

**Retinal microvascular parameters and
cardiovascular health, obesity and inflammation
in children and mid-life adults**

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Abstract

Background

The microcirculation makes up approximately three-quarters of the circulatory system but has attracted little attention as a target for cardiovascular disease (CVD) prevention, particularly in early life. Retinal microvascular parameters allow noninvasive assessment of the microcirculation, with adverse parameters (e.g., narrower retinal arterioles, wider venules) predicting CVD events in adults. In children, it is largely unknown when such microvascular changes become evident, their determinants and the underlying mechanisms.

Aims

In a large population-based sample of Australian children and mid-life adults (their parents), I aimed to determine whether:

- 1) adverse retinal vascular calibre and preclinical large arterial phenotypes covary;
- 2) body mass index (BMI) and waist-to-height ratio (WHtR; child only) over the preceding decade predict retinal vascular calibre;
- 3) inflammation mediates the association between obesity and retinal vascular calibre;
- 4) traditional CVD risk factors and large arterial phenotypes are related to retinal geometric parameters.

Methods

Participants: 1,288 11–12 year-olds (51% girls) and 1,264 parents (88% mothers).

Cross-sectional measures: Retinal vascular calibre and (child only) geometric parameters; large arterial phenotypes (pulse wave velocity, PWV; carotid arterial elasticity; carotid intima-media thickness, CIMT); blood pressure (BP); BMI; WHtR; and metabolites (e.g., LDL, HDL cholesterol and the inflammation marker Glycoprotein Acetyls, GlycA). *Biennial longitudinal measures:* BMI and (child only) WHtR. *Analysis:* In all aims, linear regression models were used, and Aim 3 used causal mediation analysis.

Results

Aim 1: In children, narrower retinal arteriolar and wider venular calibre showed modest associations with faster PWV and lower elasticity but not with CIMT. In adults, results were stronger, and there was weak evidence of an association between wider venular calibre and higher CIMT.

Aim 2: In children, higher BMI from age 4–5 years onwards was increasingly associated with adverse arteriolar calibre, but not venular calibre at 11–12 years. Effects were similar in adults. Less favourable BMI and WHtR trajectories predicted narrower arteriolar calibre.

Aim 3: Compared to children with normal BMI, those with obesity had: (i) narrower arteriolar calibre, which was not mediated by inflammation; and (ii) wider venular calibre, which was partly mediated by inflammation. In adults with obesity, this association between obesity and wider venular calibre was fully mediated by inflammation. Findings were similar for WHtR.

Aim 4 (in children only): BMI, systolic BP and PWV showed modest associations with lower arteriolar fractal dimensions, whereas only systolic BP was associated with arteriolar tortuosity. There was less evidence of associations with venular geometric parameters.

Conclusion

Preclinical phenotypes of large arteries and microcirculation have some shared, but mainly unique pathways to CVD, with shared pathways becoming more evident across the life course. The adverse impact of obesity on retinal microvasculature begins early in life, more markedly on arteriolar than venular parameters, with modest venular effects largely mediated by inflammation. In children, retinal geometric parameters did not add novel information about CVD risk beyond the vascular calibre. The microvascular parameter, retinal vascular calibre, may offer additional value to CVD prevention from childhood onwards, especially when combined with other risk assessment measures.

Declaration

I, Mengjiao Liu, certify that:

- i. the thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface,
- ii. due acknowledgement has been made in the text to all other material used,
- iii. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Date: 26/03/2020

Signed:

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Preface

Overview

This doctoral thesis includes five manuscripts published in international peer-reviewed journals. The doctoral candidate contributed to over 50% of each paper.

This thesis uses unit record data from the Longitudinal Study of Australian Children (LSAC). The study is conducted in partnership between the Department of Social Services, the Australian Institute of Family Studies and the Australian Bureau of Statistics. The findings and views reported here are those of the thesis author and should not be attributed to these government bodies.

The thesis also uses data from the Child Health CheckPoint (CheckPoint), led by principal investigator Prof Melissa Wake and project manager Dr Susan Clifford. CheckPoint data are stored at the Murdoch Children's Research Institute in Melbourne, where this PhD was undertaken. I uniquely contributed to the generation of the CheckPoint study's retinal microvascular parameters. This involved taking responsibility for all aspects of retinal vascular calibre scoring protocol development and data management. Two expert teams from Centre for Eye Research Australia in Melbourne, Australia and Zhongshan Ophthalmic Centre in Guangdong, China provided intellectual support, training and scoring assistance. Key tasks involved being the liaison between the Melbourne and China team given I am fluent in both English and Chinese, overseeing reliability testing, sending images securely between scoring teams and feedbacks between groups and helping translate and solve queries. In addition, I scored 83% (n= 1,064) of child retinal images (20–40 minutes per image) to generate retinal geometric parameters.

My supervisory team comprised a multi-disciplinary team, including Dr Kate Lycett (primary supervisor), Prof Melissa Wake, Prof David Burgner, Prof Tien Yin Wong and Prof Mingguang He. This team oversaw all aspects of the study and preparation of the five included manuscripts.

Papers arising from this thesis

Paper 1 (a systematic review and meta-analysis) is published in *Microcirculation*. I conceived and designed the study, extracted, analysed and interpreted the data and wrote the first draft of the manuscript and produced the final version. Ms Xiao assisted with screening and extracting data. Prof Wong and Prof He assisted in interpreting the results. Dr Lycett, Prof Wake and Prof Burgner provided oversight of the study and day-to-day support. All co-authors read, provided critical feedback and approved the final manuscript.

- **Liu M**, Wake M, Wong TY, He M, Xiao Y, Burgner D and Lycett K. Associations of retinal microvascular calibre with intermediate phenotypes of large arterial function and structure: A systematic review and meta-analysis. *Microcirculation*. 2019:e12557.

Paper 2 (small and large vascular phenotype associations) is published in *Microcirculation*. I conceived this study with support from my supervisory team, performed all data analyses, wrote the first draft of the manuscript and produced the final version. My supervisory team oversaw all aspects of the study and manuscript preparation. Dr Grobler, Prof Juonala, Prof Dwyer advised on statistical issues and interpretation. All authors critically reviewed the manuscript and approved the final manuscript.

- **Liu M**, Lycett K, Wong TY, Grobler A, Juonala M, He M, Dwyer T, Burgner D and Wake M. Associations of retinal microvascular calibre with large arterial function and structure: a population-based study of 11–12 year-olds and mid-life adults. *Microcirculation*. 2020:e12642.

Paper 3 (decade-long obesity-related measures and retinal vascular calibre) is published in the *International Journal of Obesity*. I conceived the study, performed all data analyses, wrote the first draft of the manuscript and produced the final version. Prof Wake advised on data drawn from LSAC's rich longitudinal data, as she was the study's health advisor for close to a decade. Prof Burgner, Prof Wong and Prof He provided intellectual support and assistance with interpretation. Dr Lycett helped create BMI trajectories and provided supervisory support. Prof Olds led CheckPoint's body composition measures. Dr Kerr, Prof Juonala, Prof Dwyer

advised on statistical issues and interpretation. All authors critically reviewed the manuscript and approved the final version.

- **Liu M**, Lycett K, Wong TY, Kerr JA, He M, Juonala M, Olds T, Dwyer T, Burgner D and Wake M. Do body mass index and waist-to-height ratio over the preceding decade predict retinal microvasculature in 11–12 year-olds and mid-life adults? *International Journal of Obesity*. 2020; 44, 1712–1722.

Paper 4 (inflammation mediates obesity and retinal vascular calibre) is published in *Scientific Reports*. I conceived the study, performed all data analyses, wrote the first draft and produced the final version. Dr Moreno-Betancur provided support for the causal mediation analysis. Prof Saffery supervised laboratory work for assays used in the study. Prof Burgner led CheckPoint's metabolomics panel. Dr Kerr and Prof Juonala advised on statistical issues and interpretation. All authors critically reviewed the manuscript and approved the final version.

- **Liu M**, Lycett K, Moreno-Betancur M, Wong TY, He M, Saffery R, Juonala M, Kerr JA, Wake M and Burgner D. Inflammation mediates the relationship between obesity and retinal vascular calibre in children and mid-life adults. *Scientific Reports*. 2020; 10, 5006.

Paper 5 (cardiovascular health and retinal geometric parameters) is published in *Microvascular Research*. I performed all data analyses, wrote the first draft of the manuscript and produced the final version. My supervisory team (Dr Lycett, Prof Wake, Prof Burgner, Prof Wong and Prof He) helped conceive the study and provided intellectual advice. Prof Olds, Prof Saffery, Dr Kerr and Prof Dwyer advised on statistical interpretation and provided critical advice. All authors critically reviewed the manuscript and approved the final version.

- **Liu M**, Lycett K, Wake M, He M, Kerr JA, Saffery R, Juonala M, Olds T, Dwyer T, Burgner D and Wong TY. Cardiovascular health and retinal microvascular geometry in Australian 11–12 year-olds. *Microvascular Research*. 2020; 129, 103966.

Co-authored publications arising from and closely related to this PhD (full texts are in Appendix C)

1. Lovern C*, **Liu M***, Lycett K, Wake M, Wong TY, He M and Burgner D. Are markers of inflammation related to retinal vascular calibre? A systematic review and Meta-analysis. This paper is almost complete and will be submitted to *JAMA Ophthalmology* for peer review.
* *Lovern C and Liu M contributed equally to this work.*
2. Dascalu J, **Liu M**, Lycett K, He M, Wong TY and Wake M. Retinal microvasculature: population epidemiology and concordance in Australian children aged 11–12 years and their parents. *BMJ Open* 2019; 9: 44-52.
3. Wang J, **Liu M**, Sung V, Lycett K, Burgner D, Wong TY and Wake M. Associations of retinal vessel calibre with hearing status in childhood and mid-life: A cross-generational population-based study. *JAMA Otolaryngology Head & Neck Surgery*. Published online 30 January 2020.

Presentations arising from this thesis

1. **Liu M**, Lycett K, He M, Wong TY, Burgner D and Wake M. The association between retinal microvascular calibre and large arterial function and structure: a population-based study of 11–12-year-old and mid-life adults. Poster presentation at Pediatric Academic Societies (PAS) 2018 Meeting, Toronto, Canada, May 2018.
2. **Liu M**. Eyes: A window to the soul? Heart! 3 Minutes Thesis competition, Murdoch Children's Research Institute, Melbourne, Australia, July 2018 (first prize).
3. **Liu M**, Lycett K, Burgner D and Wake M. Obesity and microvasculature in childhood and mid-life: A population-based study. Poster presentation at ANZOS-Breakthrough Discoveries Joint Annual Scientific Meeting 2018, Melbourne, Australia, October 2018.
4. **Liu M**, Lycett K, Burgner D and Wake M. Obesity and microvasculature in childhood and mid-life: A population-based study. Rapid-fire one-minute oral and poster presentation at Annual Scientific Meeting of the Australasian

Epidemiological Association 2018, Fremantle, Australia, October 2018.

5. **Liu M**, Wake M, Wong TY, He M, Xiao Y, Burgner D and Lycett K. Meta-analysis: Associations of retinal microvascular calibre with intermediate vascular phenotypes. Murdoch Children's Research Institute Graduate Student Symposium 2018, Melbourne, Australia, November 2018.
6. **Liu M**, Lycett K, Moreno-Betancur M, Wake M and Burgner D. Does inflammation mediate the relationship between obesity and retinal vascular calibre? Oral presentation at Annual Scientific Meeting of the Australasian Epidemiological Association 2019, Brisbane, Australia, October 2019.
7. **Liu M**, Lycett K, Moreno-Betancur M, Wake M and Burgner D. Inter-relationships between body mass index, inflammation and retinal microvasculature. Poster presentation at 11th World Congress on Development Origins of Health and Disease (DOHaD), Melbourne, Australia, October 2019.

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Figure details	Source	Permission
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Figure 2-1(b) Assessment of pulse wave velocity and carotid intima-media thickness	Yang G, Zhang Y. The carotid intimal-media thickness measurement of Ultrasound images based on an improved Chan-Vese model. 2012 <i>IEEE International Conference on Information and Automation</i> . 2012;867-870.	Yes
Figure 2-2 A vicious circle of aggravation between micro- and macrocirculation	Laurent S, Briet M, Boutouyrie P. Large and small artery cross-talk and recent morbidity-mortality trials in hypertension. <i>Hypertension</i> . 2009; 54:388-392.	Yes
Figure 5-1 Introduce Child Health CheckPoint to LSAC B cohort	Wake M, Clifford SA, York E, et al. Introducing Growing Up in Australia's Child Health CheckPoint. <i>Family Matters</i> . 2014; 94:15-23.	Yes

List of abbreviations

ARIA	Automated Retinal Image Analyser
AUC	Area under the curve
BIA	Bioelectrical impedance analysis
BIC	Bayesian information criterion
BMI	Body mass index
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
CDC	Centres for Disease Control
CEBU	Clinical Epidemiology & Biostatistics
CI	Confidence Interval
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CVD	Cardiovascular disease
CheckPoint	Child Health CheckPoint study
DAG	Directed acyclic graphs
ECG	Electrocardiogram
GlycA	Glycoprotein acetyls
HDL	High density lipoprotein
HR	Hazard ratio
ICC	Intra-class correlation coefficient
IVAN	Integrative Vessel Analysis
LDL	Low density lipoprotein
LSAC	Longitudinal Study of Australian Children
MHz	Megahertz
NMR	Nuclear magnetic resonance
OCT	Optical coherence tomography
OR	Odds ratio
PWV	Pulse wave velocity

QUARTZ	Quantitative Analysis of Retinal Vessel Topology and siZe
r	Correlation coefficient
RA	Retinal Analysis
RCT	Randomised control trial
REDCap	Research Electronic Data Capture
SEP	Socioeconomic position
SD	Standard deviation
SIVA	Singapore “I” Vessel Assessment
UK	United Kingdom
US	United States
VAMPIRE	Vessel Assessment and Measurement Platform for Images of the Retina
WHtR	Waist-height ratio
WHO	World Health Organization

Chapter 1. Introduction

Cardiovascular disease (CVD), the leading cause of death worldwide, is largely preventable. Quantifying risk has largely relied on the assessment of traditional CVD risk factors, but can be enhanced by noninvasive assessment of blood vessels, such as preclinical large arterial phenotypes. However, the microcirculation, which makes up three-quarters of the circulatory system, has received much less attention. The retinal microcirculation can be noninvasively captured via retinal imaging technique. Quantified retinal microvascular parameters (e.g., retinal vascular calibre and geometric parameters) from retinal images have been shown to predict adverse CVD events late in life. It remains unclear when and how the microvascular pathophysiology contributes to CVD pathogenesis across the life course.

Understanding this could help researchers and clinicians evaluate the utility of retinal microvascular parameters as a tool to assist CVD risk prediction and management early in life. This is important as the pathogenic process of CVD has already begun in childhood, implying opportunities for early intervention, recognised to be more effective than later.

Measuring large arterial phenotypes informs the status of preclinical cardiovascular health from macrocirculation. Looking at the association of retinal microvascular parameters with preclinical large arterial phenotypes could then help understand the correlation between the phenotypes of micro- and macro-circulation underlying the same cardiovascular system. This could also enhance our knowledge of whether assessing both phenotypic measures can improve CVD risk prediction. However, it has not been well studied, especially in children.

Obesity is a well-known risk factor for CVD risk, and a burning issue, especially in the context of Australian society. Two-thirds of Australians aged 18+ are overweight or obese, while one in 10 children is obese. Historic studies suggested that high body mass index (BMI) can predict changes to the retinal microvascular parameters. Few studies have examined how BMI across early or mid-life, or how cumulative patterns of repeated BMI over time, relate to developing retinal microvascular parameters. This could be important because distinct BMI trajectories differentially alter the risk of CVD later in life.

