor cells from the bone marrow. Cell Stem Cell 4(1): 62–72. doi: 10.1016/j.stem.2008.10.017.

Ploeger, H., Obeid, J., Nguyen, T., Takken, T., Issenman, R., de Greef, M., and Timmons, B. 2012. Exercise and inflammation in pediatric Crohn's disease. Int. J. Sports Med. 33(8): 671–679. doi: 10.1055/s-0032-1304323.

Pool, E.H., and Dunlop, G.R. 1934. Cancer Cells in the Blood Stream. Am. J. Cancer 21(1): 99–102. doi: 10.1158/ajc.1934.99.

Povsic, T.J., Sloane, R., Zhou, J., Pieper, C.F., Pearson, M.P., Peterson, E.D., Green, J.B., Cohen, H.J., and Morey, M.C. 2013. Lower levels of circulating progenitor cells are associated with low physical function and performance in elderly men with impaired glucose tolerance: a pilot substudy from the VA Enhanced Fitness trial. J. Gerontol. A. Biol. Sci. Med. Sci. 68(12): 1559–1566. doi: 10.1093/gerona/glt067.

Prince, S.A., Adamo, K.B., Hamel, M.E., Hardt, J., Gorber, S.C., and Tremblay, M. 2008. A comparison of direct versus self-report measures for assessing physical activity in adults: a systematic review. Int. J. Behav. Nutr. Phys. Act. 5(1): 56. doi: 10.1186/1479-5868-5-56.

Raitakari, O.T., Juonala, M., Kähönen, M., Taittonen, L., Laitinen, T., Mäki-Torkko, N., Järvisalo, M.J., Uhari, M., Jokinen, E., Rönnemaa, T., Akerblom, H.K., and Viikari, J.S.A. 2003. Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. JAMA 290(17): 2277–2283. doi: 10.1001/jama.290.17.2277.

Rajagopalan, S., Somers, E.C., Brook, R.D., Kehrer, C., Pfenninger, D., Lewis, E., Chakrabarti, A., Richardson, B.C., Shelden, E., McCune, W.J., and Kaplan, M.J. 2004. Endothelial cell apoptosis in systemic lupus erythematosus: a common pathway for abnormal vascular function and thrombosis propensity. Blood 103(10): 3677–3683. doi: 10.1182/blood-2003-09-3198.

Ratajczak, M.Z., Majka, M., Kucia, M., Drukala, J., Pietrzkowski, Z., Peiper, S., and Janowska-Wieczorek, A. 2003. Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle

progenitors in bone marrow and hematopoietic stem/progenitor cells in muscles. Stem Cells Dayt. Ohio 21(3): 363–371. doi: 10.1634/stemcells.21-3-363.

Rauscher, F.M., Goldschmidt-Clermont, P.J., Davis, B.H., Wang, T., Gregg, D., Ramaswami, P., Pippen, A.M., Annex, B.H., Dong, C., and Taylor, D.A. 2003. Aging, progenitor cell exhaustion, and atherosclerosis. Circulation 108(4): 457–463. doi: 10.1161/01.CIR.0000082924.75945.48.

Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. 1994. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J. Cell Biol. 127(2): 537–546.

Rehman, J., Li, J., Parvathaneni, L., Karlsson, G., Panchal, V.R., Temm, C.J., Mahenthiran, J., and March, K.L. 2004. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. J. Am. Coll. Cardiol. 43(12): 2314–2318. doi: 10.1016/j.jacc.2004.02.049.

Ribeiro, F., Ribeiro, I.P., Alves, A.J., do Céu Monteiro, M., Oliveira, N.L., Oliveira, J., Amado, F., Remião, F., and Duarte, J.A. 2013. Effects of exercise training on endothelial progenitor cells in cardiovascular disease: a systematic review. Am. J. Phys. Med. Rehabil. Assoc. Acad. Physiatr. 92(11): 1020–1030. doi: 10.1097/PHM.0b013e31829b4c4f.

Rizzo, N.S., Ruiz, J.R., Hurtig-Wennlöf, A., Ortega, F.B., and Sjöström, M. 2007. Relationship of physical activity, fitness, and fatness with clustered metabolic risk in children and adolescents: the European youth heart study. J. Pediatr. 150(4): 388–394. doi: 10.1016/j.jpeds.2006.12.039.

Roberts, K.C., Shields, M., de Groh, M., Aziz, A., and Gilbert, J.-A. 2012. Overweight and obesity in children and adolescents: results from the 2009 to 2011 Canadian Health Measures Survey. Health Rep. 23(3): 37–41.

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362(6423): 801–809. doi: 10.1038/362801a0.

Ross, R. 1995. Cell biology of atherosclerosis. Annu. Rev. Physiol. 57: 791–804. doi: 10.1146/annurev.ph.57.030195.004043.

Rowland, T.W. 1996. Developmental Exercise Physiology. Human Kinetics.

Rüegg, C., Yilmaz, A., Bieler, G., Bamat, J., Chaubert, P., and Lejeune, F.J. 1998. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. Nat. Med. 4(4): 408–414.

Rusak, M., Radzikowska, U., Glowinska-Olszewska, B., Dobrenko, E., Piotrowska-Jastrzebska, J., Dabrowska, M., Bodzenta-Lukaszyk, A., Bossowski, A., and Moniuszko, M. 2015. Endothelial progenitor cell levels in juvenile idiopathic arthritis patients; effects of anti-inflammatory therapies. Pediatr. Rheumatol. Online J. 13(1): 6. doi: 10.1186/s12969-015-0001-4.

Sandri, M., Beck, E.B., Adams, V., Gielen, S., Lenk, K., Höllriegel, R., Mangner, N., Linke, A., Erbs, S., Möbius-Winkler, S., Scheinert, D., Hambrecht, R., and Schuler, G. 2011. Maximal exercise, limb ischemia, and endothelial progenitor cells. Eur. J. Cardiovasc. Prev. Rehabil. Off. J. Eur. Soc. Cardiol. Work. Groups Epidemiol. Prev. Card. Rehabil. Exerc. Physiol. 18(1): 55–64. doi: 10.1097/HJR.0b013e32833ba654.

Sarto, P., Balducci, E., Balconi, G., Fiordaliso, F., Merlo, L., Tuzzato, G., Pappagallo, G.L., Frigato, N., Zanocco, A., Forestieri, C., Azzarello, G., Mazzucco, A., Valenti, M.T., Alborino, F., Noventa, D., Vinante, O., Pascotto, P., Sartore, S., Dejana, E., and Latini, R. 2007. Effects of exercise training on endothelial progenitor cells in patients with chronic heart failure. J. Card. Fail. 13(9): 701–708. doi: 10.1016/j.cardfail.2007.06.722.

Scalone, G., De Caterina, A., Leone, A.M., Tritarelli, A., Mollo, R., Pinnacchio, G., D'Amario, D., Lanza, G.A., and Crea, F. 2013. Effect of exercise on circulating endothelial progenitor cells in microvascular angina. Circ. J. Off. J. Jpn. Circ. Soc. 77(7): 1777–1782.

Schier, R., El-Zein, R., Cortes, A., Liu, M., Collins, M., Rafat, N., Teschendorf, P., Wu, H.-K., Heymach, J., Mehran, R., and Riedel, B. 2014. Endothelial progenitor cell mobilization by preoperative exercise: a bone marrow response associated with postoperative outcome. Br. J. Anaesth. 113(4): 652–660. doi: 10.1093/bja/aeu135.

Schisterman, E.F., Mumford, S.L., and Sjaarda, L.A. 2014. Failure to consider the menstrual cycle phase may cause misinterpretation of clinical and research findings of cardiometabolic biomarkers in premenopausal women. Epidemiol. Rev. 36: 71–82. doi: 10.1093/epirev/mxt007.

Schlager, O., Giurgea, A., Schuhfried, O., Seidinger, D., Hammer, A., Gröger, M., Fialka-Moser, V., Gschwandtner, M., Koppensteiner, R., and Steiner, S. 2011. Exercise training increases endothelial progenitor cells and decreases asymmetric dimethylarginine in peripheral arterial disease: a randomized controlled trial. Atherosclerosis 217(1): 240–248. doi: 10.1016/j.atherosclerosis.2011.03.018.

Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., Tschöpe, C., Schultheiss, H.-P., Zeiher, A.M., and Dimmeler, S. 2010. Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. PloS One 5(11): e13790. doi: 10.1371/journal.pone.0013790.

Schmidt-Lucke, C., Rössig, L., Fichtlscherer, S., Vasa, M., Britten, M., Kämper, U., Dimmeler, S., and Zeiher, A.M. 2005. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 111(22): 2981–2987. doi: 10.1161/CIRCULATIONAHA.104.504340.

Shaffer, R.G., Greene, S., Arshi, A., Supple, G., Bantly, A., Moore, J.S., and Mohler, E.R. 2006a. Flow cytometric measurement of circulating endothelial cells: the effect of age and peripheral arterial disease on baseline levels of mature and progenitor populations. Cytometry B Clin. Cytom. 70(2): 56–62. doi: 10.1002/cyto.b.20085.

Shaffer, R.G., Greene, S., Arshi, A., Supple, G., Bantly, A., Moore, J.S., Parmacek, M.S., and Mohler, E.R. 2006b. Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease. Vasc. Med. Lond. Engl. 11(4): 219–226.

Shanthi, M., Armstrong, T., Bettcher, D., Branca, F., Lauer, J., Mace, C., Poznyak, V., Riley, L., Da Costa E Silva, V., and Stevens, G. 2014. Global status report on noncommunicable diseases 2014. Available from http://www.who.int/nmh/publications/ncd-status-report-2014/en/ [accessed 7 May 2015].

Shapiro, H.M. 2005. Practical Flow Cytometry. John Wiley & Sons.

Shephard, R., and Vuillemin, A. 2003. Limits to the measurement of habitual physical activity by questionnaires. Br. J. Sports Med. 37(3): 197–206. doi: 10.1136/bjsm.37.3.197.

Shields, M. 2006. Overweight and obesity among children and youth. Health Rep. 17(3): 27–42.

Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Honma, T., Katoh, A., Sasaki, K., Shimada, T., Oike, Y., and Imaizumi, T. 2001. Mobilization of Endothelial Progenitor Cells in Patients With Acute Myocardial Infarction. Circulation 103(23): 2776–2779. doi: 10.1161/hc2301.092122.

Shi, Q., Rafii, S., Wu, M.H., Wijelath, E.S., Yu, C., Ishida, A., Fujita, Y., Kothari, S., Mohle, R., Sauvage, L.R., Moore, M.A., Storb, R.F., and Hammond, W.P. 1998. Evidence for circulating bone marrow-derived endothelial cells. Blood 92(2): 362–367.

Shiroma, E.J., and Lee, I.-M. 2010. Physical Activity and Cardiovascular Health Lessons Learned From Epidemiological Studies Across Age, Gender, and Race/Ethnicity. Circulation 122(7): 743–752. doi: 10.1161/CIRCULATIONAHA.109.914721.

Sidney, L.E., Branch, M.J., Dunphy, S.E., Dua, H.S., and Hopkinson, A. 2014. Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cells Dayt. Ohio 32(6): 1380–1389. doi: 10.1002/stem.1661.

Sieveking, D.P., Buckle, A., Celermajer, D.S., and Ng, M.K.C. 2008. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J. Am. Coll. Cardiol. 51(6): 660–668. doi: 10.1016/j.jacc.2007.09.059.

Silva, J.F.R. da, Rocha, N.G., and Nóbrega, A.C.L. da. 2012. Mobilization of endothelial progenitor cells with exercise in healthy individuals: a systematic review. Arq. Bras. Cardiol. 98(2): 182–191.

De Simone, C., Caldarola, G., Coco, V., Palumbo, S., Pocino, K., Sgambato, A., Maiorino, A., Corbi, M., Sandri, M.T., Vendittelli, F., and Capoluongo, E. 2014. Circulating endothelial cell levels in psoriatic patients and their modification after an anti-TNF-alpha (Etanercept)

treatment. J. Eur. Acad. Dermatol. Venereol. JEADV 28(5): 590–596. doi: 10.1111/jdv.12140.

Solovey, A., Gui, L., Ramakrishnan, S., Steinberg, M.H., and Hebbel, R.P. 1999. Sickle cell anemia as a possible state of enhanced anti-apoptotic tone: survival effect of vascular endothelial growth factor on circulating and unanchored endothelial cells. Blood 93(11): 3824–3830.

Solovey, A., Lin, Y., Browne, P., Choong, S., Wayner, E., and Hebbel, R.P. 1997. Circulating activated endothelial cells in sickle cell anemia. N. Engl. J. Med. 337(22): 1584–1590. doi: 10.1056/NEJM199711273372203.

Stary, H.C. 1989. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. Arterioscler. Dallas Tex 9(1 Suppl): I19–32.

Stelzer, I., Kröpfl, J.M., Fuchs, R., Pekovits, K., Mangge, H., Raggam, R.B., Gruber, H.-J., Prüller, F., Hofmann, P., Truschnig-Wilders, M., Obermayer-Pietsch, B., Haushofer, A.C., Kessler, H.H., and Mächler, P. 2014. Ultra-endurance exercise induces stress and inflammation and affects circulating hematopoietic progenitor cell function. Scand. J. Med. Sci. Sports. doi: 10.1111/sms.12347.

Strijbos, M.H., Rao, C., Schmitz, P.I.M., Kraan, J., Lamers, C.H., Sleijfer, S., Terstappen, L.W.M.M., and Gratama, J.W. 2008. Correlation between circulating endothelial cell counts and plasma thrombomodulin levels as markers for endothelial damage. Thromb. Haemost. 100(4): 642–647.

Sugihara, S., Yamamoto, Y., Matsuura, T., Narazaki, G., Yamasaki, A., Igawa, G., Matsubara, K., Miake, J., Igawa, O., Shigemasa, C., and Hisatome, I. 2007. Age-related BM-MNC dysfunction hampers neovascularization. Mech. Ageing Dev. 128(9): 511–516. doi: 10.1016/j.mad.2007.06.009.

Sutherland, D.R., Anderson, L., Keeney, M., Nayar, R., and Chin-Yee, I. 1996. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J. Hematother. 5(3): 213–226.

Sutherland, D.R., and Keating, A. 1992. The CD34 antigen: structure, biology, and potential clinical applications. J. Hematother. 1(2): 115–129.

Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J.M., and Asahara, T. 1999. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat. Med. 5(4): 434–438. doi: 10.1038/7434.

Tanaka, S., Ueno, T., Sato, F., Chigusa, Y., Kawaguchi-Sakita, N., Kawashima, M., Fujisawa, N., Yoshimura, K., Teramukai, S., Fujiwara, H., Fujita, M., and Toi, M. 2012. Alterations of circulating endothelial cell and endothelial progenitor cell counts around the ovulation. J. Clin. Endocrinol. Metab. 97(11): 4182–4192. doi: 10.1210/jc.2012-1736.

Tanha, T., Wollmer, P., Thorsson, O., Karlsson, M.K., Lindén, C., Andersen, L.B., and Dencker, M. 2011. Lack of physical activity in young children is related to higher composite risk factor score for cardiovascular disease. Acta Paediatr. Oslo Nor. 1992 100(5): 717–721. doi: 10.1111/j.1651-2227.2011.02226.x.

Tepper, O.M., Galiano, R.D., Capla, J.M., Kalka, C., Gagne, P.J., Jacobowitz, G.R., Levine, J.P., and Gurtner, G.C. 2002. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 106(22): 2781–2786.

Thijssen, D.H.J., Vos, J.B., Verseyden, C., van Zonneveld, A.J., Smits, P., Sweep, F.C.G.J., Hopman, M.T.E., and de Boer, H.C. 2006. Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. Aging Cell 5(6): 495–503. doi: 10.1111/j.1474-9726.2006.00242.x.

Thögersen, A.M., Jansson, J.H., Boman, K., Nilsson, T.K., Weinehall, L., Huhtasaari, F., and Hallmans, G. 1998. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. Circulation 98(21): 2241–2247.

Tilling, L., Chowienczyk, P., and Clapp, B. 2009. Progenitors in motion: mechanisms of mobilization of endothelial progenitor cells. Br. J. Clin. Pharmacol. 68(4): 484–492. doi: 10.1111/j.1365-2125.2009.03486.x.

Timmermans, F., Plum, J., Yöder, M.C., Ingram, D.A., Vandekerckhove, B., and Case, J. 2009. Endothelial progenitor cells: identity defined? J. Cell. Mol. Med. 13(1): 87–102. doi: 10.1111/j.1582-4934.2008.00598.x.

Timmons, B.W. 2005. Paediatric exercise immunology: health and clinical applications. Exerc. Immunol. Rev. 11: 108–144.

Timmons, B.W., Tarnopolsky, M.A., and Bar-Or, O. 2004. Immune responses to strenuous exercise and carbohydrate intake in boys and men. Pediatr. Res. 56(2): 227–234. doi: 10.1203/01.PDR.0000132852.29770.C5.

Timmons, B.W., Tarnopolsky, M.A., Snider, D.P., and Bar-Or, O. 2006. Immunological changes in response to exercise: influence of age, puberty, and gender. Med. Sci. Sports Exerc. 38(2): 293–304. doi: 10.1249/01.mss.0000183479.90501.a0.

Tkaczyk, M., Czupryniak, A., Owczarek, D., Lukamowicz, J., and Nowicki, M. 2008. Markers of endothelial dysfunction in children with idiopathic nephrotic syndrome. Am. J. Nephrol. 28(2): 197–202. doi: 10.1159/000110088.

Tongers, J., Roncalli, J.G., and Losordo, D.W. 2010. Role of endothelial progenitor cells during ischemia-induced vasculogenesis and collateral formation. Microvasc. Res. 79(3): 200–206. doi: 10.1016/j.mvr.2010.01.012.

Tortora, G.J., and Grabowski, S.R. 2002. Principles of Anatomy and Physiology, 10th Edition. *In* 10th Edition edition. John Wiley & Sons Canada, Ltd., New York.

Tremblay, M.S., Gray, C.E., Akinroye, K., Harrington, D.M., Katzmarzyk, P.T., Lambert, E.V., Liukkonen, J., Maddison, R., Ocansey, R.T., Onywera, V.O., Prista, A., Reilly, J.J., Rodríguez Martínez, M.P., Sarmiento Duenas, O.L., Standage, M., and Tomkinson, G. 2014. Physical activity of children: a global matrix of grades comparing 15 countries. J. Phys. Act. Health 11 Suppl 1: S113–125. doi: 10.1123/jpah.2014-0177.

Tremblay, M.S., Shields, M., Laviolette, M., Craig, C.L., Janssen, I., and Connor Gorber, S. 2010. Fitness of Canadian children and youth: results from the 2007-2009 Canadian Health Measures Survey. Health Rep. 21(1): 7–20.

Tremblay, M.S., Warburton, D.E.R., Janssen, I., Paterson, D.H., Latimer, A.E., Rhodes, R.E., Kho, M.E., Hicks, A., Leblanc, A.G., Zehr, L., Murumets, K., and Duggan, M. 2011. New Canadian physical activity guidelines. Appl. Physiol. Nutr. Metab. Physiol. Appliquée Nutr. Métabolisme 36(1): 36–46; 47–58. doi: 10.1139/H11-009.

Urbich, C., Aicher, A., Heeschen, C., Dernbach, E., Hofmann, W.K., Zeiher, A.M., and Dimmeler, S. 2005a. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J. Mol. Cell. Cardiol. 39(5): 733–742. doi: 10.1016/j.yjmcc.2005.07.003.

Urbich, C., and Dimmeler, S. 2004. Endothelial progenitor cells: characterization and role in vascular biology. Circ. Res. 95(4): 343–353. doi: 10.1161/01.RES.0000137877.89448.78.

Urbich, C., Heeschen, C., Aicher, A., Dernbach, E., Zeiher, A.M., and Dimmeler, S. 2003. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 108(20): 2511–2516. doi: 10.1161/01.CIR.0000096483.29777.50.

Urbich, C., Heeschen, C., Aicher, A., Sasaki, K., Bruhl, T., Farhadi, M.R., Vajkoczy, P., Hofmann, W.K., Peters, C., Pennacchio, L.A., Abolmaali, N.D., Chavakis, E., Reinheckel, T., Zeiher, A.M., and Dimmeler, S. 2005b. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat. Med. 11(2): 206–213. doi: 10.1038/nm1182.

Van Craenenbroeck, E.M.F., Conraads, V.M.A., Van Bockstaele, D.R., Haine, S.E., Vermeulen, K., Van Tendeloo, V.F., Vrints, C.J., and Hoymans, V.Y. 2008a. Quantification of circulating endothelial progenitor cells: a methodological comparison of six flow cytometric approaches. J. Immunol. Methods 332(1-2): 31–40. doi: 10.1016/j.jim.2007.12.006.

Van Craenenbroeck, E.M.F., Vrints, C.J., Haine, S.E., Vermeulen, K., Goovaerts, I., Van Tendeloo, V.F.I., Hoymans, V.Y., and Conraads, V.M.A. 2008b. A maximal exercise bout increases the number of

circulating CD34+/KDR+ endothelial progenitor cells in healthy subjects. Relation with lipid profile. J. Appl. Physiol. Bethesda Md 1985 104(4): 1006–1013. doi: 10.1152/japplphysiol.01210.2007.

Van Craenenbroeck, E.M., Beckers, P.J., Possemiers, N.M., Wuyts, K., Frederix, G., Hoymans, V.Y., Wuyts, F., Paelinck, B.P., Vrints, C.J., and Conraads, V.M. 2010a. Exercise acutely reverses dysfunction of circulating angiogenic cells in chronic heart failure. Eur. Heart J. 31(15): 1924–1934. doi: 10.1093/eurheartj/ehq058.

Van Craenenbroeck, E.M., Bruyndonckx, L., Van Berckelaer, C., Hoymans, V.Y., Vrints, C.J., and Conraads, V.M. 2011. The effect of acute exercise on endothelial progenitor cells is attenuated in chronic heart failure. Eur. J. Appl. Physiol. 111(9): 2375–2379. doi: 10.1007/s00421-011-1843-1.

Van Craenenbroeck, E.M., and Conraads, V.M. 2013. Mending injured endothelium in chronic heart failure: a new target for exercise training. Int. J. Cardiol. 166(2): 310–314. doi: 10.1016/j.ijcard.2012.04.106.

Van Craenenbroeck, E.M., Van Craenenbroeck, A.H., van Ierssel, S., Bruyndonckx, L., Hoymans, V.Y., Vrints, C.J., and Conraads, V.M. 2013. Quantification of circulating CD34+/KDR+/CD45dim endothelial progenitor cells: analytical considerations. Int. J. Cardiol. 167(5): 1688–1695. doi: 10.1016/j.ijcard.2012.10.047.

Van Craenenbroeck, E.M., Denollet, J., Paelinck, B.P., Beckers, P., Possemiers, N., Hoymans, V.Y., Vrints, C.J., and Conraads, V.M. 2009. Circulating CD34+/KDR+ endothelial progenitor cells are reduced in chronic heart failure patients as a function of Type D personality. Clin. Sci. Lond. Engl. 1979 117(4): 165–172. doi: 10.1042/CS20080564.