The underlying mechanism of how obesity is associated with adverse microvascular parameters remains unclear. Inflammation is suggested to play a key role in animal experiments, but little evidence exists from population-based studies. At the population level, the question of whether inflammatory pathways explain the association between obesity and retinal microvasculature can be addressed within a statistical model: causal mediation analysis.

This thesis drew on the population-based cross-sectional Child Health CheckPoint study nested within the Longitudinal Study of Australian Children, comprising nearly 2000 child-parent pairs at child age 11–12 years. It aimed to describe whether adverse retinal vascular calibre and preclinical large arterial phenotypes covary (Aim 1). The doctoral thesis then examined if BMI and waist-to-height ratio over the preceding decade predict retinal vascular calibre (Aim 2). The doctoral thesis further investigated the association of inflammation and retinal vascular calibre, and whether inflammation mediates the relationship between obesity and retinal vascular calibre (Aim 3). The study also measured novel retinal geometric parameters in addition to the commonly measured retinal vascular calibre. Finally, the doctoral thesis investigated whether CVD risk factors and preclinical large arterial phenotypes are associated with these new parameters (Aim 4).

1.1 Chapter summary

Chapter 2 is a review of the literature. This chapter is divided into four sections. The first section outlines why we are interested in additional assessment parameters of the microcirculation for CVD risk prediction. The second section provides information on measuring retinal microvasculature as a potential ‘window’ to the cardiovascular system and the epidemiology of retinal microvascular parameters (retinal vascular calibre and geometric parameters). The third section summarises the physiological and pathophysiological features of the retinal microvasculature and our understanding of its determinants (e.g., genetic, traditional CVD risk factors). The fourth section summarises the current knowledge of the more novel retinal geometric parameters and the fact that there is lack of evidence from children.

Chapter 3 outlines four aims of the thesis and hypotheses and what the findings could add to our understanding of the microvasculature and its role in CVD risk prediction.

Chapter 4 describes the results of my published systematic review and meta-analysis that is published in *Microcirculation* and informs Aim 1 of this thesis. The paper examines the associations of retinal microvasculature and preclinical large arterial phenotypes and highlights the need for more research on this topic early in life.

Chapter 5 describes the methods for the population-based study in which this thesis sits, the Longitudinal Study of Australian Children and its physical and biomarkers wave, the Child Health CheckPoint. It provides details of the study design and participants, study procedures, all relevant measures, and the statistical methods used. The chapter has a strong focus on the retinal microvascular parameters and outlines my unique contribution of generating these parameters.

Chapter 6 is the first of four results chapters. It provides the results of Aim 1 in the form of a paper published in *Microcirculation*. The paper examines the associations of retinal vascular calibre with preclinical large arterial phenotypes in children aged 11–12 years and mid-life adults (the children's parents).

Chapter 7 is the second results chapter. It provides the results for Aim 2 in the form of a paper published in the *International Journal of Obesity*. The paper examines whether BMI and waist-to-height ratio over the preceding decade affect retinal vascular calibre in children aged 11–12 years and mid-life adults.

Chapter 8 is the third results chapter. It provides the results for Aim 3 in the form of a paper published in *Scientific Reports*. The paper examines whether the inflammatory biomarker GlycA is associated with the retinal vascular calibre. It also examines whether GlycA mediates the association between obesity and retinal microvasculature in children aged 11–12 years and mid-life adults.

Chapter 9 is the fourth and final results chapter. It provides the results for Aim 4 in the form of a paper published in *Microvascular Research*. The paper examines whether traditional CVD risk factors and preclinical large arterial phenotypes are associated with retinal geometric parameters in children aged 11–12 years.

Chapter 10 provides a critical discussion of the results, extending on the summary provided in each results chapter. Firstly, it outlines the principal findings of each aim and the overall strengths and limitations of the study. Secondly, it interprets the findings in light of existing studies and explores a range of possible explanations for the variation in some of our findings. This is followed by describing the implications of the findings: i) from a life course perspective, ii) in terms of clinical implications, and iii) from a mechanistic perspective. Finally, additional questions are raised, which warrant further investigation. This includes questions around whether body mass or the body composition component leads to the retinal microvascular variation and future directions that artificial intelligence technology could offer a real-time retinal microvascular parameter assessment.

Chapter 11 draws together important conclusions from this thesis.

Chapter 2. Literature review

2.1 Overview

This literature review introduces parameters measured from microcirculation, henceforth referred to as microvascular parameters, and outlines their potential for assessing cardiovascular disease (CVD) risk across the life course. It summarises the current burden of CVD and argues that microvascular parameters may help evaluate CVD risk early in life. The literature review is broadly split into four sections.

The first section outlines why microvascular parameters could be used as an additional risk assessment approach for assessing CVD risk. It summarises what we know about CVD, current measures of CVD risk (e.g., traditional CVD risk factors and preclinical large arterial phenotypes) and what might be gained by assessing microcirculation.

The second section details retinal microvasculature as a window to visualise the human microcirculation, and the available techniques to quantify retinal microvascular parameters (e.g., retinal vascular calibre and geometric parameters) have high reproducibility. This section summarises the current evidence on how the retinal microvascular parameters change across the life course, as well as their association with clinical CVD events and preclinical cardiovascular health. Current evidence highlights knowledge gaps in our understanding of retinal microvascular parameters with large arterial phenotypes. This informs the first aim of the thesis.

The third section summarises the causes of retinal microvascular parameter variation by explaining the potential mechanisms and determinants. It reviews what is known about genetic and traditional CVD risk determinants of retinal vascular calibre. Obesity and inflammation are of particular focus in this thesis. They are interrelated factors implicated in the pathogenesis of CVD, but for which little evidence yet exists with regard to the microvasculature. Current gaps in research include insufficient longitudinal evidence of obesity and retinal vascular calibre, and few studies consider inflammation as a potentially important mechanism. These points will be addressed in the second and third aims of the thesis.

The fourth section summarises current evidence on retinal geometric (as opposed to calibre) parameters. These novel parameters include fractal dimension, tortuosity and branching patterns, and are postulated to offer additional CVD risk information beyond traditional retinal vascular calibre. The scant literature in this area provides the basis for the fourth aim of the thesis.

2.2 PART ONE: Microvascular parameters could offer new CVD insights

CVD is the leading cause of death globally, despite being largely preventable. Prediction relies largely on assessing traditional CVD risk factors. However, traditional CVD risk factors (such as smoking, physical inactivity, poor diet – see 2.2.3 below) explain only about half of the CVD risk – meaning that other factors are yet to be identified. Recently, the noninvasive assessment of large arteries in asymptomatic individuals was found to enhance CVD prediction, but this has not been widely used clinically due to logistic and financial barriers. Another aspect of the cardiovascular system, microcirculation, has received relatively little attention. Microcirculation has been implicated in CVD pathogenesis, and abnormalities of microcirculation have been found to coexist with macrocirculation damage. Thus, measuring microvascular parameters may offer new insights into the prediction and prevention of CVD.

In this section, I summarise what is known about CVD and the major risk assessment measures of traditional CVD risk factors and preclinical large arterial phenotypes, and explain why assessing microcirculation could offer additional value to CVD.

2.2.1 CVD remains the leading cause of death globally

2.2.1.1 *What is CVD and its burden?*

CVD includes diseases of the heart, brain and blood vessels.¹ Due to different causes, the types of CVD can be broadly classified as atherosclerotic (ischaemic heart disease or coronary artery disease, cerebrovascular disease [e.g., stroke], hypertension and peripheral vascular disease) or other types (congenital heart disease, rheumatic heart disease, cardiomyopathies and cardiac arrhythmias).¹

Across all categories, coronary artery disease and stroke account for over 80% of CVD burden.²

As the leading cause of death and years of life lost, CVD was estimated to account for 17.8 million deaths globally in 2017.³ It is also the primary cause of death in Australia: more than 1 in 4 (27%) deaths were attributed to CVD in 2017, with coronary artery disease and stroke accounting for 17.9% of all deaths.⁴ CVD is also a major economic burden for individuals and government in Australia. Premature death from CVD mostly occurs between the age of 35 and 64 years, meaning CVD affects the labour force more than any other disease.⁵ The global cost of CVD was estimated at AUD\$1,267 billion in 2010.⁶ In Australia, health expenditure on CVD was AUD\$10.4 billion in 2015-16, accounting for 8.9% of all health expenditure.⁷

2.2.1.2 CVD origins from childhood

Historically, CVD has largely been considered a disease of ageing, and prevention efforts remain heavily focus on mid to late adulthood. However, it is now firmly established that CVD has its roots in childhood,⁸ with risk factors in childhood and adolescence linked to later CVD.⁹ For example, the Cardiovascular Risk in Young Finns cohort study followed 1,809 children for 27 years since baseline (1980, age 3-18 years); they showed that childhood risk factors, such as obesity, high blood pressure and high cholesterol levels, predicted increased atherosclerosis progression rates in adulthood.¹⁰ The more recent life course approach to CVD emphasises that experiences early in life may have long-term effects on the development of CVD;¹¹ this suggests that intervention strategies should begin in childhood, in order to be effective and reduce the risk of CVD in adulthood. It is only recently that CVD public health campaigns have started to turn their focus towards children.¹²

2.2.2 CVD is preventable

Despite the burden of CVD, evidence suggests that CVD is largely preventable.¹³ Although remaining the leading cause of death (see above), the proportion of deaths from coronary artery disease and stroke had declined substantially since the peak in the late 1960s and early 1970s, when CVD was responsible for 55% of all global deaths each year.¹⁴ By 2017, this proportion had reduced to 32% of all global deaths.³ This decrease is mostly attributed to increased knowledge and action to

reduce the modifiable lifestyle risk factors,¹⁴⁻¹⁶ which will be discussed in the following section.

More recently, an increasing number of people without these modifiable lifestyle risk factors are experiencing adverse CVD events.¹⁷ Since CVD develops from childhood, long before its overt symptoms appear, the preclinical changes of cardiovascular health should be assessed and monitored. Noninvasive assessment of blood vessels is a potential way to measure cardiovascular health in people without manifest CVD across the life course.¹⁸ The preclinical phenotypes from different sites of blood vessels will be introduced in Sections 2.2.4 and 2.2.5, respectively, with their value to CVD risk management outlined.

2.2.3 Traditional risk factors are not the whole answer

Key traditional risk factors for CVD that can be easily assessed include smoking, physical inactivity, poor diet, high levels of cholesterol, glucose and blood pressure, and excess weight.¹⁹ These risk factors are modifiable, hence reducing them can lower CVD risk.^{16, 20} For example, cigarette smoking is estimated to be responsible for 10% of all deaths from CVD,²¹ while high levels of cholesterol, glucose, blood pressure collectively contributed to over 30% of deaths from coronary artery disease and stroke, data since 2000.²²⁻²⁴

However, these modifiable risk factors in total can only explain around half of the CVD risk.^{16, 20} This suggests that other important factors are being overlooked.

Recent evidence has shown that the number of people without pre-existing traditional risk factors but are diagnosed with CVD is rising. For example, up to 40% of new cases were risk-factor-free on arrival in one Australian case series.¹⁷ Such evidence suggests that residual risk may become increasingly important in the future. Consequently, other risk assessment approaches and markers are needed to measure populations that were traditionally considered asymptomatic – thereby improving the efficacy of prevention interventions.

In the following section, I introduce the noninvasive assessment of blood vessels, which could measure preclinical cardiovascular health of people with or without traditional risk factors.¹⁸ These approaches are particularly important to monitor

cardiovascular health from childhood, as CVD has its roots in childhood and its symptom may take time to manifest.

2.2.4 Large arterial phenotypes are useful but not clinically feasible

According to blood vessel size and flow dynamics, the blood circulation can be divided into two components. The macrocirculation comprises the heart and large- and medium-sized blood vessels.²⁵ The microcirculation is the circulation of blood in the smallest blood vessels (diameter less than 300 μm).²⁶ To measure preclinical cardiovascular health, most current tests assess large arteries in macrocirculation, which are introduced in this section. Preclinical assessment of microcirculation is introduced in the next section.

2.2.4.1 What are preclinical large arterial phenotypes?

Assessing the function and structure of large arteries offers a snapshot of preclinical cardiovascular health, given that variation in both has been linked to CVD. This could be particularly important for individuals without any clinical symptoms and those whose traditional CVD risk factors fall below decisional thresholds.¹⁸ Large arteries, such as the carotid artery, aorta and coronary artery, are the most commonly measured sites.¹⁸ Widely used measures are pulse wave velocity (PWV) and carotid intima-media thickness (CIMT), which assess the function and structure of large arteries.

PWV is defined as the velocity at which the pressure waves, generated by the systolic contraction of the heart, propagate along the arterial tree (Figure 2-1(a)).²⁷ It is generally accepted as a simple, noninvasive, robust, and reproducible method to determine the rigidity of the arterial wall (arterial stiffness). A higher value of PWV corresponds to lower vessel compliance and distensibility.²⁸ CIMT is defined as the thickness of the innermost two layers of an artery wall, tunica intima and tunica media (Figure 2-1(b)).²⁹ Assessing carotid arteries can identify the area of increased wall thickness, which represents a hallmark of atherosclerosis.

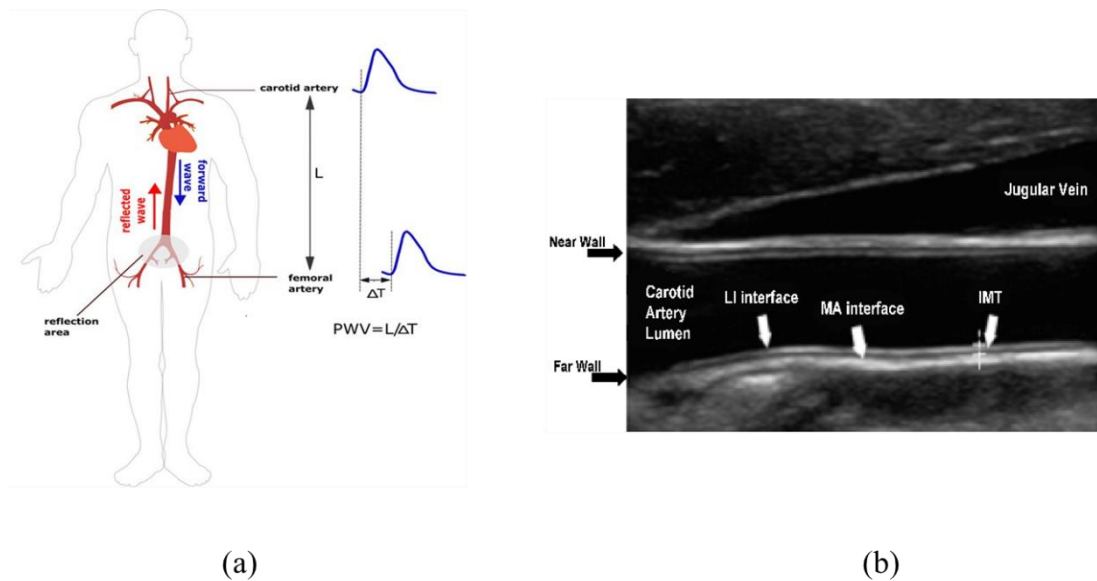


Figure 2-1 Assessment of pulse wave velocity and carotid intima-media thickness

(a) Pulse wave velocity (PWV); PWV is calculated as the surface distance between the two recording sites L (metres) divided by the time delay between two waveforms Δt (seconds); (b) B-mode ultrasound still image of the carotid artery; IMT: intima-media thickness, LI: lumen-intima, MA: media-adventitia. Figure adapted from ref.^{30,31}.