Van Craenenbroeck, E.M., Hoymans, V.Y., Beckers, P.J., Possemiers, N.M., Wuyts, K., Paelinck, B.P., Vrints, C.J., and Conraads, V.M. 2010b. Exercise training improves function of circulating angiogenic cells in patients with chronic heart failure. Basic Res. Cardiol. 105(5): 665–676. doi: 10.1007/s00395-010-0105-4.

Vasa, M., Fichtlscherer, S., Adler, K., Aicher, A., Martin, H., Zeiher, A.M., and Dimmeler, S. 2001a. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 103(24): 2885–2890.

Vasa, M., Fichtlscherer, S., Aicher, A., Adler, K., Urbich, C., Martin, H., Zeiher, A.M., and Dimmeler, S. 2001b. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ. Res. 89(1): E1–7.

Verma, S., Kuliszewski, M.A., Li, S.-H., Szmitko, P.E., Zucco, L., Wang, C.-H., Badiwala, M.V., Mickle, D.A.G., Weisel, R.D., Fedak, P.W.M., Stewart, D.J., and Kutryk, M.J.B. 2004. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. Circulation 109(17): 2058–2067. doi: 10.1161/01.CIR.0000127577.63323.24.

Vlahos, A.P., Theocharis, P., Bechlioulis, A., Naka, K.K., Vakalis, K., Papamichael, N.D., Alfantaki, S., Gartzonika, K., Mavridis, A., Michalis, L.K., and Siamopoulou, A. 2011. Changes in vascular function and structure in juvenile idiopathic arthritis. Arthritis Care Res. 63(12): 1736–1744. doi: 10.1002/acr.20613.

Volaklis, K.A., Tokmakidis, S.P., and Halle, M. 2013. Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and diseased patients. Clin. Res. Cardiol. Off. J. Ger. Card. Soc. 102(4): 249–257. doi: 10.1007/s00392-012-0517-2.

Walther, C., Adams, V., Bothur, I., Drechsler, K., Fikenzer, S., Sonnabend, M., Bublitz, B., Körner, A., Erbs, S., Busse, M., and Schuler, G. 2008. Increasing physical education in high school students: effects on concentration of circulating endothelial progenitor cells. Eur. J. Cardiovasc. Prev. Rehabil. Off. J. Eur. Soc. Cardiol. Work. Groups Epidemiol. Prev. Card. Rehabil. Exerc. Physiol. 15(4): 416–422. doi: 10.1097/HJR.0b013e3282fb2df1.

Walther, C., Gaede, L., Adams, V., Gelbrich, G., Leichtle, A., Erbs, S., Sonnabend, M., Fikenzer, K., Körner, A., Kiess, W., Bruegel, M., Thiery, J., and Schuler, G. 2009. Effect of increased exercise in school children on physical fitness and endothelial progenitor cells: a prospective randomized trial. Circulation 120(22): 2251–2259. doi: 10.1161/CIRCULATIONAHA.109.865808.

Wang, C., Li, H., Fu, P., Zhang, S., and Xiu, R. 2005. Serum C-reactive protein and circulating endothelial cells in patients with acute myocardial infarction. Clin. Hemorheol. Microcirc. 32(4): 287–296.

Weinsaft, J.W., and Edelberg, J.M. 2001. Aging-associated changes in vascular activity: a potential link to geriatric cardiovascular disease. Am. J. Geriatr. Cardiol. 10(6): 348–354.

Werner, N., Kosiol, S., Schiegl, T., Ahlers, P., Walenta, K., Link, A., Böhm, M., and Nickenig, G. 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. N. Engl. J. Med. 353(10): 999–1007. doi: 10.1056/NEJMoa043814.

Werner, N., and Nickenig, G. 2006. Clinical and therapeutical implications of EPC biology in atherosclerosis. J. Cell. Mol. Med. 10(2): 318–332.

Wiman, B., Andersson, T., Hallqvist, J., Reuterwall, C., Ahlbom, A., and deFaire, U. 2000. Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. Arterioscler. Thromb. Vasc. Biol. 20(8): 2019–2023.

Wojakowski, W., and Gminski, J. 2001. Soluble ICAM-1, VCAM-1 and E-selectin in children from families with high risk of atherosclerosis. Int. J. Mol. Med. 7(2): 181–185.

Woywodt, A., Bahlmann, F.H., De Groot, K., Haller, H., and Haubitz, M. 2002. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. Nephrol. Dial. Transplant. Off. Publ. Eur. Dial. Transpl. Assoc. - Eur. Ren. Assoc. 17(10): 1728–1730.

Woywodt, A., Blann, A.D., Kirsch, T., Erdbruegger, U., Banzet, N., Haubitz, M., and Dignat-George, F. 2006. Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. J. Thromb. Haemost. JTH 4(3): 671–677. doi: 10.1111/j.1538-7836.2006.01794.x.

Woywodt, A., Goldberg, C., Scheer, J., Regelsberger, H., Haller, H., and Haubitz, M. 2004. An improved assay for enumeration of circulating endothelial cells. Ann. Hematol. 83(8): 491–494. doi: 10.1007/s00277-004-0878-3.

Woywodt, A., Streiber, F., de Groot, K., Regelsberger, H., Haller, H., and Haubitz, M. 2003. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. Lancet 361(9353): 206–210. doi: 10.1016/S0140-6736(03)12269-6.

Wright, D.E., Bowman, E.P., Wagers, A.J., Butcher, E.C., and Weissman, I.L. 2002. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J. Exp. Med. 195(9): 1145–1154.

Wright, H.L., Moots, R.J., Bucknall, R.C., and Edwards, S.W. 2010. Neutrophil function in inflammation and inflammatory diseases. Rheumatology 49(9): 1618–1631. doi: 10.1093/rheumatology/keq045.

Xia, W.-H., Li, J., Su, C., Yang, Z., Chen, L., Wu, F., Zhang, Y.-Y., Yu, B.-B., Qiu, Y.-X., Wang, S.-M., and Tao, J. 2012. Physical exercise attenuates age-associated reduction in endothelium-reparative capacity of endothelial progenitor cells by increasing CXCR4/JAK-2 signaling in healthy men. Aging Cell 11(1): 111–119. doi: 10.1111/j.1474-9726.2011.00758.x.

Yang, Z., Xia, W.-H., Su, C., Wu, F., Zhang, Y.-Y., Xu, S.-Y., Liu, X., Zhang, X.-Y., Ou, Z.-J., Lai, G.-H., Liao, X.-X., Jin, Y.-F., and Tao, J. 2013. Regular exercise-induced increased number and activity of circulating endothelial progenitor cells attenuates age-related decline in arterial elasticity in healthy men. Int. J. Cardiol. 165(2): 247–254. doi: 10.1016/j.ijcard.2011.08.055.

Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J., and Buck, D.W. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90(12): 5002–5012.

Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T., and Ingram, D.A. 2007. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109(5): 1801–1809. doi: 10.1182/blood-2006-08-043471.

Yurchenco, P.D. 2011. Basement Membranes: Cell Scaffoldings and Signaling Platforms. Cold Spring Harb. Perspect. Biol. 3(2): a004911. doi: 10.1101/cshperspect.a004911.

Zaldivar, F., Eliakim, A., Radom-Aizik, S., Leu, S.-Y., and Cooper, D.M. 2007. The effect of brief exercise on circulating CD34+ stem cells in early and late pubertal boys. Pediatr. Res. 61(4): 491–495. doi: 10.1203/pdr.0b013e3180332d36.

Zampetaki, A., Kirton, J.P., and Xu, Q. 2008. Vascular repair by endothelial progenitor cells. Cardiovasc. Res. 78(3): 413–421. doi: 10.1093/cvr/cvn081.

Zheng, H., Fu, G., Dai, T., and Huang, H. 2007. Migration of endothelial progenitor cells mediated by stromal cell-derived factor-1alpha/CXCR4 via PI3K/Akt/eNOS signal transduction pathway. J. Cardiovasc. Pharmacol. 50(3): 274–280. doi: 10.1097/FJC.0b013e318093ec8f.

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., and Schaper, W. 2004. Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ. Res. 94(2): 230–238. doi: 10.1161/01.RES.0000110419.50982.1C.

Ziegler, B.L., Valtieri, M., Porada, G.A., De Maria, R., Müller, R., Masella, B., Gabbianelli, M., Casella, I., Pelosi, E., Bock, T., Zanjani, E.D., and Peschle, C. 1999. KDR receptor: a key marker defining hematopoietic stem cells. Science 285(5433): 1553–1558.

APPENDIX A: DESCRIPTION OF META-ANALYSIS

A review of the literature and a series of meta-analyses were designed to address the following questions in human adults, and separately in youth:

1. Is there a relationship between fitness and resting EPCs and/or CECs in healthy individuals and those with chronic disease?
2. Is there a relationship between physical activity and resting EPCs and/or CECs in healthy individuals and those with chronic disease?
3. What is the effect of an acute bout of exercise on EPCs and/or CECs in healthy individuals?
4. Does the effect of an acute bout of exercise on EPCs and/or CECs differ between healthy individuals and those with chronic disease?

Searches of the PubMed and EMBASE databases were performed using combinations of the keywords “exercise” OR “physical activity” OR “fitness” AND “endothelial progenitor cell” OR “circulating endothelial cell” OR “endothelial cells”. All relevant titles were screened based on their abstracts, and full texts were retrieved for studies involving human participants (adults and children separated) and assessment of EPCs or CECs by flow cytometry. Studies with mixed methods were included, but

only results from flow cytometric analyses were extracted for the meta-analysis. Reference lists from selected studies were examined for any additional studies not captured in the general search. The number of studies examined at each stage of the analysis is presented in the PRISMA flow diagram in Figure A-1.

Full text articles were grouped into one or more of 3 broad categories to address the aforementioned questions: (1) correlations of fitness with EPCs and/or CECs; (2) correlations of physical activity with EPCs and/or CECs; and (3) effects of acute exercise on EPCs and/or CECs. Studies were further divided into subgroups based on the populations: (1) healthy adults; (2) adults with chronic disease; and (3) children and adolescents. After exclusion, the number of studies reporting results in the context of exercise on CECs in adults and youth, as well as those examining EPCs in youth was insufficient for pooling results. As such, data extraction and meta-analyses were only performed on studies examining EPCs in adult populations (study findings summarized in tables A-1 and A-2).

Data extraction for correlation studies included sample size, statistical test performed (Pearson or Spearman), and test statistic (Pearson correlation coefficient or Spearman's rho). Data extraction for acute exercise studies included information on sample size, groups assessed (experimental vs. control groups), exercise protocol (type,

intensity, duration), number of time points assessed, measures of central tendency for EPCs at each time point (mean or median), variance in reported values at each time point (standard deviation, range, interquartile range, or 95% confidence intervals). Because of the differences in blood sampling time points, the meta-analyses performed only included resting or baseline samples compared with post-exercise samples (within 1-hr of exercise completion). All data were extracted from the text or tables of the article; data were not extracted when presented in the form of figures alone.

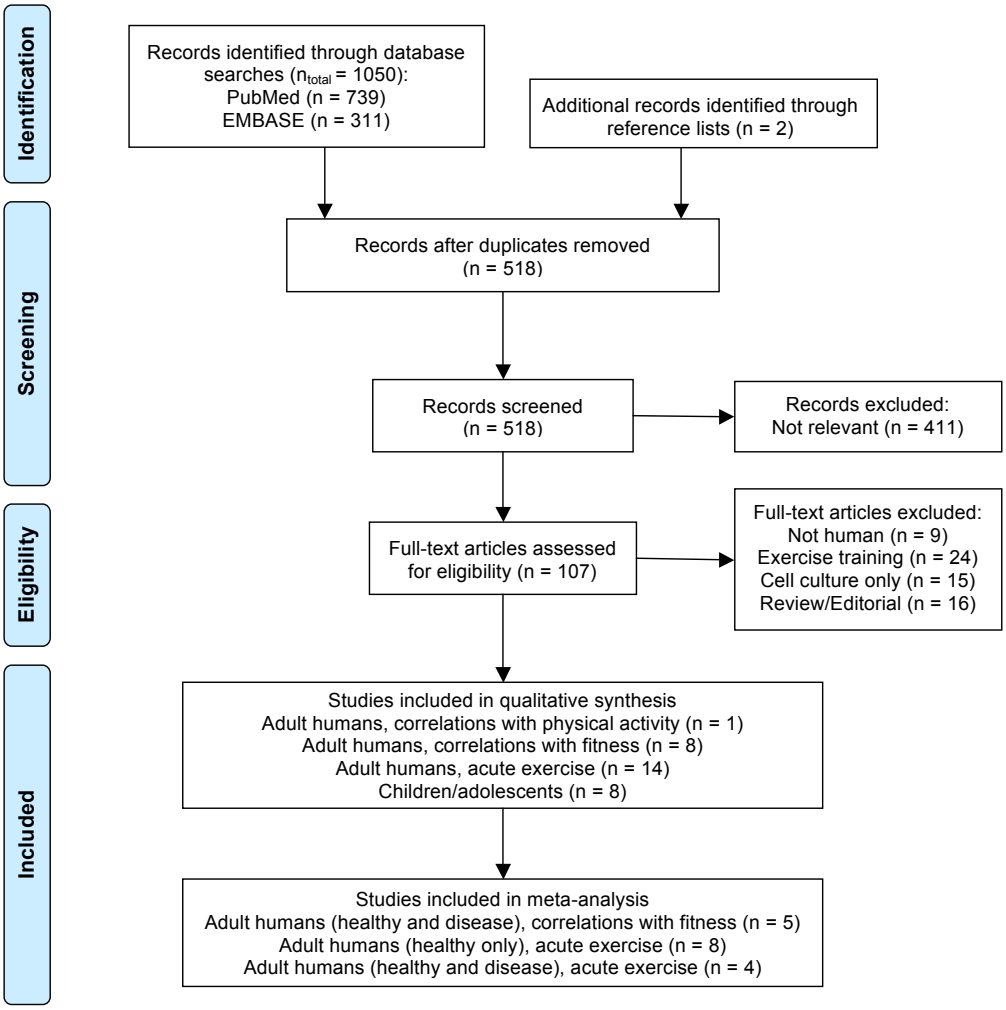

Figure A-1 *PRISMA flow diagram of the database search, screening, and inclusion/exclusion of studies in the meta-analysis (Moher et al. 2009).* After assessing full-text articles, 43 articles were examined for data extraction, of which 17 met the criteria for inclusion in the meta-analyses.

The data extraction process revealed that only 1 of the retrieved full-text articles examined the relationship between EPCs and physical activity, and as such, this was not examined further by meta-analysis. Of the 8 papers assessing correlations of EPCs with fitness, only 5 reported the necessary statistics for inclusion in the meta-analysis. Thus, of the 8

planned meta-analyses (4 questions × 2 age groups), 3 were conducted data from studies examining the relationship between fitness and EPCs in adults (n = 5), the effects of acute exercise on EPCs in healthy adults (n = 8 in healthy adults only, 4 with both healthy and chronic disease – healthy extracted), and differences in exercise-induced changes in EPCs in healthy adults vs. adults with chronic disease (n = 4). It is important to note that when EPCs were examined using multiple definitions in a single study, data from each definition was included in the analysis.

Subgroup analyses were performed in the healthy adults in an effort to reduce the heterogeneity within the pooled data that may be linked to differences in exercise protocols between studies (Figure A-2). Specific exercise intensity (i.e., % of max heart rate or $\dot{V}O_{2max}$) was not always provided, as such, studies were broadly grouped into two distinct exercise stimuli: (1) constant workload, moderate-intensity exercise (n = 6, Figure A-3A), and (2) graded workload, maximal exercise (n = 8, Figure A-3B). A second subgroup analysis was performed using exercise time, since there is some evidence to suggest that EPC response may be more affected by exercise time than intensity (Laufs et al. 2005). For this analysis, studies were grouped as: (1) short protocols (< 20-min, n = 7; Figure 1.3A); (2) mid protocols (20-180 min, n = 4; Figure 1.3B); and (3) long protocols (> 180 min, n = 3; Figure 1.3C).

To examine the effects of acute exercise on EPCs (questions 3 and 4 above), standardized mean differences (i.e., a measure of effect size) were calculated to account for the heterogeneity in selection of monoclonal antibodies and units for reporting EPCs between studies (Borenstein et al. 2009). Standardized mean difference (SMD) was calculated using Hedges g:

$$g = \frac{\bar{x}_{post} - \bar{x}_{pre}}{s_{pooled}}$$

Where $\bar{x}_{post}$ is the mean post-exercise, $\bar{x}_{pre}$ is the mean pre-exercise value, and s_{pooled} is the pooled standard deviation of pre- and post-values, calculated as follows:

$$s_{pooled} = \sqrt{\frac{(n_{post}-1)s_{post}^2 + (n_{pre}-1)s_{pre}^2}{n_{post}+n_{pre}-2}}$$

Where n represents the sample size in each group. For the second analysis comparing EPC responses between disease and control groups, the standard deviation of the difference in post-exercise and pre-exercise values within each group was calculated as:

$$s_{difference} = \sqrt{s_{post}^2 + s_{pre}^2 - 2 \times r \times s_{post} \times s_{pre}}$$

Where s is the reported standard deviation at pre- and post-exercise time points, and r is the correlation between pre- and post-scores. Since these correlations were seldom reported, r was fixed at 0.5. Analysis was repeated with r values of 0.0 and 1.0 to assess the effect on calculated

standard deviation values, which resulted in minimal, non-significant changes in calculated standard deviations. Hedges g values were corrected to account for inherent bias of small samples (Borenstein et al. 2009), and both fixed and random effects models as well as forest plots were generated using MedCalc 15.2.2.

Fixed or random effect model selection was based on assessments of heterogeneity across studies using Cochran's Q and I^2 statistic. A significant Cochran's Q ($p < 0.10$) and moderate I^2 statistic (>40%) were interpreted as significant heterogeneity, resulting in selection of the random effects model (Higgins et al. 2003, Ryan and Cochrane Consumers and Communicaton Review Group 2014). To address the aforementioned questions, pooled standardized mean difference values for the fixed effects or random effects models were interpreted using Cohen's criteria for effect size where values of <0.2 indicate a trivial effect, 0.2 – 0.49 a small effect, 0.5 – 0.79 is a medium effect, and ≥ 0.8 is a large effect (Cohen 1988).

Finally, forest plots were generated using the standardized mean difference or correlation (marker) value and 95% confidence interval (tails) for each study. The size of each marker corresponds to the weight assigned to the study in the overall model. The size of diamond markers used for the fixed and random effects models are indicative of the precision of the estimate (Higgins et al. 2003, Borenstein et al. 2009).

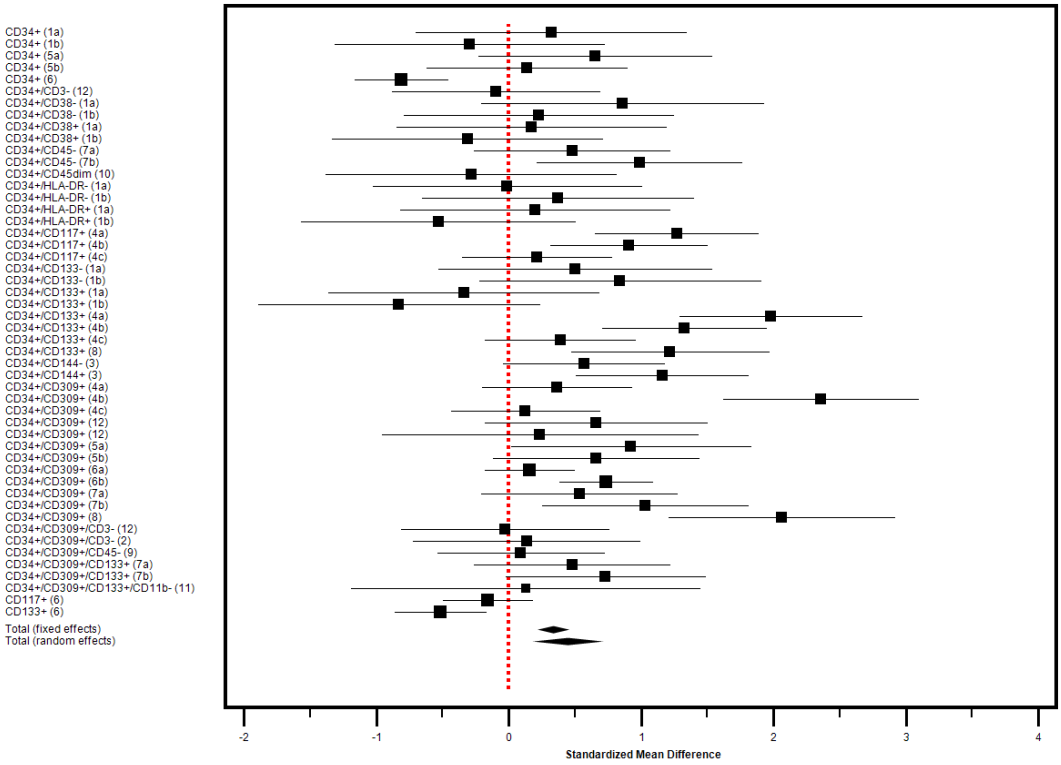

Figure A-2 *Effects of acute exercise on EPCs in healthy adults.* This pooled analysis represents the data from all 12 studies included in the meta-analysis, with no distinction between exercise protocols. Data are sorted by cell definition to illustrate the fact even identical EPC cell definitions demonstrated large variability, suggesting that EPC cell definition alone cannot explain the significant degree of heterogeneity between studies (Q = 223.2, p<0.001; I^2 = 78.1 [lower, upper 95% CI: 71.4, 83.1]). This finding prompted further investigation into the potential effects of different exercise protocols on EPC responses. Despite the heterogeneity, the pooled SMD of the random effects model suggested a small *effect* (SMD_{pooled} = 0.447 [0.246, 0.647], p<0.001), favouring an increase in EPCs with acute exercise. (1) a: Marathon (range: 159 – 254 min), b: half-marathon (range: 69 – 91 min) (Bonsignore et al. 2002); (2) Maximal exercise test on a cycle ergometer (25 W step per 3-min stage) (Adams et al. 2004); (3) Maximal exercise test on a cycle ergometer or treadmill (no additional details provided) (Rehman et al. 2004); (4) a: 30-min running at 100% velocity at anaerobic threshold (~82% $\dot{V}O_{2max}$), b: 30-min running at 80% of velocity at anaerobic threshold (~68% $\dot{V}O_{2max}$), c: 10-min running at 80% of velocity at anaerobic threshold (~68% $\dot{V}O_{2max}$) (Laufs et al. 2005); (5) Maximal exercise test on a cycle ergometer (25 W step per 2-min stage) from a: PBMC samples (n = 11), and b: whole blood samples (n = 14) (Van Craenenbroeck et al. 2008b); (6) Marathon (mean ± SEM: 4 h 11 min ± 4 min), a: EPCs expressed as cells/mL blood, and b: EPCs expressed as a % of $CD309^+$ cells (Adams et al. 2008); (7) 30-min of cycling at a workload equivalent to 80% of lactate threshold in a: South Asian men, and b: white European men (Cubbon et al. 2010); (8) Maximal exercise test on a treadmill (12% incline, 3.5 km/h); (9) Maximal exercise test on a treadmill (Bruce protocol) (Scalone et al. 2013); (10) Ultra-endurance cycling race consisting of 550-km (7,000-m altitude) over 4 days, 8-h competition alternating with 8-h rest (Stelzer et al. 2014); (11) 30-min treadmill running (heart rate maintained >140 bpm) (Chang et al. 2015); (12) Maximal exercise test on a cycle ergometer (ramp protocol, 10 or 20 W/min stages) (Van Craenenbroeck et al. 2010a).