2.2.4.2 Assessing large arteries enhances CVD risk prediction

Mounting evidence has shown the value of these preclinical assessments in CVD risk management. A systematic review with data from 17,635 individuals (10% of whom had experienced CVD events) has provided evidence that PWV improves model fit and reclassifies risk for future CVD.³² The pooled hazard ratios (HRs) per one standard deviation (SD) higher in $\log_e$ PWV increased the risk for coronary artery disease by 23% (95% CI 1.11 to 1.35) and the risk for stroke by 28% (95% CI 1.16 to 1.42), after adjusting for traditional CVD risk factors.³² Interestingly, the predictive value was stronger in younger than older subjects.³² This review found that adding PWV into risk prediction models also increased the number of participants who were correctly classified, particularly among younger individuals at intermediate risk, and improved the overall 10-year classification by 13%.³²

Systematic reviews and meta-analyses also show that CIMT improves CVD risk prediction.^{33, 34} For example, Lorenz and colleagues pooled results from eight studies with a total of 37,097 participants and found that the age- and sex-adjusted

overall estimates of the relative risk of myocardial infarction and stroke were 1.15 (95% CI 1.12 to 1.17) and 1.18 (95% CI 1.16 to 1.21) respectively, per 0.10 mm common CIMT difference.³³ Den Ruijter and colleagues had similar findings when pooling data from 14 population-based cohorts with 45,828 individuals of median follow-up of 11 years.³⁴ They additionally compared the predictive value of CIMT with traditional risk prediction models and found that, for people with intermediate risk, adding CIMT to the model may improve the 10-year classification by 3.6% (95% CI 2.7 to 4.6).³⁴

2.2.4.3 Limitations of assessing large arterial phenotypes clinically

Although the preclinical large arterial phenotypes do improve CVD risk prediction, they are not yet performed routinely in clinical settings. There are several major concerns. Firstly, there are no unified measurement methods.³⁵ Lack of standardisation across studies limits data synthesis and meta-analyses.³⁴ Secondly, patients have been shown to report feelings of discomfort during the process of measurement; for example, it is not easy to assess the pulsation from obese patients.³⁶ Thirdly, there are cost-effectiveness concerns. Den Ruijter and colleagues found that adding a single CIMT measure to routine evaluation of coronary artery disease risk resulted in small health benefits: 1-3% low absolute risk of myocardial infarction in asymptomatic 50–59 year old men and women but with an additional cost of US\$100-300 per test.³⁷ It also takes time for the health benefits to outweigh the initial CIMT measurement costs – the cost-effectiveness will be acceptable for men within 30 years and for women within 10 years.³⁷ Fourthly, these measures only assess the preclinical status of large arteries without considering other components of the circulation.

Because of these concerns, the assessment of preclinical large arterial phenotypes has not been widely used in practice. With the development of technology, the phenotypic measures of small vessels from microcirculation can be assessed and quantified. One way to measure this is through quantification of microvascular parameters, which may add value to CVD management and are introduced in the following section.

2.2.5 Microvascular parameters may offer a way forward

The small vessels in the microcirculation have attracted much less attention in terms of their potential value to CVD prevention. This seems surprising, given that at any given time, only 25% of our blood is in the macrocirculation, with the remaining 75% circulating through the microcirculation³⁸ and shouldering distinct responsibilities in the body. The microvasculature has been essentially invisible due to technical limitations for a long time. Only recently has the potential value of microvascular parameters been uncovered with the development of advanced visualisation and quantification technologies.

In this section, I first introduce the physiologic function of microcirculation. Secondly, I review the pathophysiological connection between micro- and macrocirculation and their shared mechanisms, such as hyperglycaemia, dyslipidaemia and hypertension. This knowledge then informs the aims of this thesis by raising the possibility that the microcirculation may provide additional information for CVD risk assessment.

2.2.5.1 General features of microcirculation

The primary role of human microcirculation is to optimise the exchange of gases, nutrients and metabolites between blood and surrounding tissues. In other words, its main function is to meet metabolic demands for organs, tissues, and most cells of the body through blood flow. A key secondary role of the microcirculation is to avoid large fluctuations in hydrostatic pressure via capillary modulation. This is vital as 70–90% of the systemic arterial pressure may be delivered directly to the microcirculation. This makes the microcirculation essential in determining the local and overall peripheral resistance and hence influencing blood pressure.^{39, 40} A well-modulated microcirculation is required for the vascular system to fulfil its fundamental role.

Microvasculature comprises the small arterioles, venules and capillaries with diameters from about five to a few hundred μm .⁴¹ In the microvasculature, multiple abnormalities may occur in the structure, including rarefaction of arterioles, capillaries and venules, decreased arteriolar diameter, and increased wall-to-lumen ratio of small arteries.⁴² These structural changes of microvasculature are not only

related to microvascular disease but also macrovascular disease, such as coronary artery disease.⁴³

2.2.5.2 Cross-talk between micro- and macrocirculation

Abnormalities in microcirculation may lead to macrocirculation damage, which may happen in a vicious cycle, as depicted in Figure 2-2. Microcirculation plays an important role in stabilising permeability, vascular tone, and hemostasis.⁴⁴ Structural alterations of the microvasculature (as is common in diabetes) can result in the elevation of peripheral resistance and increased blood pressure.⁴⁵ Higher blood pressure increases large arterial stiffness, which may in turn alter blood pressure and flow patterns, leading to an increase in pressure and flow pulsatility.⁴⁶ The increase in pulsatility will cause damage to the microcirculation.⁴⁷ Furthermore, increased arterial stiffness favours the development of arterial structural changes, such as increased CIMT.⁴⁵ These changes may eventually lead to adverse CVD events.

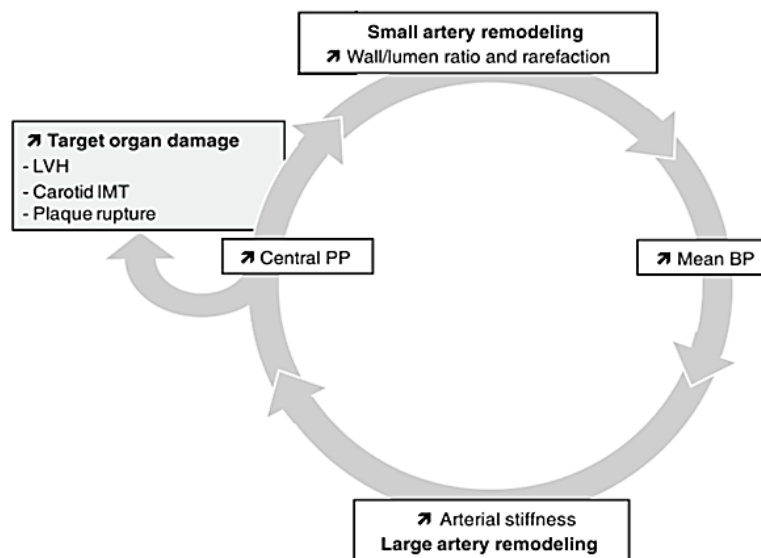


Figure 2-2 A vicious cycle of aggravation between micro- and macrocirculation

Abbreviations: BP, blood pressure; IMT, intima-media thickness; LVH, left ventricular hypertrophy; PP, pulse pressure. Image initially published in ref.⁴⁸; reproduced with permission.

2.2.5.3 Micro- and macrovascular disease frequently coexist

Historically, diseases have tended to be classified based on distinct parts of the body.⁴³ For example, hypertension mainly influences large arteries, such as increased arterial stiffness, while diabetes primarily leads to small vessel damage, such as diabetic retinopathy. The two conditions were classified based on changes in the structure and function of large and small arteries, respectively.⁴³ With the recognition of ‘cross-talk’ between micro- and macrocirculation, these diseases were found to be interrelated. In particular, concurrent arterial damage can be seen in both small arteries (e.g., in heart, brain, retina, kidneys) and large arteries in hypertensive individuals. Similarly, long-term follow-up studies in patients with type 2 diabetes have shown that microvascular disease complications (e.g., diabetic retinopathy) are associated with future risk for macrovascular disease (e.g., myocardial infarction).⁴⁹ Additionally, the coexistence of both micro- and macrovascular disease was associated with a 2.06-fold higher 10-year risk of death than people who had only one disease.⁴⁹

2.2.5.4 Micro- and macrovascular damage may share mechanisms

Risk factors for CVD are well recognised, as outlined in Section 2.2.3. It has been found that microvasculature is also determined by these risk factors, and shared mechanisms may initiate and promote both micro- and macrovasculature abnormality.⁵⁰ For instance, hyperglycaemia, dyslipidaemia, hypertension and hyperinsulinemia could lead to endothelial dysfunction, which presents in both small and large vascular damage. Hypercholesterolemia-induced inflammatory events have been seen not only in large arteries, such as coronary arteries, but also in the microvasculature.⁵¹ Thus, examining microcirculation could provide additional information when discussing the risk of CVD.

2.3 PART TWO: Retinal microvasculature — a window to the cardiovascular system

Although microcirculation is known to be important in cardiovascular health, its role is not fully elucidated. The retina is a unique ‘window’ that allows the local microvasculature to be visualised directly and noninvasively. It is now realised that

the retinal imaging technique offers an exceptional opportunity for the investigation of not only the retinal but also, by inference, the systemic microvasculature. Retinal microvascular parameters can be quantified from a photograph of the retina that shows its microvasculature. These parameters have been found to predict CVD events, especially coronary artery disease and stroke. New hand-held retinal cameras and auto-scoring deep learning system mean that measuring retinal microvascular parameters is becoming ever simpler and with excellent reproducibility. It moves the application of retinal microvascular parameters towards a viable CVD risk assessment approach across the life course.

However, it remains unclear when in the life course retinal vascular and cardiovascular health begin to co-vary. Understanding this could help researchers and clinicians assess the utility of retinal microvascular parameters as a tool to assist with CVD risk prediction and management.

In this section, I provide a detailed literature review on measuring microvascular parameters from the eyes; how these parameters change across the life course; the associations of retinal microvascular parameters with clinical CVDs; and preclinical cardiovascular health, measured by large arterial phenotypes.

2.3.1 Microvasculature can be seen from the retina

Advanced technology has been developed to allow microcirculation to be measured in animal, clinical and epidemiological studies.⁵² The assessment of microcirculation can be performed on different types of organs and tissue, according to the technique and device used. For example, Laser Doppler Flowmetry can measure blood flow of microcirculation on any area of intact skin, while videomicroscopy can provide a direct view of microcirculation through organs with a thin epithelial layer, such as the sublingual region.⁵³ However, these assessments are not ideal for studying CVD risk due to equipment limitations.⁵³ The major sites of target organ damage from CVD are microcirculation of the heart, brain and kidneys, which are difficult to measure noninvasively. Thus, the assessment of microcirculation should be a proxy for target organ damage in these areas. Researchers have found that assessing the retinal microcirculation from the eyes could be the window to study CVD risk, given

the physiological features of the human retina and the fact that assessment is easy to undertake.

2.3.1.1 Characteristics of the human retina

Physiologically, ocular structures of the retina are optically transparent for image formation, meaning that the retinal tissue can be visualised from the outside and is accessible for imaging noninvasively (Figure 2-3).⁵⁴ Additionally, the retina is a highly metabolically active tissue with a double blood supply. This function allows direct noninvasive observation of the circulation.⁵⁴

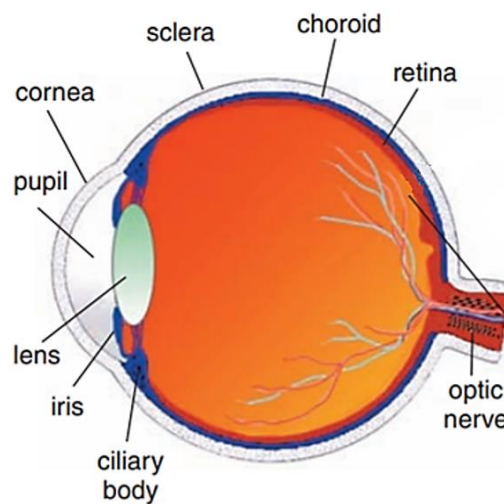


Figure 2-3 A human eye structure (adapted from ref.⁵⁵)

2.3.1.2 Retinal microvasculature is a surrogate for systemic microvasculature

The typical features of the retina provide researchers with a spot to look into the subtle variations of retinal microvasculature noninvasively. There are several reasons to support the assessment of retinal microvasculature in CVD-related research. One of the essential reasons is the homology between the retinal microvasculature and other microvascular beds. For example, retinal vascular signs vary with hypertensive arteriolar changes in the brain and myocardium.^{56, 57} There is also a close relationship between retinal and cerebral microvasculature, as they have a common origin: the internal carotid artery. The retinal arteriole pathological

changes parallel the small cerebral arteries, which cause white matter lesions and may lead to stroke.⁵⁸

Investigating retinal microvascular variation could also provide a proxy for other vascular beds that are difficult to assess, such as coronary microvasculature. It has been shown that retinal arteriolar narrowing is strongly associated with the presence and severity of coronary artery occlusion and reduced myocardial perfusion.⁵⁹⁻⁶¹ Thus, retinal microvasculature has become a surrogate marker to examine the changes in systemic microcirculation. Retinal microvascular parameters, quantified using computer-based software, can help to understand the variation in retinal microvasculature with the associations of CVD.

2.3.2 Standard microvascular parameters can be quantified from retinal images

2.3.2.1 Retinal imaging technique

The most common way to measure retinal microvasculature is through imaging techniques. There are two widely used retinal imaging techniques: fundus photography and optical coherence tomography (OCT). Both techniques image the back of the eye. The invention of fundus photography occurred in the mid-19th century.⁶² This simple procedure involves an experienced operator taking photos, with or without mydriasis (e.g., dilation of the pupil), while the patient's chin and forehead are positioned against small cushioned rests. Figure 2-4 shows a retinal image taken by fundus photography.

Retinal images taken by fundus photography have been used for a long time to follow, diagnose and treat eye diseases, such as macular degeneration and glaucoma. In the 1890s, retinal vascular 'sign' was first described by Marcus Gunn in three patients with kidney disease and hypertension and 12 patients with stroke.⁶³ From that, more and more evidence showed that changes seen on retinal images are associated with many systemic diseases such as diabetic retinopathy from diabetes, hypertensive retinopathy from long-term hypertension and multiple sclerosis.⁵⁴ Nowadays, the retinal image is used as a key method to monitor diabetic retinopathy. Ophthalmologists read the abnormal signs on the image (e.g.,

microaneurysms, haemorrhages, hard exudates and cotton-wool spots) to evaluate the severity of microvascular damage.⁶⁴

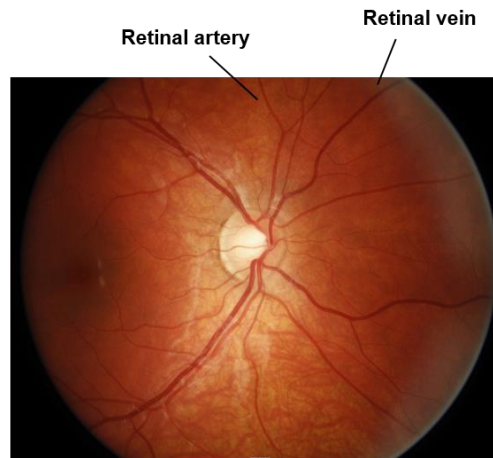


Figure 2-4 Retinal image taken by fundus photography

The other retinal imaging technique, OCT, was invented in 1991.⁶⁵ This technique offers three-dimensional information on retinal microvasculature and the most recently developed OCT angiography even allows the retinal capillary circulation to be visualised.⁶⁶ The human retina is supplied by both retinal vessels and choriocapillaris (e.g., the large capillary network beneath the retina). OCT angiography may provide extra information on the development of CVD than retinal vessels, which is under investigation.⁶⁷

Evaluation of retinal microvascular parameters most commonly relies on images obtained from fundus photography; this is how we analysed microvascular parameters in this PhD project. Although OCT can also extract data on retinal microvascular parameters, it is less studied than fundus photography. The data extraction is not straightforward, as the analysis of retinal microvasculature requires direct and indirect signs of the vessels;⁶⁷ there are differences between retinal microvascular parameters analysed from OCT and retinal fundus images; furthermore, how these parameters perform in CVD risk assessment has been less studied.^{68, 69} Lastly, the cutting-edge artificial intelligence based on deep learning algorithms discovered the CVD risk information contained in retinal fundus images (see next section). In this thesis, the retinal microvascular parameters discussed later are analysed from retinal fundus images.