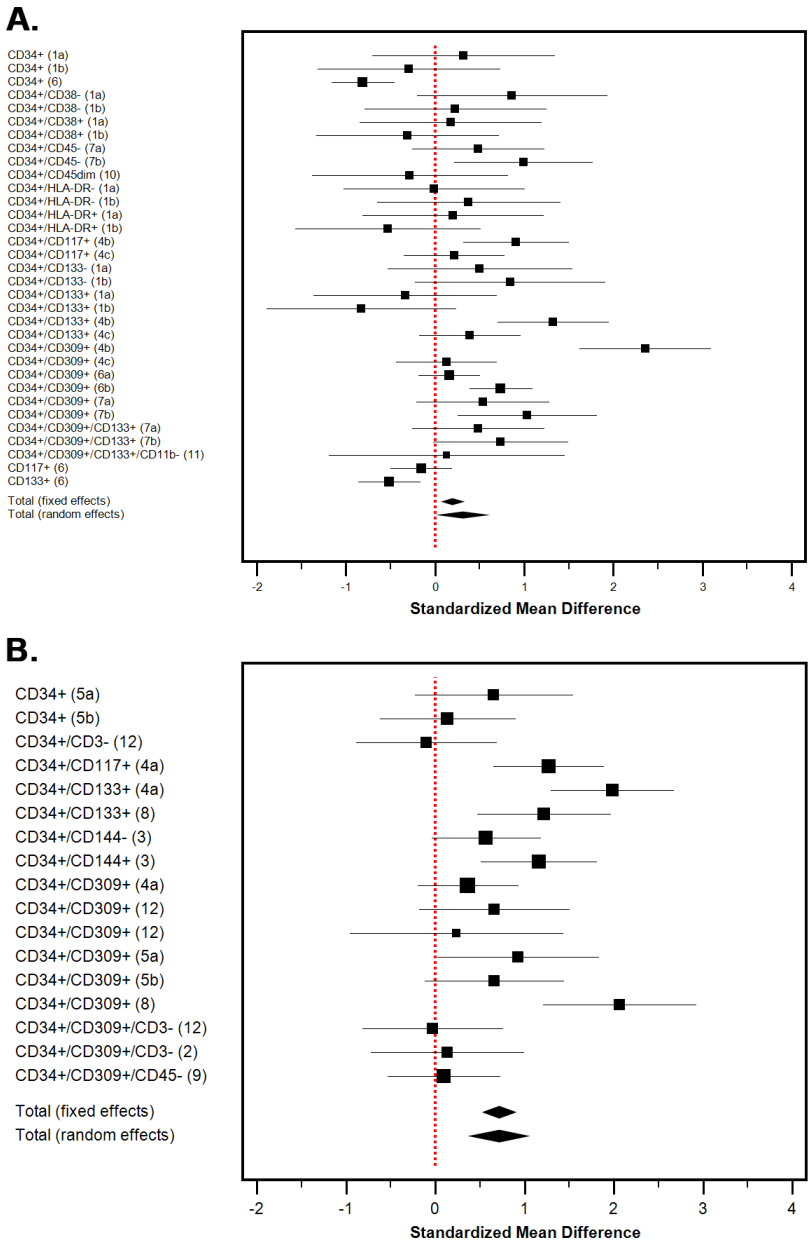

Figure A-3 *Effects of an acute bout of (A) constant workload, moderate-intensity and (B) graded workload, maximal exercise on EPCs in healthy adults.* A significant degree of heterogeneity was observed between studies for both types of exercise (Q_A = 146.6, p<0.001; I^2_A = 78.2 [lower, upper 95% CI: 67.8, 84.2]; Q_B = 51.1, p<0.001; I^2_B = 68.7 [48.3, 81.0]). Both types of exercise led to an increase in EPCs, with the pooled SMD from the random effects model revealing a significant small effect ($SMD_{pooled\text{-}A}$ = 0.309 [0.068, 0.550], p = 0.012) of constant workload exercise, while the graded workload exercise demonstrated a significant medium effect ($SMD_{pooled\text{-}B}$ = 0.714 [0.400, 1.022], p < 0.001). References are identical to Figure A-2.

Table A-1. Summary of selected studies examining the effects of acute exercise on CECs.

Reference	Participants (n [males]; Age; Health status)	Exercise Protocol	CEC Definition	Δ CECs
Mutin et al, 1999	n = 14 [n/s]; 44–85 y; Effort angina	Maximal exercise (cycle ergometer: 30 W increments, 3 min/stage)	Immunomagnetic beads for S-Endo 1; fluorescence microscopy – rosetted cells with ≥10 beads, between 20-50 µm	↔
O'Sullivan, 2003	n_{fit} = 18 [18]; n/s; Healthy $n_{sedentary}$ = 16 [16]; n/s; Healthy	Maximal exercise test (cycle ergometer: 30 W increments, 3 min/stage)	10 µL smears of cresyl violet and vWF stained cells, compared with ECs from rat aortas	↔
Shaffer et al, 2006b	n_{young} = 9 [9]; 33 y; Healthy n_{old} = 13 [13]; 66 y; Healthy $n_{old\text{-}PAD}$ = 15 [15]; 69 y; PAD	Young – maximal exercise (treadmill; Bruce protocol); Old – submaximal exercise (treadmill, Gardner protocol)	Flow cytometry: (1) $CD146^+$ (2) $CD146^+CD34^+$ (3) $CD146^+CD31^+$ (4) $CD146^+CD34^+CD31^+$ (5) $CD34^+CD31^+CD133^-$	↑ $CD146^+$ in young; ↔ for all others
Boos et al, 2008b	n = 31 [26]; 40–75 y; Effort angina	Maximal exercise (treadmill; Bruce protocol)	Immunomagnetic beads for CD146; fluorescence microscopy – rosette cells with ≥4 beads, between 10-50 µm, and Ulex Europeus Lectin$^+$	↑
Möbius-Winkler et al, 2009	n = 28 [28]; 32.4 ± 2.3 y; Healthy	Submaximal exercise (cycle ergometer; 240-min at 70% of anaerobic threshold)	Flow cytometry: $CD146^+$	↔ up to 90-min; ↑ at 120- to 210-min
Sandri et al, 2011	n_H = 17 [16]; 31.3 ± 3.4 y; healthy n_D = 23 [22]; 61.4 ± 2.1 y; POAD	Maximal exercise (treadmill; 3.5 km/h at 12% incline)	Flow cytometry: $CD146^+$	↑ in healthy at 2 h; ↔ in POAD
Schier et al, 2014	n = 60; 60.3 ± 10.3 y; Pre-major thoracic surgery	Maximal exercise (cycle ergometer: increments n/s, 3 min/stage)	Flow cytometry: (1) $CD45^-CD133^-CD146^+$ (2) $CD45^-CD133^-CD31^+$ (3) $CD45^-CD133^-CD146^+CD31^+$	↔ for all definitions

Change in CEC reported for pre- to within 1-hour post-exercise, unless otherwise specified. Age reported as range or mean ± SD. Maximal exercise test time range was 8-12 minutes. ECs – endothelial cells; n/a – not applicable; n/s – not specified; PAD – Peripheral artery disease; PAOD – Peripheral arterial occlusive disease; vWF – von Willebrand Factor.

Table A-2. Summary of selected flow cytometry-based studies examining the effects of acute exercise on EPCs in healthy adults and adults with chronic disease.

Reference	Participants (n [males]; Age; Health status)	Exercise Protocol	EPC Definition	Δ EPCs
Bonsignore et al, 2002	n_{HM} = 8 [8]; Healthy n_{M} = 8 [8]; Healthy Total sample age: 41.3 ± 13.4 y	Half (HM) or full marathon (M)	(1) $CD34^+$ (2) $CD34^+CD38^-$ (3) $CD34^+CD38^+$ (4) $CD34^+HLA\text{-}DR^-$ (5) $CD34^+HLA\text{-}DR^+$ (6) $CD34^+CD133^-$ (7) $CD34^+CD133^+$	↔ for all definitions immediately post-HM and M ↑ $CD34^+$ at morning post-race for HM and M ↑ $CD34^+HLA\text{-}DR^-$ at morning post-race for M
Adams et al, 2004	$n_{Healthy}$ = 11 [n/s]; 59.0 ± 4.4 y; healthy $n_{CAD\text{-}I}$ = 16 [n/s]; 65.0 ± 1.4 y; ischemic CAD $n_{CAD\text{-}NI}$ = 12 [n/s]; 60.0 ± 2.1 y; nonischemic CAD	Maximal exercise (cycle ergometer; 25 W increments, 3 min/stage)	$CD3^-CD34^+CD309^+$	↔ in all 3 groups from 2- to 8-h post-exercise ↑ in ischemic CAD group only at 24- and 48-h post-exercise
Rehman et al, 2004	n = 22 [16]; 54 ± 10 y; Healthy	Symptom-limited exercise (cycle ergometer or treadmill; duration n/s, 9.5 ± 2.4 METs)	$CD133^+$VE-Cadherin$^+$	↑
Laufs et al, 2005	n = 25 [25]; 28.4 ± 6.5 y Healthy	Running exercise: (1) 30-min at 100% of anaerobic threshold (2) 30-min at 80% of anaerobic threshold (3) 10-min at 80% of anaerobic threshold	(1) $CD34^+CD309^+$ (2) $CD34^+CD133^+$ (3) $CD34^+CD117^+$	↑ for all definitions with 30-min at both 100% and 80% of anaerobic threshold ↔ for all definitions with 10-min at 80% of anaerobic threshold
Van Craenenbroeck et al, 2008b	n_1 = 11 [6]; 20 – 30 y; Healthy n_2 = 14 [9]; 20 – 50 y; Healthy	Maximal exercise test (cycle ergometer; 20 W increments, 1 min/stage)	(1) $CD34^+$ (2) $CD34^+CD309^+$	↔ $CD34^+$ in both groups ↑ $CD34^+CD309^+$ in both groups

Reference	Participants (n [males]; Age; Health status)	Exercise Protocol	EPC Definition	Δ EPCs
Adams et al, 2008	n = 68 [n/s]; 57 ± 6 y; Healthy	Marathon	(1) $CD34^+CD309^+$ (2) Fraction of $CD34^+$ expressing $CD309^+$ (3) $CD34^+$ (4) $CD133^+$ (5) $CD117^+$	↔ $CD34^+CD309^+$ ↑ for fraction of $CD34^+$ expressing $CD309^+$ ↓ in all other definitions
Möbius-Winkler et al, 2009	n = 28 [28]; 32.4 ± 2.3 y; Healthy	Submaximal exercise (cycle ergometer; 240-min at 70% of anaerobic threshold)	(1) $CD34^+$ (2) $CD34^+CD309^+$ (3) $CD133^+$ (4) $CD34^+CD309^+$	↑ $CD34^+$ at 180- to 240-min ↑ $CD34^+CD309^+$ at 310- to 240-min ↑ $CD133^+$ at 150- to 210-min ↑ $CD133^+CD309^+$ at 210- to 240-min
Van Craenenbroeck et al, 2010a	$n_{Healthy}$ = 10 [8]; 56.5 ± 2 y; Healthy $n_{CHF\text{-}Training}$ = 21 [18]; 61.3 ± 2.2 y; CHF $n_{CHF\text{-}Control}$ = 17 [12]; 63.4 ± 3 y; CHF	Maximal exercise (cycle ergometer; 10 or 20 W increments, 1 min/stage)	(1) $CD34^+CD309^+CD3^-$ (2) $CD34^+CD3^-$	↔ for both definitions in all 3 groups
Cubon et al, 2010	$n_{European}$ = 15 [15]; 28.3 ± 1.3 y; Healthy n_{Asian} = 15 [15]; 30.3 ± 1.3 y; Healthy	Submaximal exercise (cycle ergometer; 30-min at 80% of lactate threshold)	(1) $CD34^+CD45^-$ (2) $CD34^+CD309^+$ (3) $CD34^+CD133^+CD309^+$	↑ in all 3 definitions for both European and Asian men Δ European > Asian
Sandri et al, 2011	n_H = 17 [16]; 31.3 ± 3.4 y; Healthy n_D = 23 [22]; 61.4 ± 2.1 y; POAD	Maximal exercise (treadmill; 3.5 km/h at 12% incline)	(1) $CD34^+CD309^+$ (2) $CD133^+CD309^+$	↑ in healthy and POAD at 6- to 24-h for both definitions Δ Healthy > POAD at 12- to 24-h

Reference	Participants (n [males]; Age; Health status)	Exercise Protocol	EPC Definition	Δ EPCs
Van Craenenbroeck et al, 2011	$n_{Healthy\text{-}Young}$ = 4 [4]; 20.3 ± 0.9 y; Healthy $n_{Healthy\text{-}Old}$ = 4 [4]; 71.5 ± 3 y; Healthy n_{CHF} = 7 [5]; 65.1 ± 3.5 y; CHF	Maximal exercise test (cycle ergometer; workload n/s)	(1) $CD3^-CD34^+CD309^+$ (2) $CD3^-CD34^+$	↑ $CD3^-CD34^+CD309^+$ in young and old healthy ↔ $CD3^-CD34^+CD309^+$ in CHF ↑ $CD3^-CD34^+$ in all 3 groups
Scalone et al, 2013	$n_{Healthy}$ = 20 [10]; 59 ± 8 y; Healthy n_{CAD} = 20 [10]; 63 ± 8 y; CAD n_{MVA} = 20 [10]; 61 ± 10 y; MVA	Maximal exercise (treadmill; Bruce protocol)	$CD34^+CD309^+CD45^-$	↑ at 24-h post-exercise in whole group Δ in Healthy > CAD > MVA
Stelzer et al, 2014	n = 7 [4]; 39.6 ± .8 y; Healthy	Submaximal ultra-endurance exercise (bicycle; 8-h cycling alternating with 8-h rest for 550-km at 7,000-m altitude)	$CD34^+CD45^{dim}$	↔
Chang et al, 2015	n = 5 [5]; 26 – 36 y; Healthy	Submaximal exercise (treadmill; 30-min at a heart rate ≥ 140 bpm)	CD309+CD11b-CD34+CD133+	↔ at 10-min post-exercise ↑ at 24-h post-exercise

Change in EPCs reported for pre- to within 1-hour post-exercise, unless otherwise specified. Age reported as range or mean ± SD. Maximal exercise test time range was 8-12 minutes. CAD – coronary artery disease; CHF – chronic heart failure; MVA – microvascular angina; n/a – not applicable; n/s – not specified; PAD – Peripheral artery disease; PAOD – Peripheral arterial occlusive disease.

APPENDIX B: FLOW CYTOMETRY ANALYSIS BY EPC DEFINITION

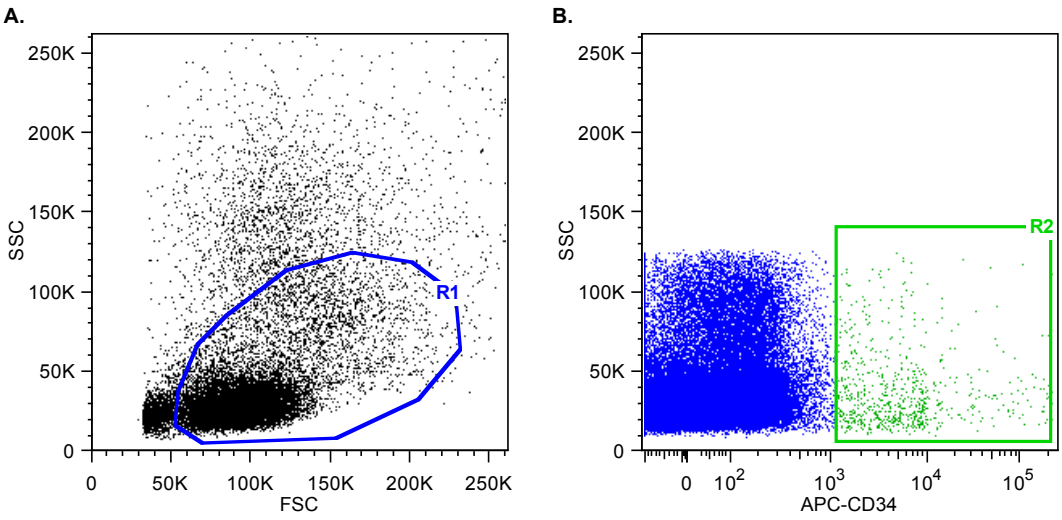

Figure B-1 *Gating strategy for CD34$^+$ cells based on the cell definition of Kinik et al., 2005.* PBMCs were identified based on forward scatter and side scatter properties (A, R1), followed by gating for CD34$^+$ cells from a CD34 vs. side scatter plot (B, R2). Cell counts were expressed as a percentage of total PBMCs (R1).

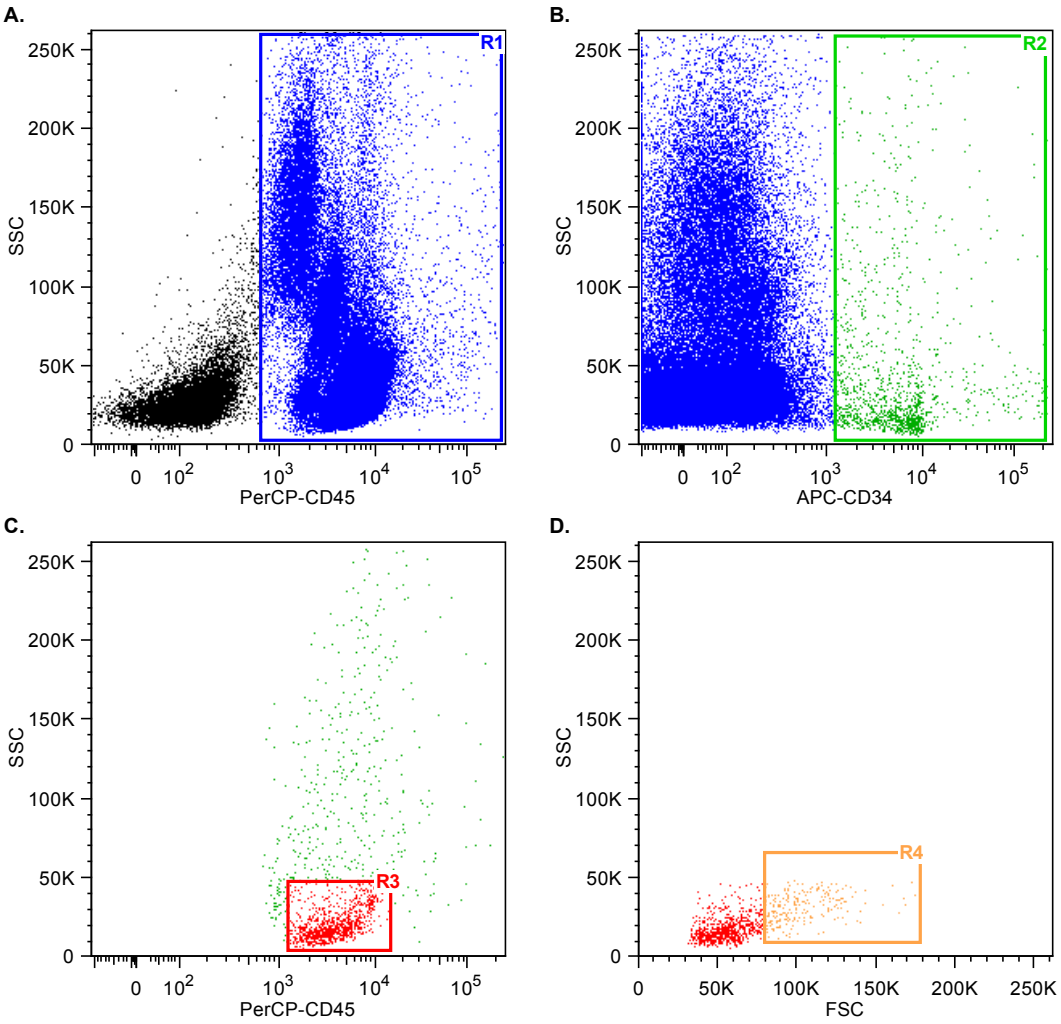

Figure B-2 *Gating strategy for CD45^{dim}CD34^{+} cells based on the cell definition of Zaldivar et al., 2007.* Gating was performed in accordance with the published ISHAGE protocols (Sutherland, 1996). First, CD45^{+} cells were gated from the CD45 vs. side scatter plot (A, R1), followed by gating of CD34^{+} cells from the CD34 vs. side scatter plot (B, R2). Next, CD45+ cells with low side scatter were identified from a CD45 vs. side scatter plot (C, R3). Finally, lymphocytes with typical side scatter and slightly higher forward scatter properties were gated from a forward scatter vs. side scatter plot (D, R4). Cell counts were expressed as cells per microliter of blood.

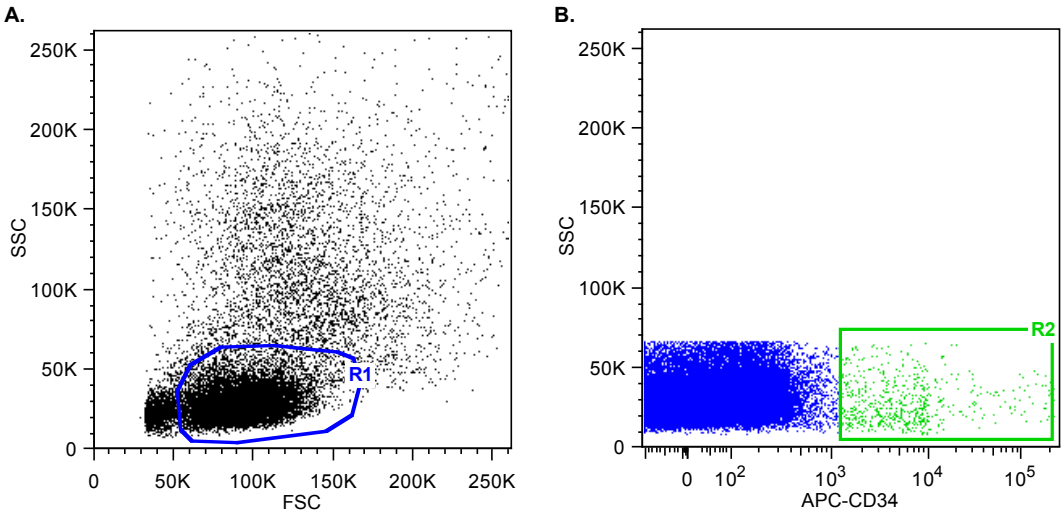

Figure B-3 *Gating strategy for CD34$^+$ cells based on the cell definition of Park et al., 2012 and Walther et al. 2008; 2009.* Lymphocytes were gated from a plot of forward scatter vs. side scatter (A, R1), followed by gating for CD34$^+$ cells from a CD34 vs. side scatter plot (B, R2). Cell counts according to Park et al. were expressed as a percentage of lymphocytes (R1); and as cells per milliliter of blood according to Walther et al.