2.3.2.2 Deep learning system uncovers CVD risk from retinal images

Deep learning system consists of a neural network of interconnected layers of nonlinear processing units.⁷⁰ It is the most commonly used machine learning method for data extraction and transformation and has attracted tremendous research interest in medicine, especially ophthalmology.⁷¹ Recently, Peng and colleagues developed a deep learning system, which unexpectedly predicted traditional CVD risk factors from retinal fundus images.⁷² The system was trained with data from 284,335 United Kingdom (UK) Biobank participants and 236,234 United States (US) EyePACS participants. The study also validated the system in two independent datasets from the UK Biobank (n= 12,026) and the EyePACS database (n= 999). The results showed that the system could accurately predict a series of traditional CVD risk factors, such as age, smoking and systolic blood pressure, with area under the receiver operating characteristic curve (AUC) up to 0.97.⁷² The deep learning system also predicted the risk of major adverse cardiac events within five years, with an AUC of 0.70.⁷² These findings provide further support that retinal images contain CVD risk information.⁷³ In the next section, I outline the methods of measuring retinal microvascular parameters from retinal fundus images.

2.3.2.3 Microvascular parameters quantified from retinal images

The image quantification technique was developed to provide more information from retinal images – specifically, to quantify the microvascular parameters of the retinal microvasculature. Parr and Spear first proposed calculating the central retinal equivalent for arteries as early as 1974.⁷⁴ This was extended to retinal veins by Hubbard and colleagues in 1999.⁷⁵ Over the last two decades, computer-based software has been developed based on the proposed algorithm to include retinal vessel diameters measured in epidemiological and clinical studies (Table 2-1). The principle of vessel diameter analysis is to measure the red blood cell column width inside the vessels.⁷⁶ In addition to vessel diameters, advanced systems can analyse other features of the retinal microvasculature, such as geometric parameters: fractal dimension, tortuosity and branching patterns. The semi-automated systems only allow vessels in a specific range to be analysed, while the fully automated systems can analyse vessels across the whole retina automatically.

Table 2-1 Retinal microvascular parameter analytic platforms

Software	Research & development organisation	Feature	Microvascular parameters analysed
Retinal Analysis (RA)	Department of Ophthalmology & Visual Sciences, University of Wisconsin, US	Semi-automated	Retinal arteriolar calibre, retinal venular calibre
Integrative Vessel Analysis (IVAN)	University of Wisconsin, US	Semi-automated	Retinal arteriolar calibre, retinal venular calibre, arteriole-to-venule ratio
Singapore “I” Vessel Assessment (SIVA)	Singapore Eye Research Institute in collaboration with the National University of Singapore, Singapore	Semi-automated	Retinal arteriolar calibre, retinal venular calibre, arteriole-to-venule ratio, fractal dimension, tortuosity, branching patterns
Vessel Assessment and Measurement Platform for Images of the Retina (VAMPIRE)	University of Dundee in collaboration with the University of Edinburgh, UK	Semi-automated	Retinal arteriolar calibre, retinal venular calibre, arteriole-to-venule ratio, fractal dimension, tortuosity, branching angles, bifurcation
Quantitative Analysis of Retinal Vessel Topology and siZe (QUARTZ)	Collaborated work by researchers from the National University of Sciences and Technology, Pakistan, St. Georges University of London, and Kingston University London, UK	Fully automated	Retinal arteriolar width, retinal venular width, tortuosity
Automated Retinal Image Analyser (ARIA)	Chinese University of Hong Kong, China	Fully automated	Retinal arteriolar calibre, retinal venular calibre, tortuosity, symmetry of branches and bifurcation angles

It has been acknowledged that the absolute (raw) measurements of the retinal microvascular parameters have poor to moderate correlations across different software.⁷⁷ This may be due to differences in vessel tracing algorithm protocol and retinal vessel border definition.⁷⁷ However, the strength of associations between traditional CVD risk factors and retinal microvascular parameters are similar.⁷⁸ This means that although the absolute value analysed from different software cannot be used interchangeably, their findings in CVD-related research are comparable. Thus, in this thesis, we assessed the retinal microvascular parameters in two systems: Integrative Vessel Analysis (IVAN, University of Wisconsin, Madison, US) and Singapore “I” Vessel Assessment (SIVA, Singapore Eye Research Institute in collaboration with the National University of Singapore, Singapore). These systems have been validated in large population-based cohort studies with robust reproducibility.

The IVAN software was developed and validated in the Atherosclerosis Risk in Communities Study, a large population-based cohort study, which examined the retinal vascular signs of atherosclerosis in 11,114 middle-aged adults.⁷⁵ The software measures arteriolar and venular diameters (retinal vascular calibre) from retinal images. It offers two unique features: the assessment is quick (around 2–5 minutes per image scoring), and results are of substantial reproducibility. In several large population-based epidemiological studies, the intra- and inter-grader correlation coefficient ranged from 0.67 to 0.99, proving this image scoring tool to be a highly precise and reliable research tool for objective assessment of structural retinal vascular changes.⁷⁹

The SIVA software was developed to provide more information on retinal vessel architecture, in addition to retinal vascular calibre, which has been increasingly used in recent years.⁸⁰ SIVA measures a wider range of vessels on the retinal image and additional retinal geometric parameters, such as: 1) fractal dimension, which summarises the whole branching pattern of the retinal microvascular tree; 2) tortuosity, which is the relative length variation between the curvatures of the vessel and the shortest distance of the vessel path; 3) branching angle, which is the first angle subtended between two daughter vessels at each bifurcation.⁸¹

These parameters could inform more geometric variation of the microvasculature. SIVA is a semi-automated software that requires the assistance of trained graders to assess each image. The reproducibility of each new parameter is high in adults, with intraclass correlation coefficient (ICC) ranging from 0.78 to 0.99.⁸²

The retinal microvascular parameters analysed from these tools provide us with an opportunity to examine the characteristics of retinal microvasculature across the life course. How these parameters change with age is outlined in the following section. These parameters can help us understand how cumulative microcirculatory changes related to an individual's lifetime exposure to lifestyle and environmental factors. Thus, retinal microvascular parameters could serve as a tool to measure preclinical cardiovascular health across the life course. The evidence of retinal microvascular parameters with CVD in adults and with cardiovascular health in different life stages will be shown in Sections 2.3.4 and 2.3.5, respectively.

2.3.3 Retinal microvascular parameters evolve across the life course

Before examining the associations of retinal microvascular parameters with CVD, researchers have tried to understand the natural history of these parameters. Most of the evidence relates to retinal vascular calibre, with less evidence on the more novel geometric parameters. The overall trend for both retinal vascular calibre and geometric parameters is that they decrease with age (e.g., smaller arteriolar and venular calibre, lower fractal dimension and straighter vessel tortuosity). Details are summarised below.

2.3.3.1 Retinal vascular calibre across the life course

Retinal vascular calibre has been assessed in generally healthy participants across the life course, including one study in infancy. In 20 healthy weight Australian infants born at term, Kandasamy and colleagues showed that on average retinal arteriolar and venular calibre was 85.5 μm and 130.0 μm respectively.⁸³ Two large cohorts ($n > 1,000$) were conducted separately in Australian and Dutch children aged about six years. In these cohorts retinal arteriolar and venular calibre were nearly double that of Kandasamy's newborn data (mean arteriolar calibre 159.0 to 159.6 μm , mean venular calibre 219.0 to 222.3 μm).^{84, 85} Several studies in mid-childhood (e.g., age 10–12 years), including the Child Health CheckPoint study, on which this

thesis is based, suggest that there is little change in the natural history of retinal arteriolar and venular calibre at age 11–12 years (mean arteriolar calibre 149.3 to 159.5 μm , mean venular calibre 217.2 to 231.1 μm).⁸⁶⁻⁸⁹ To date, there are no studies in children aged 13–18 years.

There is also a dearth of studies in early- and mid-adulthood. The Child Health CheckPoint study, one of the few studies to consider mid-adulthood, showed that at mean age 44 years, retinal arteriolar and venular calibre are lower than what was reported in mid-childhood.⁸⁹ In late adulthood (age range 59–78 years), findings are mixed. Some studies suggest that retinal arteriolar and venular calibre are larger in adults than in children,^{90, 91} while others suggest the contrary, with smaller calibre measurements in adults compared to children.^{92, 93}

In order to visualise the overall life course trend, I plotted 15 population-based studies with large sample sizes (e.g., $n > 1,000$) into a graph (Figure 2-5).⁸⁹ From the fitted two exploratory linear regression models on the graph, we can see that from childhood to adulthood, venular calibre narrows slightly at a rate of approximately -1.2 μm (95% CI -0.37 to 0.14) per decade, while arteriolar calibre decreased slowly at a rate of -0.02 μm (95% CI -0.21 to 0.17) per decade.⁸⁹

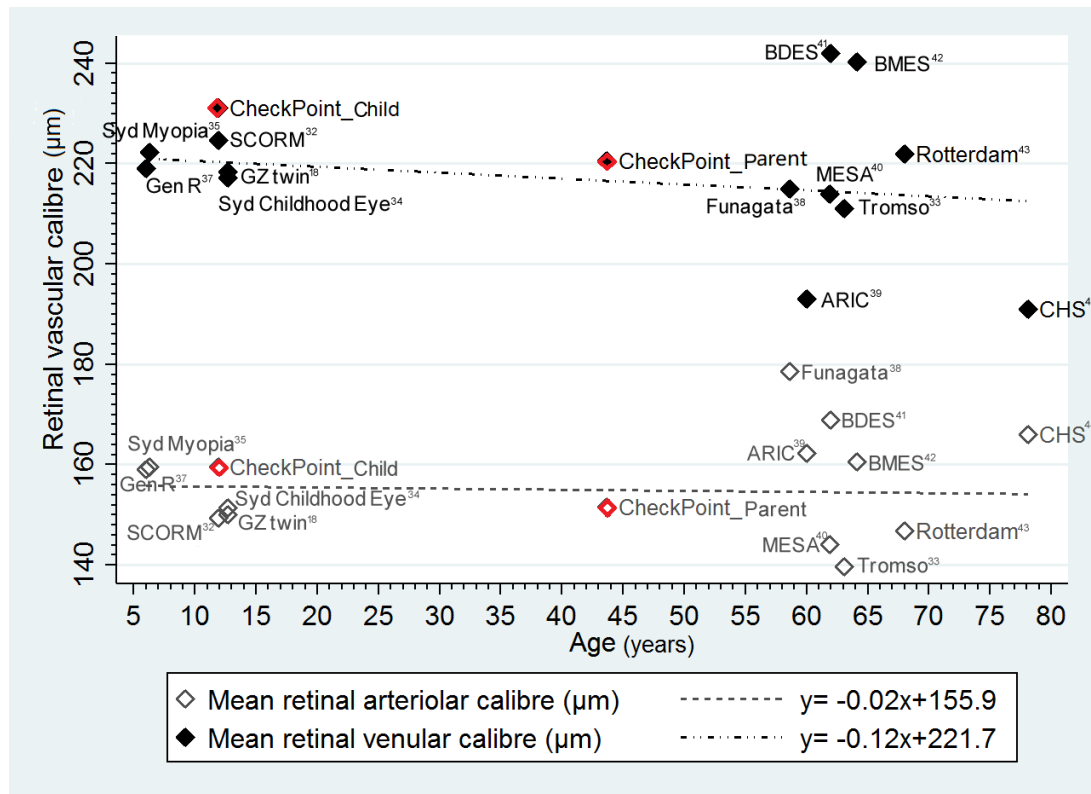


Figure 2-5 Retinal arteriolar and venular calibre change with age

The figure is adapted from ref.⁸⁹, reported by our team. Full texts can be seen in Appendix C. Abbreviations: Gen R, Generation R Study; Syd Myo, The Sydney Myopia Study; SCORM, Singapore Cohort Study of the Risk Factors for Myopia; CheckPoint_Child, Child Health CheckPoint data on children; Syd Chi, The Sydney Childhood Eye Study; GZ twin, The Guangzhou twin eye study; CheckPoint_Parent, Child Health CheckPoint data on parents; ARIC, Atherosclerosis Risk In Communities; MESA, Multi-Ethnic Study of Atherosclerosis; BDES, Beaver Dam Eye Study; Troms, The Tromsø Eye Study; BMES, Blue Mountains Eye Study; Rotterdam, The Rotterdam Study; CHS, Cardiovascular Health Study.