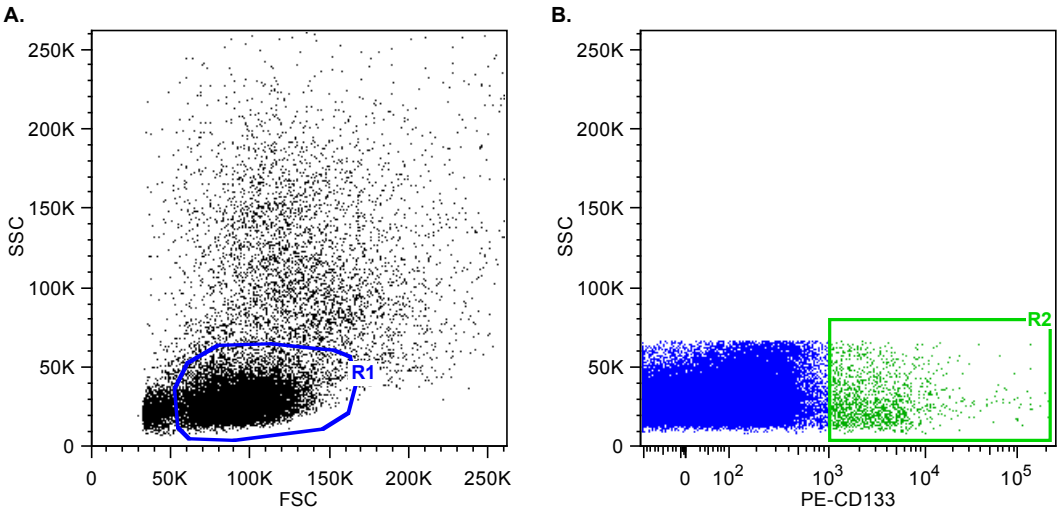

Figure B-4 *Gating strategy for CD133$^+$ cells based on the cell definition of Park et al., 2012 and Walther et al., 2008.* Lymphocytes were gated from a plot of forward scatter vs. side scatter (A, R1), followed by gating for CD133$^+$ cells from a CD133 vs. side scatter plot (B, R2). Cell counts according to Park et al. were expressed as a percentage of lymphocytes (R1); and as cells per milliliter of blood according to Walther et al.

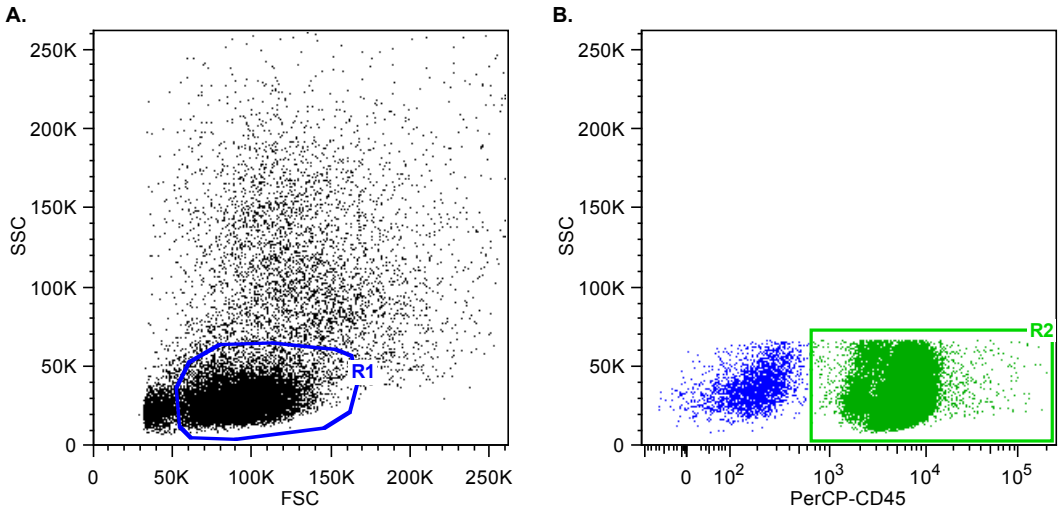

Figure B-5 *Gating strategy for CD45$^+$ cells based on the cell definition of Walther et al., 2008.* Lymphocytes were gated from a plot of forward scatter vs. side scatter (A, R1), followed by gating for CD45$^+$ cells from a CD45 vs. side scatter plot (B, R2). Cell counts were expressed as cells per milliliter of blood.

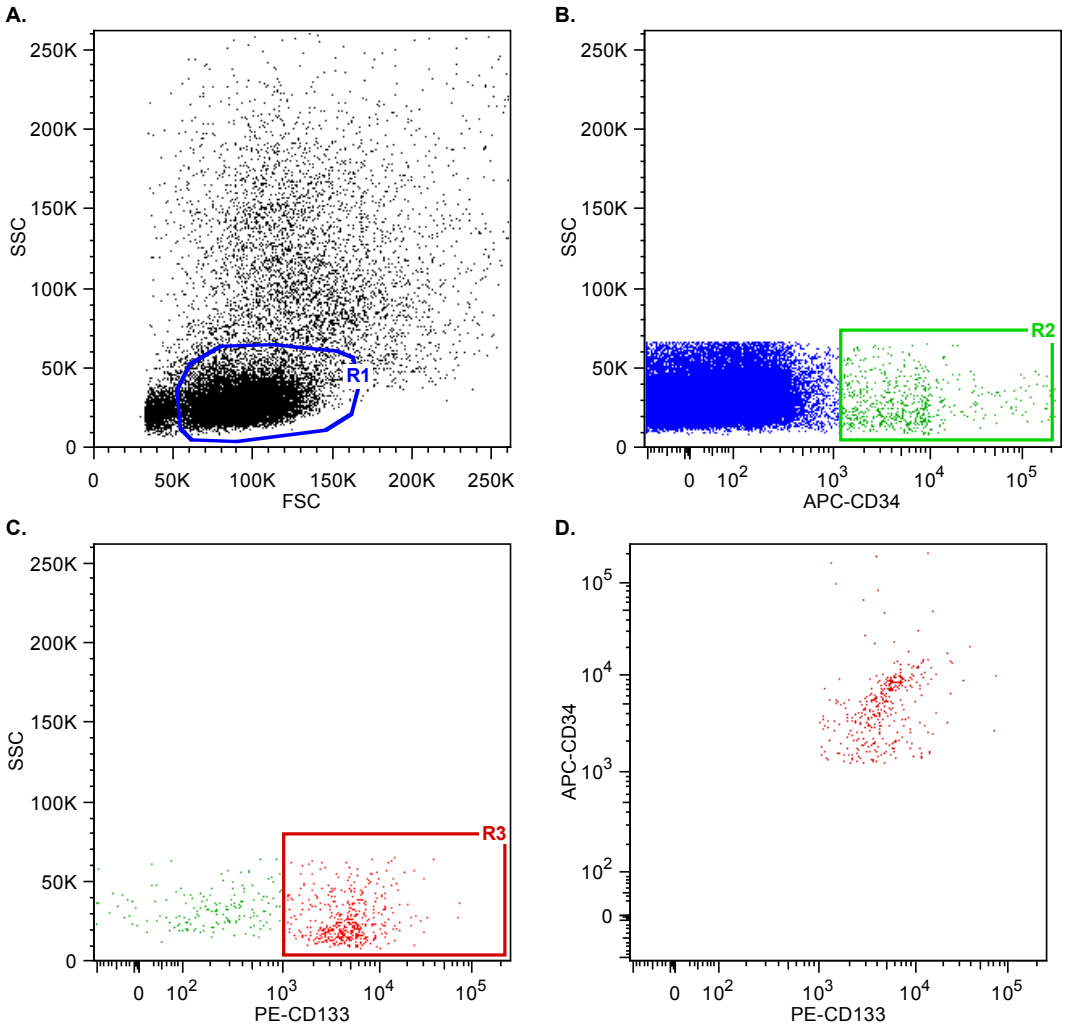

Figure B-6 *Gating strategy for CD34$^+$CD133$^+$ cells (D) based on the cell definition of Park et al., 2012.* Lymphocytes were gated from a plot of forward scatter vs. side scatter (A, R1), followed by gating for CD34$^+$ cells from a CD34 vs. side scatter plot (B, R2); finally, CD133$^+$ cells were gated from a CD133 vs. side scatter plot (C, R3). Cell counts were expressed as a percentage of lymphocytes (R1).

APPENDIX C: SUPPLEMENTARY DATA AND FIGURES

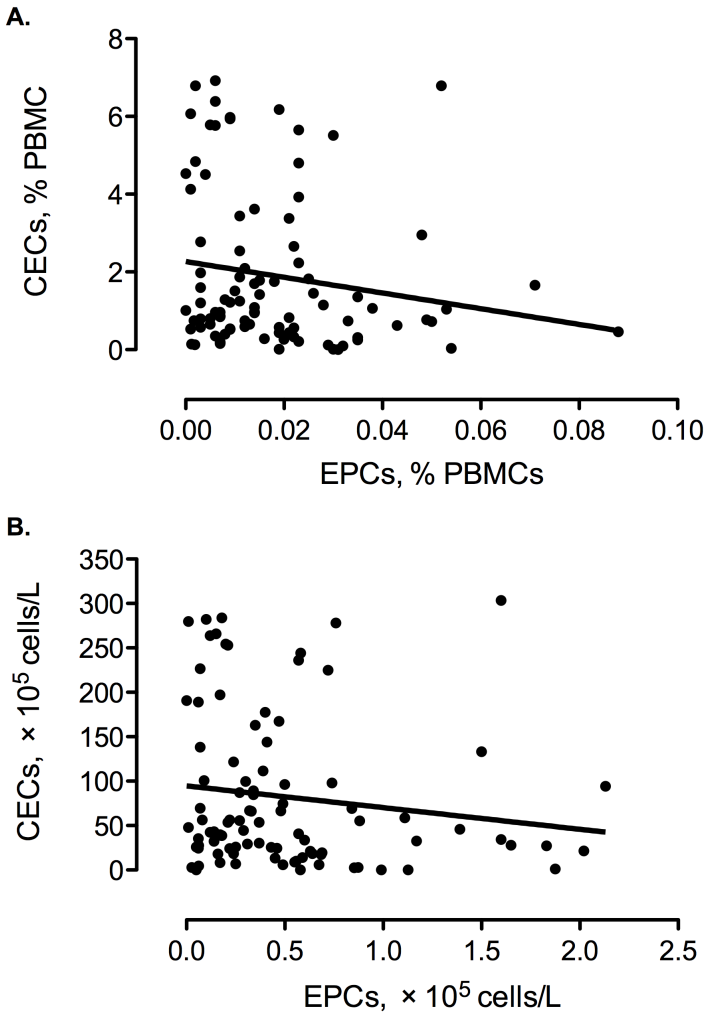

Figure C-1 The relationship between CEC and EPC proportions (A) and concentrations (B) were assessed using Spearman correlations. A trend towards a negative link between CECs and EPCs was observed for both proportions (ρ = -0.200 [BC_a 95% lower and upper CI: -0.410, 0.034], p = 0.053) and concentrations (ρ = -0.183 [-0.385, 0.063], p = 0.077).