2.3.3.2 Retinal geometric parameters across the life course

Much less is known about the natural history of retinal geometric parameters compared to the vascular calibre. Only one study was conducted in children. Among 10-year-old Malaysians (n= 166), mean arteriolar and venular fractal dimension were 1.22 and 1.19 respectively, and mean arteriolar and venular simple tortuosity were 1.09 and 1.10 respectively.⁹⁴ Two studies in adults – the Flemish Study on Environment, Genes and Health Outcomes (n= 240, age range 19–86 years)⁸¹ and the Australian Heart Eye Study (n= 1,187 adults, mean age 61 years)⁹⁵ – suggested that the fractal dimension and tortuosity decrease with age (e.g., become sparser in

fractal dimension and straighter in vessels trace). For example, in the Flemish study, linear regression considering other CVD risk factors showed that fractal dimension (β coefficient -0.002, 95% CI -0.021 to -0.008) and simple tortuosity (β coefficient -0.006, 95% CI -0.011 to -0.001) decreased with every decade of age increase.⁸¹

2.3.4 Retinal microvascular parameters are implicated in CVD

2.3.4.1 Retinal vascular calibre and CVD

The association of retinal vascular calibre (quantified via computer-based software IVAN) with CVD has been widely studied in population-based cohorts. The first study to look at this association was the Atherosclerosis Risk in Communities Study, a prospective study that investigated the aetiology of atherosclerosis and its clinical sequelae.⁹⁶ A total of 10,470 participants (mean age 60 years) without CVD events had retinal photos taken in 1993–1995.⁹⁷ At its follow-up, roughly 16 years later, there were 6,515 CVD events and deaths. In both sexes, those with a higher risk of death and stroke were participants with wider retinal venular calibre (HR 1.13, 95% CI 1.08 to 1.18; HR 1.18, 95% CI 1.07 to 1.31; per one SD increase) and narrower arteriolar calibre (HR 1.06, 95% CI 1.01 to 1.11; HR 1.14, 95% CI 1.03 to 1.26; per one SD decrease).⁹⁷

This finding has been replicated in different cohorts and confirmed in a recent meta-analysis.⁹⁸ The individual participant meta-analysis examined associations between retinal vascular calibre and the risk of coronary artery disease 5 to 14 years later.⁹⁸ Of the 22,159 participants (mean age 62 years) from the six population-based prospective studies included in the meta-analysis, 2,219 (10.0%) had experienced incident coronary artery disease events.⁹⁸ Wider retinal venular and narrower arteriolar calibre were each associated with an increased risk of coronary artery disease in women but not men. In women, the pooled multivariable-adjusted HRs were 1.16 (95% CI 1.06 to 1.26) per 20 μ m increase in venular calibre, and 1.17 (95% CI 1.07 to 1.28) per 20 μ m decrease in arteriolar calibre.⁹⁸

The researchers used the same six population-based cohorts to examine whether retinal vascular calibre is associated with the incidence of stroke.⁹⁹ They found that 945 (4.5%) patients had experienced an incident stroke.⁹⁹ From the meta-analysis, wider retinal venular, but not arteriolar, calibre predicted stroke (pooled HR 1.15,

95% CI 1.05 to 1.25, per 20 μm increase in venular calibre), with no substantial differences between the sexes.⁹⁹

2.3.4.2 Retinal geometric parameters and CVD

In addition to retinal vascular calibre, novel geometric parameters have also been shown to be associated with CVD. For example, in a population-based cohort of 3,303 middle-aged participants, those with suboptimal fractal dimension (lowest and highest quartiles) had a 50% higher 14-year coronary artery disease mortality than those with optimal fractal dimension, after adjusting for traditional CVD risk factors.¹⁰⁰ In the Australian Heart Eye Study survey, 104 (8.9%) of the 1,680 participants had arterial fibrillation; participants in the second tertile of fractal dimension compared to the highest group had a 92% higher likelihood of having atrial fibrillation after adjusting for potential confounders.¹⁰¹ Similarly, in a case-control study with acute ischemic stroke patients (557 cases, 557 controls), decreased fractal dimension and increased tortuosity predicted ischemic stroke events.¹⁰² The results showed that a one SD decrease in the arteriolar and venular fractal dimension was associated with a higher risk of stroke (odds ratio [OR] 2.28, 95% CI 1.80 to 2.87; OR 1.80, 95% CI 1.46 to 2.23), while a one SD increase in arteriolar and venular tortuosity was associated with a 56% and 49% higher risk of stroke (OR 1.56, 95% CI 1.25 to 1.95; OR 1.49, 95% CI 1.27 to 1.76).¹⁰²

2.3.5 Retinal microvasculature and large arterial phenotypes relationships (Aim 1)

Physiologically, the retinal microvasculature and large arteries share elements of pulsatile transmission of pressure and flow as part of the circulatory system, although they represent different sections of the vascular tree.⁴³ Retinal vascular calibre, like large arterial phenotypes, are influenced by traditional CVD risk factors,^{79, 103, 104} but the degree and rate that the small and large vascular phenotypes impact on one another is unclear. Understanding the association between the two kinds of preclinical phenotypes could help us determine whether damage to both parts of the vascular tree usually happens in unison, or whether variation in each profile occurs at different rates. This knowledge also help us understand whether assessing both could improve CVD risk prediction. In addition, the common

measures of retinal microvasculature and large arteries have both advantages and disadvantages in usage, see Table 2-2. Exploring their association may inform the practical utilisation.

Table 2-2 Comparison between micro- and macrovascular measures

Measure	Description	Vessel site	Device	Clinically availability of device	Advantages	Limitations
Pulse wave velocity (PWV)	PWV is the velocity at which the blood pressure pulse propagates through the circulatory system, usually an artery or a combined length of arteries. It measures arterial stiffness.	Large arteries; most commonly carotid and femoral artery.	Magnetic resonance imaging, vascular ultrasound, echotracking, mechanotransducers and tonometric devices. ²⁷	MRI and ultrasound are available.	Highly reproducible; widely validated as the gold standard for measuring arterial stiffness.	The device is expensive; measurement requires some skills; measurement causes discomfort. ¹⁰⁶
Carotid intima-media thickness (CIMT)	CIMT is the thickness of the inner two layers of the carotid artery—the intima and media. It measures the extent of atherosclerosis.	Carotid artery.	High resolution ultrasound or magnetic resonance imaging	Available.	Radiation-free; relatively easy to acquire regardless of patient anatomy; relatively inexpensive, and perform on widely available equipment.	Only presents arterial wall and fails to capture the complexity of plaque features but only; not easy to perform in children.
Retinal microvascular parameters	Parameters quantified from retinal fundus images, including retinal vascular calibre, branching angle, tortuosity, and fractal dimension. The variation of retinal microvascular parameters indicates the health of systemic microvasculature.	Retinal blood vessels.	Fundus camera	Available.	Low cost technology; highly reliable; measurement without contact; can be used in mass screening.	The scoring process is semi-automated including subjective decisions.

Several studies have explored the association between retinal vascular calibre and large arterial phenotypes, such as CIMT and PWV, but their findings are inconsistent. Therefore, I conducted a systematic review to synthesise current literature and pooled the research findings to quantify the associations using meta-analysis methods. The systematic review included 26 studies, with the most commonly measured arterial phenotypes being PWV (9 studies) and CIMT (10 studies). The systematic review and meta-analysis publication can be found in Chapter 4. The findings are summarised in the following section.

2.3.5.1 Summary of findings from systematic review to inform Aim 1

Nine studies examined the association of retinal vascular calibre and PWV. Six studies showed that narrower retinal arteriolar calibre was associated with higher PWV. Four studies reported the association of retinal venular calibre and PWV,⁸⁵ of which two showed significant associations. It is noteworthy that one study was performed in children aged six.⁸⁵ This study suggested that wider venular calibre was associated with higher PWV (β coefficient 0.04, 95% CI 0.01 to 0.08, per SD increase in retinal venular calibre).⁸⁵ Other measures of arterial function, such as arterial compliance and arterial distensibility, showed significant associations with narrowing arteriolar calibre but not venular calibre.^{107, 108}

All studies that measured the association of retinal vascular calibre and CIMT were restricted to late adulthood. Of the 10 studies, seven found narrowing retinal arteriolar calibre or lower arteriole-to-venule ratio was associated with increased CIMT.¹⁰⁹⁻¹¹⁵ Five studies reported the association between retinal venular calibre and CIMT, but only two found that wider retinal venular calibre was significantly associated with greater CIMT.^{115, 116} However, other structural measures, such as aorta calcification and carotid plaque, were not associated with retinal arteriolar calibre or venular calibre.¹⁰⁹

2.3.5.2 Summary of meta-analysis findings to inform Aim 1

Five adult studies with eligible data were included in the meta-analysis, with the pooled results showing that narrower arteriolar calibre was associated with faster PWV (correlation coefficient (r) -0.17, 95% CI -0.25 to -0.10).¹¹⁷ The random-

effects meta-analysis showed little evidence of an association between retinal venular calibre and PWV ($r = -0.03$, 95% CI -0.23 to 0.07).¹¹⁷

The meta-analysis also included five adult studies examining CIMT, with results showing that narrower arteriolar calibre was associated with greater CIMT ($r = -0.05$, 95% CI -0.09 to -0.02).¹¹⁷ As with PWV, there was little evidence of an association between retinal venular calibre and CIMT.¹¹⁷

2.3.5.3 Limited knowledge of small and large vascular phenotype associations across the life course

Studies examining the association between retinal vascular calibre and large arterial phenotypes have predominately focused on adults in the late stage of the life course. They show that large arteries may share some pathophysiologic processes with the microvasculature late in life, but when this shared association begins is still unknown. What is known is that CVD risk factors in childhood are related to adverse changes in preclinical large arterial phenotypes in later life.^{118, 119} Thus, examining the association in younger populations may help to understand the nature of the relationship between retinal microvascular parameters and preclinical large arterial phenotypes.

If the two kinds of vascular phenotypes already co-vary prior to emergent disease, this would suggest that the relationship is not solely the result of dysregulation, potentially advancing the understanding of CVD pathogenesis. With technology and artificial intelligence putting retinal imaging into the mainstream clinical and population research realm, microvascular status could potentially enter precision prediction models for future CVD.¹⁸ Before this could take place, we would need to understand the small vessels' unique and shared contributions with more traditional large artery measures.

Most previous studies have only assessed *one* aspect of large arterial health (e.g., functional or structural phenotypes), but not both in the same individuals.¹⁶

Assuming that large arterial dysfunction precedes structural changes, retinal microvascular associations with large arterial function might emerge earlier than with large arterial structure.¹⁹ Thus, investigating the association between large

arterial function and structure together with retinal microvasculature in the same participants could help us understand the temporal sequence in the association.

In this PhD project, I have a unique opportunity to explore these research questions using data from the Child Health CheckPoint study. The first aim of this thesis will examine associations between retinal vascular calibre and preclinical large arterial phenotypes in Australian children aged 11–12 years and mid-life adults (the children's parents).

2.4 PART THREE: Contributors to retinal microvascular variation

Although retinal microvascular parameters could be a potential risk predictor for CVD, the underlying mechanisms of retinal microvascular alterations are yet to be fully elucidated. It is postulated that multiple processes can lead to changes in retinal microvascular parameters, such as genetics and the shared traditional CVD risk factors.

In the next section, I first outline the current understanding of the physiological and pathophysiological features of the retinal microvasculature. Secondly, the potential determinants of retinal microvascular parameters are reviewed. I then further focus on two important factors, obesity and inflammation, that inform Aims 2 and 3 of this thesis.

2.4.1 Mechanisms of retinal microvascular variation

2.4.1.1 Physiological features of retinal microvasculature

The retinal blood vessels are different from the large blood vessels, which consist of three layers with collagen and elastin filaments. There are two layers of retinal blood vessels. One is the endothelium, which is the common structural component of all small blood vessels throughout the body.¹²⁰ The other is the perivascular cells (e.g., pericytes), which are smooth muscle cells or mural cells that envelop the surface of the vascular tube.¹²¹ The retina has the highest pericyte density in the body.¹²¹ The key physiological functions of the endothelium are maintaining antithrombotic surface and the vasomotor tone, while pericytes can control microvascular remodelling and angiogenic switching.¹²⁰

Retinal vessels present a high adaptation ability and may present acute and chronic variation in response to stimuli. The acute adjustments involve changes in the vascular smooth muscle tone, while the chronic adjustments involve changes to the wall structure.¹²² Under systemic disease or chronic conditions, such as diabetes and hyperglycaemia, the loss of pericytes change the vascular structure and result in changes to vessel diameters.¹²¹ How these changes happen is described in the following section.

2.4.1.2 Pathophysiological changes of retinal microvasculature

Depending on the source of abnormality, the pathophysiological changes can be grouped as functional and structural. Functional abnormality is the dysfunction of the endothelium, while structural abnormalities are vascular remodelling and vascular rarefaction. Functional changes may lead to structural changes.

Functional decline in the endothelium is a common feature of microvascular pathophysiology.¹²³ Endothelial dysfunction results in reduced angiogenic capacity, impaired vasodilatory function and abnormal expression of adhesion molecules, with the latter amplifying the interaction between circulating factors and inflammatory cells.¹²³ The impaired endothelial function can contribute to the development of microvascular insulin resistance, which results in impaired capillary recruitment.¹²⁴ The reduction in capillary density can cause chronic tissue hypoxia, leading to sustained inflammation.¹²⁵ Beyond that, the functional abnormality influences the vascular structure and perivascular inflammation, altering the release and clearance of inflammatory cytokines.¹²⁶ The structural changes following the functional abnormality include thickening of basement membranes, stiffness of resistance arterioles and a decrease in lumen size.¹²⁷

The pathophysiological structural changes can be observed by measuring retinal microvascular parameters. With the exception of genetic factors discussed in the following section, the traditional CVD risk factors influence the variation of retinal microvascular parameters to varying degrees, as seen in Section 2.4.3.

2.4.2 Genetic factors and retinal microvascular parameters

2.4.2.1 Heritability of retinal microvascular parameters

The heritability of retinal vascular calibre has been reported with substantial correlation within families. For example, the parent-child correlations for retinal arteriolar and venular calibre in the Beaver Dam Eye Study (n= 341, age range 43–86 years) were 0.24 and 0.27 respectively;¹²⁸ in the Flemish Study on Environment, Genes and Health Outcomes (n= 174, aged 20+ years) the correlations were 0.13 and 0.20;¹²⁹ and in the Strabismus Amblyopia and Refractive Error in Singaporean Children study (n= 304, parent: mean age 40 years, child: mean age 9 years) they were 0.12 and 0.31.¹³⁰ Our Child Health CheckPoint team (n= 1,186 pairs, mean age 44 and 12 years) found similar parent-child correlations (0.22 and 0.23 for arteriolar and venular calibre).⁹⁰ Our stratified analyses also showed little difference between mother-child pairs (0.20 and 0.22 for arteriolar and venular calibre) and father-child pairs (0.32 and 0.29 for arteriolar and venular calibre).⁸⁹ The correlations of retinal vascular calibre for twins are higher than for parent-child pairs, with higher correlations observed in monozygotic twins (0.60 to 0.8 and 0.63 to 0.88 for arteriolar and venular calibre) than dizygotic twins (0.25 to 0.54 and 0.28 to 0.72 for arteriolar and venular calibre).^{88, 128, 131, 132} Similarly, in 67 pairs of monozygotic twins (mean age 70 years) the correlation coefficients of retinal geometric parameters were moderate to high (0.52 and 0.64 for arteriolar and venular fractal dimension, 0.71 and 0.69 for arteriolar and venular tortuosity).¹³³ The heritability of tortuosity was also lower in dizygotic twin pairs (correlation coefficient 0.10 to 0.56).¹³⁴

2.4.2.2 Genes in relation to retinal microvascular parameters

Genome-wide association studies have identified several genetic loci associated with retinal vascular calibre.^{131, 135} For example, the Beaver Dam Eye Study found that retinal arteriolar and venular calibre were linked to multiple genetic loci – some loci were unique for arterioles or venules, while others were common to both.¹³⁶ Furthermore, these genetic loci were found to overlap with genes that determined the pathophysiological process, such as hypertension, endothelial dysfunction and vasculogenesis.¹³⁶ Congruently, another study with 24,000+ multiethnic participants

from seven cohorts extended this finding; the single-nucleotide polymorphism rs201255422 was associated with retinal arteriolar calibre, as well as both systolic and diastolic blood pressure.¹³⁷ Thus, genetic factors can determine the retinal microvascular parameters and may be a fundamental factor in the association of retinal microvascular parameters with CVD risk factors.

2.4.3 Traditional CVD risk factors and retinal vascular calibre

As described in Section 2.2.3, smoking, poor diet, lack of physical activity, high blood pressure, high cholesterol and excess weight are well-established risk factors associated with CVD. In this section, the associations of these factors with retinal vascular calibre will be reviewed. Evidence on the retinal geometric parameters will be presented in Section 2.5

2.4.3.1 Smoking and retinal vascular calibre

Smoking is related to worse retinal vascular calibre in middle-age and older adults. Cross-sectionally, the Rotterdam study (n= 5,674, mean age 59 years) found that compared with non-smokers, current smokers had wider arteriolar (mean difference 5.0 μm , 95% CI 3.9 to 6.0) and venular calibre (10.0 μm , 95% CI 8.3 to 11.4). Former smokers also had wider arteriolar calibre (1.3 μm , 95% CI 0.4 to 2.3) and venular calibre (2.5 μm , 95% CI 1.2 to 3.9), but to a lesser extent.¹⁰⁹ The Blue Mountains Eye Study (n= 3,654, age 49 years+) confirmed the cross-sectional association of current and past smokers with wider arteriolar and venular calibre at baseline analysis.¹³⁸ The association persisted at the five-year follow-up, with heavy smokers showing the biggest change in venular calibre.¹³⁸ Hence, long-term smoking was found to have cumulative effects on retinal microvasculature. Fortunately, a Japanese study has shown that this cumulative effect can be mitigated by giving up smoking. Those who had ceased smoking for over 10 years had similar mean retinal arteriolar (128.4 vs. 128.3 μm) and venular calibre (195.8 vs. 195.4 μm) to participants who had never smoked.¹³⁹

2.4.3.2 Dietary intake and retinal vascular calibre

Some evidence showed that both the consumption of specific foods and long-term dietary intake patterns may influence retinal microvascular variation. For example, the population-based cross-sectional Sydney Childhood Eye Study (n= 888, mean

age 12.7 years) showed that consumption of higher long-chain omega-3 polyunsaturated fatty acid, fish and/or dairy food was associated with wider retinal arteriolar and narrower venular calibre (e.g., a better retinal microvascular profile), while consumption of soft drinks and carbohydrates was associated with the reverse.¹⁴⁰⁻¹⁴² In adults with higher cardiovascular risk, high consumption of low-fat milk, yoghurt, and cheese, and low consumption of red meat have also been associated with wider arteriolar and narrower venular calibre.¹⁴³ However, this finding was not replicated in a randomised control trial (RCT) in overweight adults with high CVD risk. The RCT assessed the influence of fruit and vegetable consumption (2/4/7 portions per day) on changes in retinal vascular calibre in 62 participants (mean age 57 years).¹⁴⁴ The intervention of fruit and vegetable consumption for 12 weeks did not change either retinal vascular calibre or the participant's overweight condition (body weight, total body fat, blood pressure or lipid profile).¹⁴⁴

In addition to specific food groups, overall food consumption patterns may have a cumulative risk for retinal microvasculature. Our study group generated dietary trajectories based on the 24-hour dietary recall survey of the Longitudinal Study of Australian Children from 2–3 to 10–11 years. We found that compared with children who followed an 'always healthy' trajectory, those who followed a 'never healthy' trajectory had similar retinal vascular calibre.¹⁴⁵ In contrast, the association was evident in an adult study. In the Irish Nun Eye Study (n= 1,233, mean age 76.3 years), unhealthy dietary patterns (defined as high intake of sugar and sweets, chips, high-fat dairy products and french fries) were associated with wider venular and narrower arteriolar calibre.¹⁴⁶ The discrepancy between the child and adult studies highlights the cumulative risk of food consumption patterns for worse retinal microvasculature and informs a window of opportunity for preventive intervention from childhood.

2.4.3.3 Physical activity, sedentary behaviour and retinal vascular calibre

The benefits of physical activity and negatives of sedentary lifestyles (often due to screen time) on retinal vascular calibre have been shown in both children and adults. The Sydney Childhood Eye Study (n= 1,492) found that, in children aged six years,

those who spent more time doing outdoor activities (the highest tertile) had wider retinal arteriolar calibre (mean difference 2.2 μm , 95% CI 0.65 to 3.71) than those in the lowest tertile (p for trend <0.01); and children who spent more time watching TV had narrower arteriolar calibre (highest vs. lowest quartile: mean difference 2.3 μm , 95% CI 0.73 to 3.92, p for trend <0.01).¹⁴⁷

Analogous associations were seen in adults. For instance, in the Australian Diabetes, Obesity and Lifestyle Study, a population-based cross-sectional study (n= 2,024, age 25+ years), those who watched TV for at least two hours per day had 4.71 μm wider venular calibre (95% CI 1.37 to 8.04) compared to those who watched less than two hours of TV per day.¹⁴⁸ As with the Atherosclerosis Risk in Communities Study (n= 12,492, mean age 60 years), they found that participants with higher levels of physical activity were about 10% less likely to have wider venular calibre (OR= 0.91, 95% CI 0.83 to 0.99).¹⁴⁹

2.4.3.4 Blood pressure and retinal vascular calibre

As discussed in Section 2.2.5.1, the key role of microcirculation is to regulate blood pressure, which is reflected in the association between blood pressure and retinal microvascular parameters. This finding is consistent across children and adults, with elevated blood pressure shown to have major impacts on retinal vascular calibre.⁸⁰

In a recent systematic review and meta-analysis by Kochli and colleagues, all 12 cross-sectional studies in children aged 4.6 to 16.2 years (n= 13,412) showed associations of high blood pressure with adverse arteriolar calibre, but less evidence with venular calibre.¹⁵⁰ They pooled six studies with eligible data into meta-analysis and showed that with each unit higher of systolic and diastolic blood pressure, retinal arteriolar calibre narrowed by 0.63 μm (95% CI -0.92 to -0.34) and 0.60 μm (95% CI -0.95 to -0.25) respectively.¹⁵⁰ However, the meta-analysis did not support the associations with retinal venular calibre (pooled β coefficient -0.07, 95% CI -0.21 to 0.06; -0.06, 95% CI -0.14 to 0.03).¹⁵⁰ In adults, analogous findings were observed across several studies, including the Atherosclerosis Risk in Communities study,¹⁵¹ Multi-Ethnic Study of Atherosclerosis,¹⁵² the Rotterdam study¹⁰⁹ and the Beaver Dam Eye Study.¹⁵³

Using Mendelian randomisation, Li and colleagues have shown a causal relationship between elevated blood pressure and retinal arteriolar narrowing.¹⁵⁴ In their Asian cohort of 6,528 participants (mean age 58.1 years), a genetic risk score of blood pressure was used as an instrument. The Mendelian randomisation model presented that each 10 mmHg increase in diastolic blood pressure and mean arterial blood pressure was associated with a 4.5 μm decrease (95% CI -7.9 to -1.0, $p=0.01$) in retinal arteriolar calibre, but not associated with venular calibre.¹⁵⁴

2.4.3.5 Blood cholesterol levels and retinal vascular calibre

It is consistent in the adults' but not children's studies that higher levels of LDL cholesterol, total cholesterol and triglycerides, and lower levels of HDL cholesterol were related to poor retinal microvascular parameters. In children with a median age of six years ($n=4,145$), there is little evidence to support the association of blood cholesterol levels with retinal vascular calibre.¹⁵⁵ The association was not evident in children aged 11 years either.¹⁵⁶

In adults, the Atherosclerosis Risk in Communities study ($n=8,794$, mean age 60 years) found that each interquartile range increase of triglycerides was associated with 0.5 μm (95% CI -0.9 to -0.1) narrower arteriolar calibre; and each interquartile range increase of LDL cholesterol was associated with 0.9 μm (95% CI 0.5 to 1.2) wider venular calibre.¹⁵⁷ Similarly, the Multi-Ethnic Study of Atherosclerosis ($n=5,979$, age 45–84 years) found that per SD (15 mg/dL) higher HDL cholesterol was associated with 1.13 μm narrower retinal venular calibre ($p=0.001$), while per SD (31 mg/dL) higher LDL cholesterol was associated with 0.63 μm wider venular calibre ($p=0.02$).⁹³

2.4.3.6 Obesity and retinal vascular calibre

Obesity is a well-known risk factor for CVD risk¹⁵⁸ and a burning issue, especially in the context of Australian society. Two thirds (67%) of Australians aged 18+ are overweight or obese, while one in 10 children is obese.¹⁵⁹ Thus, I am particularly interested in the association of obesity with retinal microvasculature. There is a wealth of evidence showing that obesity has substantial impacts on poor retinal microvasculature. This is discussed in detail in the following section, where I review

associations of obesity-related measures with retinal vascular calibre and build the rationale for Aim 2 of this thesis.

2.4.4 Obesity and retinal vascular calibre (Aim 2)

2.4.4.1 Wealth of cross-sectional evidence on obesity-related measures and retinal vascular calibre

Meta-analyses in adults and children have found consistent results of high body mass index (BMI) with poorer retinal vascular calibre. Boillot and colleagues' meta-analysis of 12 cross-sectional studies with more than 44,000 individuals (nine in adults and three in children)¹⁰⁴ showed that a higher BMI was associated with narrower arteriolar calibre and wider venular calibre in both adults and children.¹⁰³ Specifically, a one kg/m² increase in BMI was associated with a difference of 0.07 µm (95% CI -0.08 to -0.06) in arteriolar calibre and 0.22 µm (95% CI 0.21 to 0.23) in venular calibre in adults.¹⁰⁴ Similar results were found in children.¹⁰⁴ Kochi and colleagues' recent systematic review and meta-analysis of 13 studies in children observed similar findings.¹⁵⁰ In their meta-analysis, eight studies with 5,003 participants were included: results showed that in children a one kg/m² higher BMI was associated with -0.37 µm (95% CI -0.50 to -0.24) lower retinal arteriolar calibre and 0.35 µm (95% CI 0.07 to 0.63) wider venular calibre.¹⁵⁰ The effect of BMI on retinal vascular calibre is larger in children than in adults, based on the two meta-analyses.

2.4.4.2 Less longitudinal evidence of obesity-related measures and retinal vascular calibre

Given that obesity in youth persists into adulthood,^{160, 161} the question of whether long-term BMI/weight status adversely impacts the microvasculature warrants attention. To the best of our knowledge, only eight studies have examined this question: four in adults and four in children.¹⁶²⁻¹⁶⁵ The four children's studies had inconsistent results (see Table 2-2). In a Belgium cohort, Van Aart and colleagues studied the relationship between changes in BMI and other obesity-related factors and retinal vascular calibre.¹⁶² They did not find an association between BMI and retinal microvasculature over a seven-year period; however, higher body fat was associated with retinal vascular calibre. In contrast, a UK study by Tapp and

colleagues showed that BMI changes over eight years, especially at 5–5.5 and 8.5–10 years, were associated with wider venular calibre.¹⁶⁴ Similarly, in Singaporean children, Kurniawan and colleagues showed that a one kg/m² higher BMI at age 7–9 years predicted -0.17 μ m (95% CI -0.83 to -0.12) narrower arteriolar calibre and 0.76 μ m (95% CI 0.26 to 1.26) wider venular calibre five years later.¹⁶³ Inversely, arteriolar and venular calibre at baseline weakly predicted BMI at follow-up.¹⁶³ Hence, this longitudinal study suggests that BMI is more likely to influence retinal vascular calibre on the causal pathway, rather than the other way around. Our study team has looked at whether longitudinal BMI pattern is associated with retinal vascular calibre in a small sample (n= 187, mean age 14 years), but did not observe significant findings.¹⁶⁶

Studies in adults consistently show that there are long-term associations between BMI and retinal vascular calibre. Both Saito and colleagues (900 Japanese participants, mean age 59 years)¹⁶⁷ and Shankar and colleagues (2,089 American participants, mean age 60 years)¹⁶⁸ showed that middle-aged participants with wider retinal venular calibre at baseline were at higher risk of incident obesity at follow-up 5–15 years later. Gong and colleagues explored the associations of longitudinal BMI variability with retinal vascular calibre over five years in an elderly Chinese cohort (n= 3,494, mean age 59 years):¹⁶⁵ findings showed that higher BMI over time was associated with retinal microvascular alterations. Klein and colleagues found that in adults of similar age (n= 4,573, mean age 64 years) increasing BMI over five years had more profound influences on retinal vascular calibre in participants who had normal weight status, than those who were overweight or obese at baseline.¹⁵³ Although there is no evidence for middle-aged adults, based on current evidence for children and older adults, there might be a cumulative impact of higher BMI on retinal microvasculature across the life course.

Table 2-3 The association between BMI and retinal vascular calibre in longitudinal childhood studies

Study Ref.	Country, study	Sample size (female)	Mean age(y)	Analysis of BMI changes (two-time points)	Evidence of association	
					Retinal arteriolar calibre	Retinal venular calibre
2017; Van Aart. et al. ¹⁶²	Belgium, The IDEFICS study	171 (46.2%)	Start: 5.3y (2008) Follow-up: 12.4y (2015)	2008–2015 2010–2015 2011–2015 2012–2015 2013–2015	No significant association was seen for BMI changes and arteriolar calibre.	No significant association was seen for BMI changes and venular calibre.
2017; Hanvey. et al. ¹⁶⁶	Australia, The PEAS Kids Growth Study	187 (54.5%)	Start: 2 weeks (2000) Follow-up: 14 years (2014)	first 2 years of life (at 2 weeks and 2, 4, 8, 12, 18, and 24 months), 4, 6.5, 10 and 14 years	* High normal or consistently overweight trajectories were not associated with arteriole-to-venule ratio.	
2014; Kurniawan, E.D. et al. ¹⁶³	Singapore, The SCSRFM Study	421(50.8%)	Start: 7.9 (2001) Follow-up: 11.9(2006)	2001–2006	<i>Cross-sectional analysis</i> BMI was associated with narrower arteriolar calibre at both 2001 and 2006 visit. <i>Longitudinal analysis</i> Changes in BMI were not associated with arteriolar calibre.	<i>Cross-sectional analysis</i> BMI was associated with wider venular calibre at both 2001 and 2006 visit. <i>Longitudinal analysis</i> Each 1 kg/m ² increase in BMI was associated with a significant increase in venular calibre (0.597 µm; p = .044), but not with arteriolar calibre.
2013; Tapp, R.J. et al. ¹⁶⁴	UK, The ALSPAC	1067 (51%)	From 2–10y	2–5y 5–5.5y 6.5–7y 7–8.5y 8.5–10y	No association between BMI changes and arteriolar diameters.	Positive association between BMI change at 5–5.5 (β= 0.18, 95% CI 0.01 to 0.35) and 8.5–10 years (β= 0.05, 95% CI -0.003 to 0.56) with venular diameter.

Abbreviations: The IDEFICS study; The SCORM Study, The Singapore Cohort Study of Risk Factors for Myopia; The ALSPAC, Avon Longitudinal Study of Parents and Children; BMI, body mass index.

2.4.4.3 Limited knowledge of longitudinal obesity patterns with retinal microvasculature across the life course

Although previous studies clearly demonstrated that higher BMI is associated with worse retinal microvasculature, few studies have examined how BMI across early or mid-life, or cumulative patterns of repeated BMI over time – relates to developing retinal microvascular parameters. This could be important, since distinct BMI trajectories may differentially alter the risk of CVD later in life.^{169, 170} For example, a longitudinal study over 23 years found that a high-increasing childhood BMI trajectory was associated with poorer indicators for adult preclinical cardiovascular health (e.g., CIMT and left ventricular mass index), compared to a normal BMI trajectory.¹⁷¹ If retinal microvascular parameters are surrogate markers of preclinical cardiovascular health observed from microcirculation, longitudinal impacts of BMI would be embedded in worse retinal microvasculature. Such research may help to identify if people with typical BMI patterns have different microvascular profiles; targeting these people would contribute to early CVD risk management.

In addition, most studies have focused on BMI. Of course, BMI does not comprehensively represent obesity,¹⁷² as it does not distinguish between fat and muscle.¹⁷³ Thus, we need studies that also explore other obesity-related measures, such as waist-height ratio (WHtR),¹⁷⁴ which has been found to be more predictive of CVD.^{155, 165, 175}

Furthermore, if retinal vascular calibre changes reflect cumulative life course responses to systemic risk factors,⁸⁰ and if adiposity tracks strongly through life,¹⁷⁶ then associations should be larger in adults than in children. However, this is yet to be investigated. If there is a gradient in the risk with age, this adds further weight to the importance of early obesity prevention.