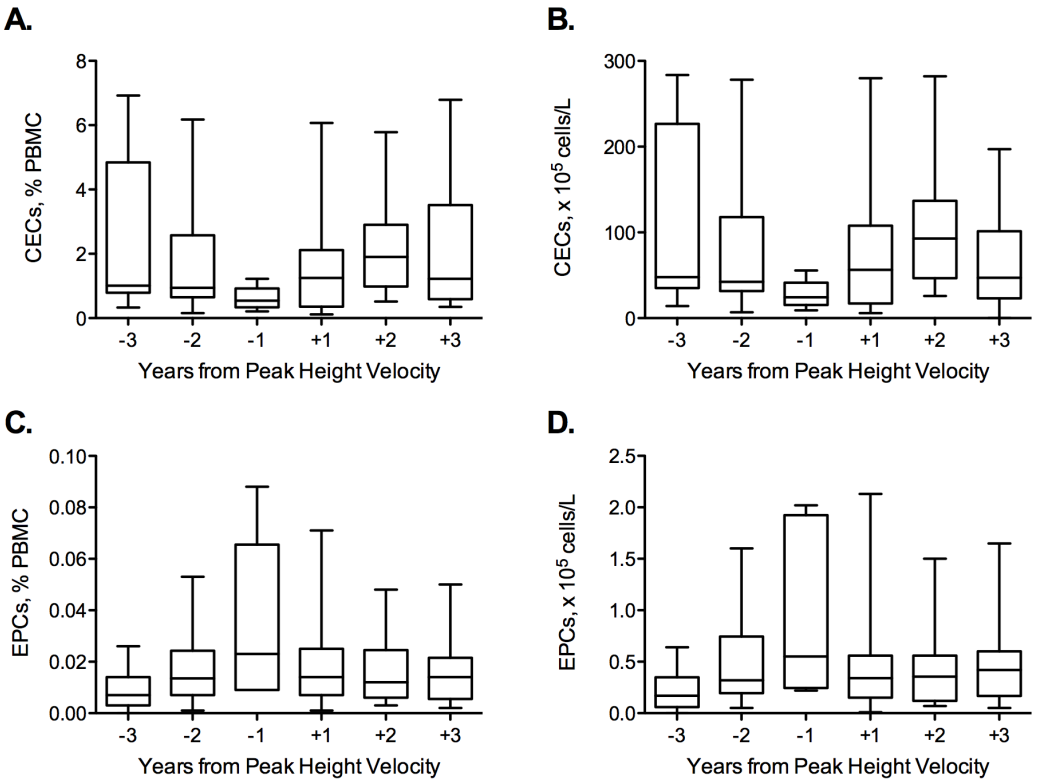

Figure C-2 To assess the effects of puberty on CECs and EPCs, participants were grouped in 1-year increments based on estimated years from peak height velocity. Kruskal-Wallis tests revealed no differences across YPHV for CEC proportions (A; $p = 0.158$), and concentrations (B; $p = 0.092$), as well as EPC proportions (C; $p = 0.182$), and concentrations (D; $p = 0.129$). Paired comparisons, corrected for multiple comparisons (significance set at $p < 0.01$), revealed a trend towards lower CECs, and higher EPCs at -1 YPHV relative to -3 ($p = 0.029 – 0.054$), -2 ($p = 0.043 - 0.070$) , +2 ($p = 0.007 – 0.20$), and +3 YPHV ($p = 0.070 – 0.308$). Data are presented as median, and interquartile range; whiskers represent minimum and maximum values.

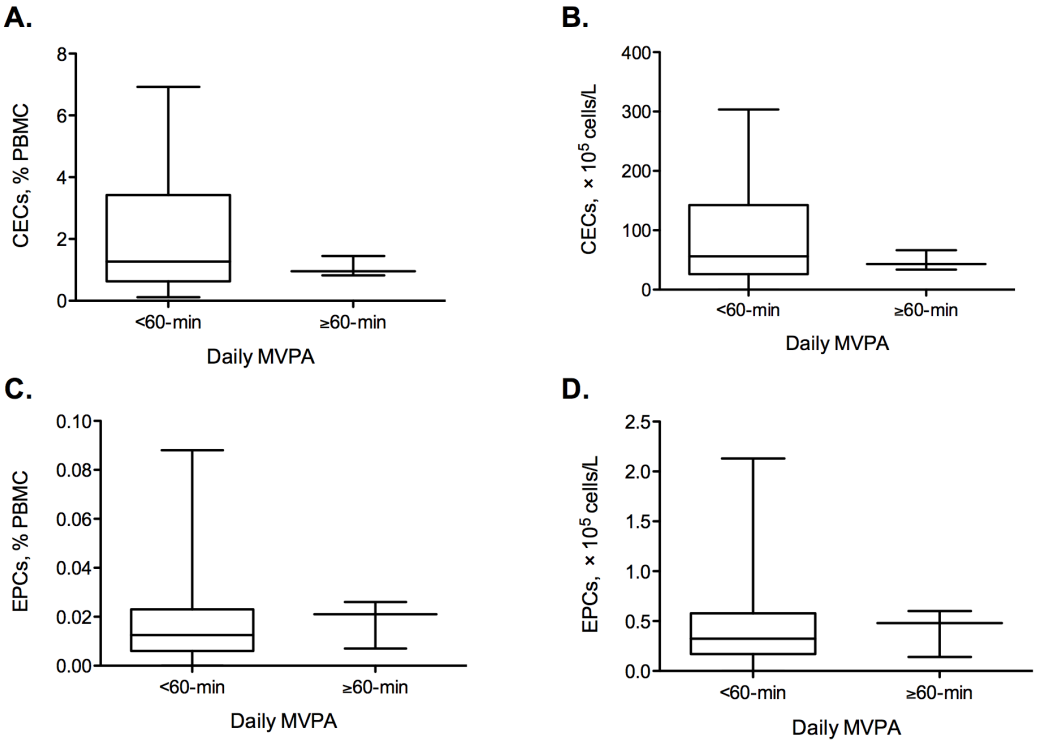

Figure C-3 CECs and EPCs were examined in children meeting and not meeting the Canadian physical activity guidelines of 60-min of daily MVPA. Mann Whitney U tests suggested no difference between groups for CEC proportions (A; U = 91.5, p = 0.665), and concentrations (B; U = 91.0, p = 0.656), as well as EPC proportions (C; U = 85.5, p = 0.552), and concentrations (U = 97.5, p = 0.787). These results should be interpreted with caution as only 3 participants (4%) of 82 were meeting the physical activity recommendations. Data are presented as median and interquartile range; whiskers represent the minimum and maximum values.

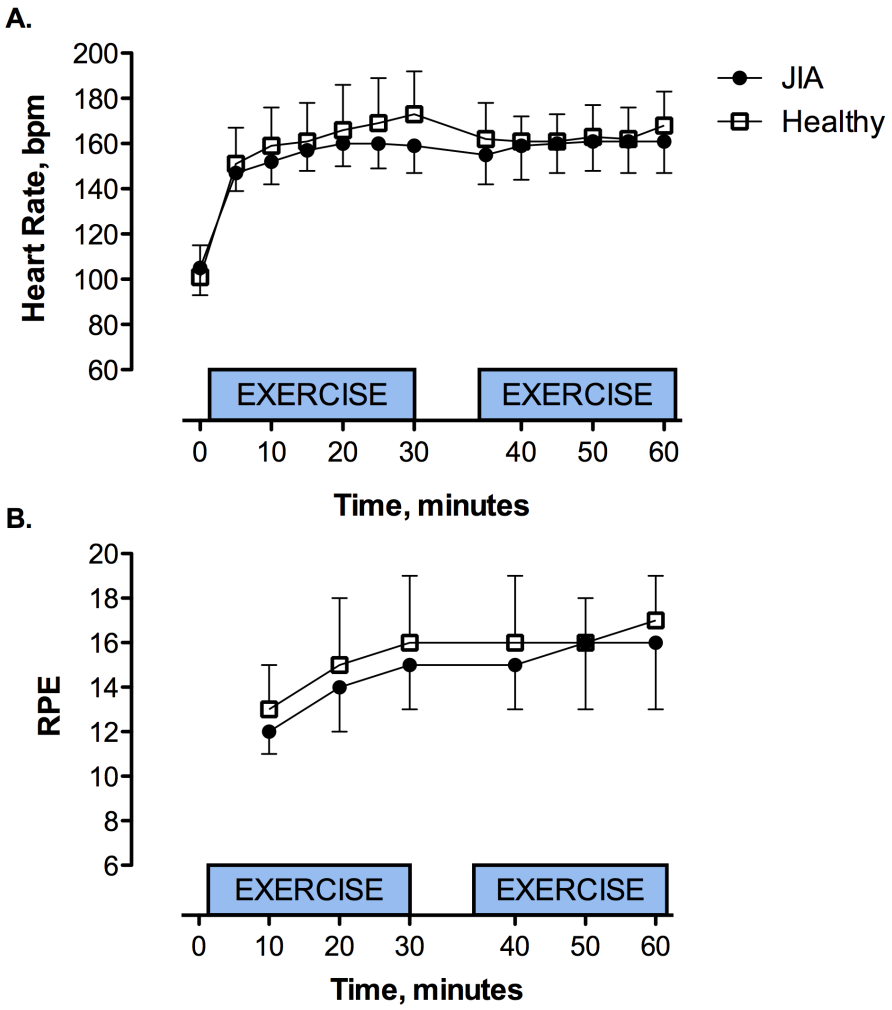

Figure C-4 Heart rate (A) and ratings of perceived exertion (B) were similar in children with JIA (circles) and healthy controls (squares) throughout the MICE protocol, as determined by two-way ANOVA ($p = 0.487$ and 0.341, respectively). Heart rate was measured at rest and every 5 minutes of the exercise, while Borg's ratings of perceived exertion was assessed every 10 minutes of exercise.

APPENDIX D: COPYRIGHT PERMISSION FORMS

Chapter 3: Manuscript Permission from Wolters Kluwer for Publication in Medicine & Science in Sports & Exercise

Copyright Clearance Center RightsLink®

Home | Create Account | Help

Wolters Kluwer Health

Lippincott Williams & Wilkins

Requesting permission to reuse content from an LWW publication.

Title: Circulating Endothelial Cells in Children: Role of Fitness, Activity, and Adiposity

Author: JOYCE OBEID, THANH NGUYEN, RACHEL WALKER, et al

Publication: Medicine & Science in Sports & Exercise

Publisher: Wolters Kluwer Health, Inc.

Date: Jan 1, 2014

Copyright © 2014, (C) 2014 American College of Sports Medicine

LOGIN

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already **a RightsLink user** or want to learn more?

This reuse is free of charge. No permission letter is needed from Wolters Kluwer Health, Lippincott Williams & Wilkins. We require that all authors always include a full acknowledgement. Example: AIDS: 13 November 2013 - Volume 27 - Issue 17 - p 2679-2689. Wolters Kluwer Health Lippincott Williams & Wilkins© No modifications will be permitted.

McMaster University

J. Obeid <obeidj@mcmaster.ca>

Thesis Inquiry [Incident: 150404-000124]

1 message

Wolters Kluwer Rights and Permissions <journalpermissions@lww.com> Tue, Apr 7, 2015 at 6:07 AM
Reply-To: Wolters Kluwer Rights and Permissions <journalpermissions@lww.com>
To: obeidj@mcmaster.ca

Recently you requested personal assistance from our on-line support center. Below is a summary of your request and our response.

If you are receiving this in response to a request you made, a summary is below. If you have not made a request, the following is a communication on behalf of your LWW Sales Representative.

Thank you for allowing us to be of service to you.

To access your question from our support site, click here.

Subject

Thesis Inquiry

Discussion Thread

Response Via Email (Delayna S.) 04/07/2015 06:07 AM

Dear Joyce,

If you are the author of the original content then I am happy for you to go ahead and reuse this within your thesis without requesting any permissions at all. I understand that you would possibly need to copy and paste to get the article into the correct format and I am happy for you to do so and use this email as proof of permission.

I have attached the author's permissions document to this email to provide you with more information on what you can do with your content without requesting permissions.

Kind regards,
Delayna

Delayna Spencer
Permissions Assistant
Medical Research
Wolters Kluwer

250 Waterloo Road, London, SE1 8RD, England, United Kingdom
+44 (0)207 981 0518 Direct Line
+44 (0)207 981 0562 Fax
Delayna.Spencer@wolterskluwer.com
www.wolterskluwerhealth.com

Wolters Kluwer