In Aim 2 of this thesis, I will examine whether retinal vascular calibre is predicted by firstly, BMI and WHtR at multiple time points, and secondly, BMI and WHtR trajectories, all spanning the preceding decade in two generations: children aged 11–12 years and mid-life adults.

2.4.5 Inflammation and retinal vascular calibre (Aim 3)

Inflammation is well known for its role in the pathogenesis of CVD, given its contribution to the earliest steps of atherogenesis.¹⁷⁷ The elevated levels of some inflammatory biomarkers, such as C-reactive protein (CRP) and interleukin-6 (IL-6), have been shown to predict adverse CVD events.^{178, 179} It is noteworthy that therapy targeting inflammatory markers has been shown to lower the rate of recurrent CVD events in a clinical trial.¹⁸⁰ The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), a large RCT with 10,061 patients (mean age 61 years) of previous myocardial infarction, targeted interleukin-1 β using Canakinumab for a median follow-up of 3.7 years. The RCT found that the treatment group was 15% less likely to have secondary CVD events.¹⁸⁰

Inflammatory processes have also been implicated in microvascular health variation through animal experiments.^{181, 182} For instance, an experiment found that after injecting lipid hydroperoxide into the vitreous of rats, the number of leukocytes increased in the retinal microvasculature, and led to a widening retinal venular diameter.¹⁷⁹ However, a limited number of population-based studies have explored whether inflammatory biomarkers are associated with the retinal microvasculature in humans. Of the existing studies, results are inconsistent.^{93, 109, 155-157, 183-194} Moreover, while obesity is the key driver of inflammation (Figure 2-6),¹²⁵ most studies examining the association of inflammation and microvasculature have failed to consider the role of obesity.^{183, 194} If the role of inflammation in the association of obesity and microvasculature can be understood, it could provide a potential therapeutic target to maintain stable and healthy microcirculation, and could even help lower the risk of developing CVD.

In this section, I will first summarise the current evidence on inflammation and retinal microvascular parameters from a systematic review and meta-analysis. I will then illustrate the gaps in the current evidence to inform Aim 3 of this thesis.

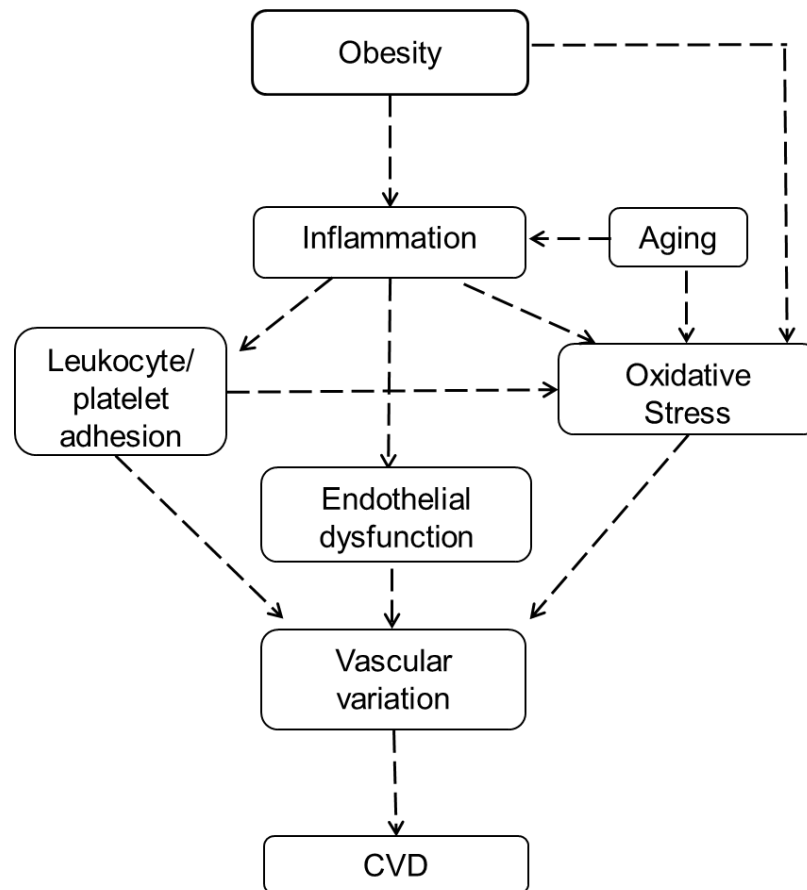


Figure 2-6 Mechanism of obesity, inflammation and CVD

2.4.5.1 Inflammation and retinal vascular calibre – a systematic review and meta-analysis

Several studies have examined associations between different markers of inflammation and retinal vascular calibre, with inconsistent findings. There was no systematic reviews or meta-analyses of inflammation and retinal microvascular parameters in either the general population or those with CVD and related conditions. Investigating this association will help us understand whether we can observe the association of inflammation and retinal vascular variation at the population level. In addition, we can see whether there are any differences in this association among people with different health conditions. My colleague (Ms Claire Lovern) and I, therefore, performed a systematic review and meta-analysis to quantify the associations of various inflammation markers with retinal vascular calibre.

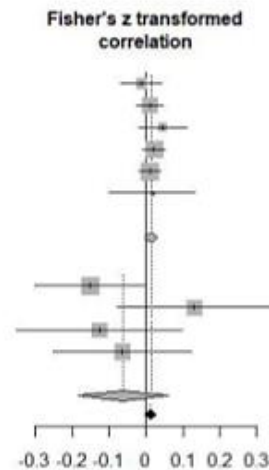
We identified studies in Medline, Embase and PubMed on the following topics: 1) retinal vascular calibre; 2) inflammatory markers; and 3) observational studies. Studies drawn from general population samples and individuals with disease conditions ('patients') were included. The search was limited to studies published in English or Chinese, with no limitations on the date of publication, meaning all studies up to February 2020 were included.

Of the 2,383 studies identified, 28 met inclusion criteria (general population 19, patients 9). Most data were cross-sectional from mid-life adults (43 to 68 years), while three studies focused on children. Various inflammation markers were studied, most commonly CRP (20 studies) and white blood cell count (8 studies). Studies that reported either correlation coefficients or linear regression coefficients were eligible for meta-analysis. Thus, data were pooled using meta-analysis from 10 of 20 studies measuring CRP (general population 6, patients 4) and four of eight studies measuring white blood cell count (all in the general population).

Findings from our narrative review (for all studies) and meta-analysis (of studies that could be combined) were highly consistent. The meta-analysis showed little evidence of association between CRP with retinal arteriolar calibre ($r = 0.01$, 95% CI -0.00 to 0.03 in the general population, and $r = -0.06$, 95% CI -0.18 to 0.06 in patients, Figure 2-7(A)). The association with venular calibre was only seen in the general population group ($r = 0.08$, 95% CI 0.04 to 0.11), but not in the patient group ($r = 0.03$, 95% CI -0.06 to 0.12, Figure 2-7(B)). White blood cell count was associated with both arteriolar and venular calibre, with stronger associations seen for venular calibre ($r = 0.18$, 95% CI 0.04 to 0.32). The association with arteriolar calibre was in the unexpected direction ($r = 0.05$, 95% CI 0.01 to 0.09). Findings from the three studies in children were mixed.

(A) Retinal arteriolar calibre

Study citation no.	Total	ZCOR (95% CI)	Weight
General population			
Buttner, M-2019	1682	0.02 (-0.03, 0.07)	12.3
Gishti.O-2015	4145	0.14 (0.11, 0.17)	14.8
Daïen, V-2013	1224	0.13 (0.07, 0.19)	11.1
Myers, E-2012	4600	0.08 (0.05, 0.11)	15.0
De Jong, FJ-2007	5279	0.10 (0.07, 0.12)	15.2
Tien, YW-2006	5940	0.04 (0.01, 0.07)	15.4
Klein,R-2006	396	0.01 (-0.09, 0.11)	6.4
Random effects model	23266	0.08 (0.04, 0.11)	p < 0.001
Heterogeneity: $I^2 = 84\%$			
Patients			
Beltrami M-2015	172	-0.02 (-0.17, 0.13)	3.5
Bachmayer, C-2013	92	0.12 (-0.09, 0.33)	2.0
Okada, M-2013	79	0.03 (-0.20, 0.25)	1.8
Brazionis, L-2010	112	0.03 (-0.16, 0.21)	2.4
Random effects model	454	0.03 (-0.06, 0.12)	p = 0.55
Heterogeneity: $I^2 = 0\%$			
Overall Random effects model			
	23720	0.07 (0.04, 0.11)	p < 0.001
Heterogeneity: $I^2 = 75\%$			



(B) Retinal venular calibre

Study ID	Total	ZCOR (95% CI)	Weight
General population			
Buttner, M-2019	1682	-0.01 (-0.06, 0.04)	10.2
Gishti.O-2015	4145	0.01 (-0.02, 0.04)	21.8
Daïen, V-2013	1224	0.04 (-0.02, 0.10)	7.6
De Jong, FJ-2007	5279	0.02 (-0.01, 0.05)	26.3
Tien, YW-2006	5940	0.01 (-0.02, 0.04)	28.6
Klein,R-2006	396	0.02 (-0.08, 0.11)	2.6
Random effects model	18666	0.01 (-0.00, 0.03)	p = 0.07
Heterogeneity: $I^2 = 0\%$			
Patients			
Beltrami M-2015	172	-0.15 (-0.30, 0.00)	1.1
Bachmayer, C-2013	92	0.13 (-0.08, 0.34)	0.6
Okada, M-2013	79	-0.13 (-0.35, 0.10)	0.5
Brazionis, L-2010	112	-0.06 (-0.25, 0.12)	0.7
Random effects model	455	-0.06 (-0.18, 0.06)	p = 0.33
Heterogeneity: $I^2 = 39\%$			
All participants Random effects model			
	19121	0.01 (-0.01, 0.03)	p = 0.19
Heterogeneity: $I^2 = 10\%$			



Figure 2-7 Associations of CRP and retinal vascular calibre

2.4.5.2 Limited knowledge of inflammation and retinal microvasculature across the life course

Our systematic review and meta-analysis suggest that inflammation is a key mechanism of variation in retinal vascular calibre, which can be observed from population-based studies. In adults, inflammatory markers have been most consistently associated with venular rather than arteriolar calibre.^{155, 183, 194} Less is known about the associations of inflammatory markers and microvasculature in children; further, we do not know how inflammation contributes to retinal microvascular variation across the life course.

Given that retinal microvascular parameters are suggested to reflect cumulative lifespan exposures, chronic inflammation markers should be considered in analysing this association. Previous studies were more likely to perform with acute-phase reactants, such as high-sensitivity CRP. A newly described inflammatory marker, glycoprotein acetyls (GlycA), is suggested to be more indicative of chronic and cumulative inflammation in adults and in early life.^{195, 196} However, data on GlycA are sparse, and the association with retinal microvascular parameters has not been studied.

2.4.5.3 Limited knowledge of obesity-induced inflammation with retinal microvasculature across the life course

In Section 2.4.4, we noted that obesity, one of the leading risk factors for CVD, has adverse effects on retinal microvasculature even in childhood, and that inflammation is thought to play a key role in this relationship (Figure 2-6 above).¹²⁵ Whether the impact of obesity on retinal microvasculature occurs via inflammation has not been discussed in population-based studies. It is also unclear whether the inflammation pathway between obesity and retinal microvasculature could become more important with age. Understanding the magnitude of this association would inform the extent to which inflammation intervention could help lower the adverse impacts of obesity on microvasculature among different populations.

To address these knowledge gaps, Aim 3 of this thesis will examine associations of the chronic inflammation marker GlycA with retinal microvasculature, as well as the extent to which the association between obesity and retinal microvasculature is mediated through GlycA. The analyses will explore these associations across the life course based on two generations: children aged 11–12 years and mid-life adults.

2.5 PART FOUR: Less is known about retinal geometric parameters than vascular calibre (Aim 4)

As discussed in Section 2.3.2, advanced software now allows the overall assessment of retinal vascular architecture on a large scale. The additional retinal microvascular parameters captured via the novel SIVA software include fractal dimension, vessel tortuosity and branching patterns. In contrast to the retinal vascular calibre, which only contains information on vessel diameters, SIVA captures novel parameters that

reflect geometric features across the overall retinal microvasculature, which could be important.

It is assumed that the design of the retinal vascular tree follows the optimisation principle, based on the theory of minimum work.⁵ This was originally postulated by Murray in 1926 and has later been validated for the retinal tree.¹⁹⁷ It is hypothesised that when the architecture of the vascular tree is compromised, the efficiency of metabolic transport will be affected, which is reflective of wider microcirculation damage.¹⁹⁸ These changes may increase the risk of future diseases such as CVD. However, retinal geometric parameters have been far less studied than retinal vascular calibre.

2.5.1 Traditional CVD risk factors and retinal geometric parameters

Section 2.3.4.2 mentioned that retinal geometric parameters predict CVD in adults. There are also studies examining the traditional CVD risk factors with these novel geometric parameters in adults. However, limited evidence exists for children. In adults, retinal geometric parameters have been associated with higher blood pressure, cholesterol levels, BMI and levels of inflammation.^{6,8}

In the Singapore Malay Eye Study (n= 1,913, mean age 57.6 years), adults with elevated blood pressure showed cross-sectional associations with lower fractal dimension, lower arteriolar tortuosity and higher venular tortuosity. Lower HDL cholesterol level was significantly associated with higher venular tortuosity.¹⁹⁹ The inverse associations of higher blood pressure and lower fractal dimension are consistent across multi-ethnic groups.^{200, 201} A Chinese study (n= 2,169, mean age 51.9 years) further showed that lower fractal dimension was not only seen with prehypertension status (systolic blood pressure of 120–139 mmHg and diastolic blood pressure of 80–89 mmHg; β coefficient -0.008, 95% CI -0.013 to -0.002) and hypertension status (-0.013, 95% CI -0.022 to -0.005), but also observed in individuals with hypertension history (-0.007, 95% CI -0.012 to -0.002).²⁰²

The association of BMI with retinal geometric parameters is less understood; of the few studies available, findings are mixed. One study of 166 Malaysian children (mean age 9.6 years) found that children with obesity had slightly higher venular fractal dimension and tortuosity (mean difference: fractal dimension 0.015 (95% CI

0.001, 0.030), tortuosity 0.006 (95% CI 0.001, 0.012)).⁹⁵ On the contrary, the Sydney Childhood Eye Study (n= 2,353, median age 12.7 years) showed that higher BMI was not significantly associated with retinal fractal dimension.²⁰³ Likewise, the Child Heart and Health Study in England (CHASE) (n= 986, aged 10–11 years) did not observe a significant association between waist circumference or fat mass index and arteriolar tortuosity.²⁰⁴ Alternatively, they found that levels of triglyceride, total and LDL cholesterol, and diastolic blood pressure (for one SD increase 3.7% (95% CI 1.2 to 6.4), 3.3% (0.9 to 5.8), 3.1% (0.6 to 5.6), and 2.3% (0.1 to 4.6), respectively) were positively associated with increased arteriolar tortuosity.²⁰⁴

Very few studies have investigated inflammation and retinal geometric parameters, and the findings are inconsistent. The only child study, the CHASE study (n= 1,642, mean age 9.8 years), did not suggest that CRP was associated with arteriolar tortuosity.²⁰⁴ In the Lothian Birth Cohort 1936 Study (n= 655, mean age 72.5 years) CRP was weakly correlated with venular tortuosity (correlation coefficient $r = 0.12$, $p < 0.01$), but not correlated with other geometric parameters (e.g., arteriolar and venular fractal dimension, or arteriolar tortuosity) in older adults.⁷⁸ However, no other studies have investigated other inflammatory biomarkers with retinal geometric parameters.

2.5.2 More research is needed on retinal geometric parameters from childhood

Although it is suggested that retinal geometric parameters would provide additional information beyond the vascular calibre, there is not enough evidence to support this, especially in children. In Aim 4 of this thesis, I will investigate whether CVD risk factors and preclinical large arterial phenotypes present similar associations with retinal geometric parameters in the same cohort. If they provide additional information beyond the vascular calibre, this may suggest that measuring the overall architectural variation would be useful. Equally, if they do not provide any novel information or have similar patterns of association, this suggests that the measurement of vascular calibre alone may be enough.

Chapter 3. Thesis aims & hypotheses

3.1 Aims

In a large population-based sample of Australian 11–12 year-olds and mid-life adults, this thesis aims to examine whether:

- 1) retinal vascular calibre variations associate with preclinical large arterial phenotypes;
- 2) BMI and waist-to-height ratio (WHtR; child only) over the preceding decade predict retinal vascular calibre;
- 3) inflammation mediates any association seen between obesity and retinal vascular calibre; and
- 4) traditional CVD risk factors and large arterial phenotypes affect retinal geometric parameters.

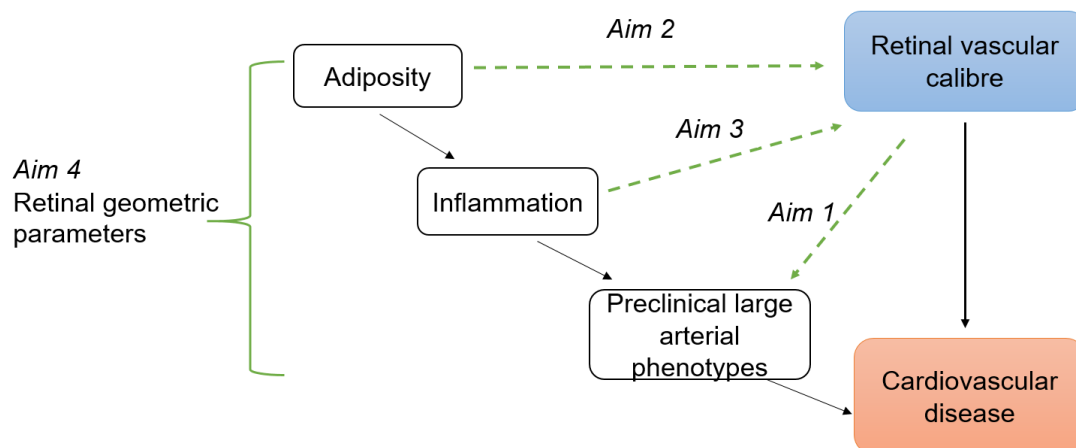


Figure 3-1 PhD thesis aims illustration

3.2 Hypotheses

It is hypothesised that:

Aim 1: narrower retinal arteriolar calibre and wider retinal venular calibre will be associated with poorer large artery functional and structural phenotypes, and associations will be stronger in mid-life adults than children;

Aim 2 a): associations between BMI and/or WHtR across multiple time points and retinal vascular calibre will be evident from early childhood, with stronger associations in mid-life;

Aim 2 b): being persistently overweight or obese across the preceding decade will carry greater impairment on retinal vascular calibre, compared to those who are always with normal weight status;

Aim 3 a): higher levels of inflammation will be associated with adverse retinal vascular calibre, and associations will be stronger for venular than for arteriolar calibre;

Aim 3 b): inflammation will mediate the association between obesity and retinal vascular calibre; and

Aim 4: CVD risk factors and preclinical large arterial phenotypes will be associated with retinal geometric parameters in mid-childhood.

3.3 What can this project add?

Through the literature review, it became apparent that most research to date has focused on adults late in the life course (e.g. > 55 years), with less evidence relating to early-to-mid adulthood and few studies in childhood. It is well-known that CVD has its roots in childhood and progresses with age.²⁰⁵ In this PhD project, I have the opportunity to examine the associations in two generations: mid-childhood and mid-life. In our cohort, the same measure was assessed on the same day by the same staff, ensuring less measurement errors. This cross-generational design may enable us to efficiently infer temporal strengthening of associations with age – data that would otherwise take decades to observe. No studies have included these two age groups (ie, childhood and mid-life) to examine the life course variation. By doing so, we can examine whether associations strengthen with age early in life, as is the case with the life course epidemiology of CVD development.

This PhD project is based on a longitudinal study of parents and children that has followed up participants for over 10 years. This gives me a unique opportunity to explore the association between cumulative patterns of repeated obesity-related measures over time with retinal microvascular parameters. This could be important,

since long-term adiposity may have a cumulative impact on CVD.^{167, 168} If people with specific patterns of adiposity trajectories (e.g., always obese for a decade) have poorer retinal microvasculature than people with normal weight status, the findings will have important public health implications. Such findings highlight the subclinical effects of long-term obesity. Moreover, interventions are warranted to target those with worse microvasculature, as microvascular abnormality will further contribute to the development of CVD.

With the rich source of data, I can explore one of the important mechanisms underlying retinal microvascular variation: inflammation. The inflammation pathway has been suggested as a key mechanism for the obesity-associated microvascular abnormality in experiments. In Aim 3 of this PhD, the mediation analysis approach can inform, at the population level, whether inflammation likely lies on the pathway of obesity and retinal microvascular parameters. Such a finding will be meaningful for a potential therapeutic target. If targeting inflammation pathways can reduce the adverse effect of obesity, then intervention could help maintain a stable and healthy microcirculation in the eye and/or other vascular beds (e.g., in the kidney or brain), which may eventually help lower the risk of developing CVD.

Our study also measured novel retinal geometric parameters that are associated with CVD events in adults. There is also evidence showing that microvascular geometric parameters have been associated with CVD risk factors and preclinical large arterial phenotypes in adults;^{101, 207} however, findings from the few children's studies are mixed.^{95, 208} Examining whether the novel retinal geometric parameter presents similar associations as retinal vascular calibre can inform if they provide additional information beyond vascular calibre by mid-childhood.

Chapter 4. Associations of retinal vascular calibre with large arterial phenotypes: A systematic review and meta-analysis

4.1 Overview

Chapter 4 describes a systematic review and meta-analysis, providing a comprehensive summary of current evidence on the associations of retinal vascular calibre with large arterial phenotypes. The findings showed that retinal vascular calibre was modestly associated with large arterial function and weakly associated with large arterial structure, with stronger evidence in patients with cardiometabolic disease. Except for one study in children, other studies were in old adults. This review highlights the needs for more research on childhood associations, which is undertaken in the first aim of this thesis (Chapter 6).

This systematic review is published in *Microcirculation*, with the published paper and supplementary materials forming this chapter. Preclinical large artery functional and structural phenotypes are referred to as intermediate phenotypes of large arterial function and structure in this paper.

- **Liu M**, Wake M, Wong TY, He M, Xiao Y, Burgner D and Lycett K. Associations of retinal microvascular calibre with intermediate phenotypes of large arterial function and structure: A systematic review and meta-analysis. *Microcirculation* 2019:e12557.

4.2 Published paper

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REVIEW

Microcirculation WILEY

Associations of retinal microvascular caliber with intermediate phenotypes of large arterial function and structure: A systematic review and meta-analysis

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Abstract

Objective: Intermediate phenotypes of microcirculation (retinal microvascular caliber) are associated with cardiovascular (CV) risk factors and independently predict CV events. However, the effect of microcirculation variation on the vascular system is unclear. We conducted a systematic review and meta-analysis of observational studies to quantify associations of retinal microvascular caliber (arteriolar, venular caliber, arteriole-to-venule ratio) and preclinical CV measures (large arterial function and structure). **Methods:** We identified studies in MEDLINE, EMBASE, and PubMed (1946 to March 2018) studying (a) general population samples and (b) patients with cardiometabolic disease. Study-specific correlation estimates were combined into meta-analysis where possible.

Results: Of 1294 studies identified, 26 met inclusion criteria (general population 16, patients 10), of which five studies were included in meta-analysis. Most studied middle-aged adults cross-sectionally, with one childhood study. Large arterial function and structure were predominantly assessed by pulse wave velocity and carotid intima-media thickness, respectively. Only arteriolar caliber was consistently associated with arterial function and structure, with stronger associations observed in cardiometabolic patients. Narrower (worse) arteriolar caliber was associated with faster (poorer) pulse wave velocity (correlation coefficient (r) -0.17 , 95% CI -0.25 to

Abbreviations: AVR, arteriole-to-venule ratio; CIMT, carotid intima-media thickness; CV, cardiovascular; CVD, cardiovascular disease; PWV, pulse wave velocity.

Protocol registration: The protocol was registered online at PROSPERO (ID CRD 42017064425).

*David Burgner and Kate Lycett contributed equally to this work.

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–0.10) and greater (poorer) intima-media thickness (r –0.05, 95%CI –0.09 to –0.02) across all adult participants.

Conclusions: Retinal arteriolar, but not venular caliber, was modestly associated with large arterial function and weakly associated with large arterial structure, with stronger evidence in patients with cardiometabolic disease. This suggests that pre-clinical changes in large arteries and the microcirculation have some shared but mainly unique pathways to associate with cardiovascular disease.

KEYWORDS

carotid intima-media thickness, microcirculation, pulse wave velocity, retinal imaging, retinal microvascular caliber

1 | INTRODUCTION

Quantifying CVD risk in asymptomatic individuals largely relies on assessment of traditional risk factors but can be enhanced by non-invasive assessment of vascular phenotypes.^{1–4} Vascular phenotypes include measures of large arterial stiffness, most commonly by PWV,⁵ and structural changes to the arterial wall indicative of early atherosclerosis, often by CIMT.^{6,7} These phenotypes are widely used in population-based studies as they predict adverse CVD events and can be considered “intermediate” between traditional risk factors and CVD itself. For example, a one unit increase in aortic PWV and a standard deviation unit increase in CIMT (eg, poorer cardiovascular health) have been shown to be associated with a 14% and 37% increased risk of adverse CVD events, respectively.^{5,8}

In addition to these large arterial phenotypes, the microcirculation has increasingly been recognized for its role in CVD pathogenesis.^{9,10} Technological advances now allow non-invasive assessment of the microcirculation in large studies, most commonly by quantification of the retinal microvasculature.¹¹ Adverse phenotypes of the retinal microcirculation (narrower retinal arteriolar and wider venular caliber) have also been associated with adverse CVD outcomes.^{12,13} For instance, the large Atherosclerosis Risk in Communities Study showed that, over a 16 years follow-up period, generally healthy subjects with narrower arteriolar caliber and wider venular caliber had a higher risk of ischemic stroke in both sexes (hazard ratio 1.10, 95% CI 1.00 to 1.20 and 1.14, 95%CI 1.03 to 1.26, respectively).¹² The arteriole-to-venule ratio (AVR) also predicted CVD events in some earlier studies.^{9,10}

Both large arterial and retinal microvascular intermediate phenotypes are individually recognized as adding value to CVD prediction, yet the extent to which the two are related is largely unknown. Physiologically, they are part of the one circulatory system, sharing elements of pulsatile transmission of pressure and flow,¹⁴ but they capture different profiles of the vascular tree. They are both influenced by traditional CVD risk factors, such as age, hypertension, dyslipidemia, and smoking,^{11,15,16} yet the degree and rate that they impact on one another are unclear. Examining their association could help us understand whether damage to both parts of the vascular tree usually happens in unison or whether variation in each profile occurs at different rates.

This knowledge could enhance our understanding of whether assessing both could improve cardiovascular risk prediction. In addition, identifying if and when in the life course they co-vary would inform prediction models and the ages that could benefit most. There may be a certain point in the life course where the two deteriorate in unison.

Several studies have examined associations of retinal microvascular caliber with large arterial phenotypes such as PWV^{17,18} and CIMT,^{19,20} but this association has not been comprehensively synthesized across studies or specific populations. We therefore performed a systematic review and meta-analysis to quantify the associations of retinal microvascular caliber with various intermediate large arterial phenotypes.

2 | MATERIALS AND METHODS

2.1 | Protocol registration

The systematic review conforms to the standards of quality for reporting Meta-analysis of Observational Studies in Epidemiology²¹ and the PRISMA checklist.²² The protocol was prospectively registered with the International Prospective Register of Systematic Reviews (CRD 42017064425) on July 5, 2017.²³

2.2 | Sources and electronic search strategy

We searched MEDLINE, EMBASE, and PubMed databases in March 2018 for studies reporting the association of retinal microvascular caliber and intermediate phenotypes of large arterial function or structure. The search strategy was developed together with content experts and the institution librarian. MeSH terms, keywords, and search limits for the following three topic areas were combined: (a) retinal microvascular caliber, (b) intermediate large arterial phenotypes, and (c) observational studies (Table S1). The search was limited to studies published in English or Chinese between 1946 and March 7, 2018.

2.3 | Study selection

All studies identified were uploaded to the web-based systematic review software package “Covidence” (Veritas Health Innovation,

Australia).²⁴ Two reviewers (ML, YX) independently screened abstracts and titles to identify relevant studies. Each reviewer then independently screened full texts using the pre-defined inclusion criteria. If conflicts arose between the reviewer's decisions, a discussion took place between reviewers to resolve the conflict. However, if a consensus was not reached, then a third independent reviewer (KL) was available to make the final decision. Studies were included if they were observational designs, had retinal microvascular caliber quantified from digital retinal photographs via computer-assisted methods, and intermediate large arterial functional (eg, PWV, arterial elasticity) or structural phenotypes (eg, CIMT, calcium scores). Additional studies were sought by handsearching the reference lists of relevant studies. We excluded conference abstracts and study protocols without original data.

2.4 | Data extraction and quality assessment

Data were extracted independently by each reviewer from included full texts into EpiData software (version 3.1). These data included the first author, year of publication, study population, study name, sample size, age of participants, measurement methods, statistical methods, correlation coefficients, main estimates and confidence intervals (CI), and estimates of the association with covariates if applicable. To avoid over-adjustment by potential intermediate variables and to improve comparability between studies, we preferentially extracted minimally adjusted results (eg, age- and sex-adjusted).

Each reviewer (ML, YX) also independently assessed the methodological quality of relevant studies to assess risk of bias using a

modified version of the Ottawa-Newcastle scale checklist.²⁵ Briefly, the checklist considers bias arising from three domains: (a) selection of study population; (b) comparability of study groups; and (c) adequacy of outcome assessment. Studies can receive a maximum of 10 stars comprising a total of five for the study group selection, two for comparability between groups, and three for the outcome. The quality of studies is defined as follows: high (9-10 stars), moderate (7-8 stars), and low (≤ 6 stars).

2.5 | Data synthesis and meta-analysis

Study characteristics and main findings (eg, magnitude of association and level of statistical evidence) were compiled into tables. In order to conduct a meta-analysis, we aimed to extract correlation coefficients or standardized β from unadjusted or minimally adjusted regression models given that these are directly comparable. When raw regression coefficients were presented, we were able to calculate the standardized β . This meant that we could directly compare associations across studies given that the correlation coefficient and a standardized β from an unadjusted model are equal.²⁶ This also allowed us to include studies where retinal microvascular caliber was assessed as the outcome, rather than the exposure, given that standardized β avoids the issue of directionality. We then transformed correlation coefficients to Fisher's z scores and pooled estimates using the random-effects model of z transformed correlations.²⁷ The 95% CIs of the pooled weighted Fisher's z scores were calculated, after which all the values were back-transformed to the metric of the correlation coefficients to facilitate interpretation.²⁷ Statistical heterogeneity was investigated using the I^2 square index (I^2), which

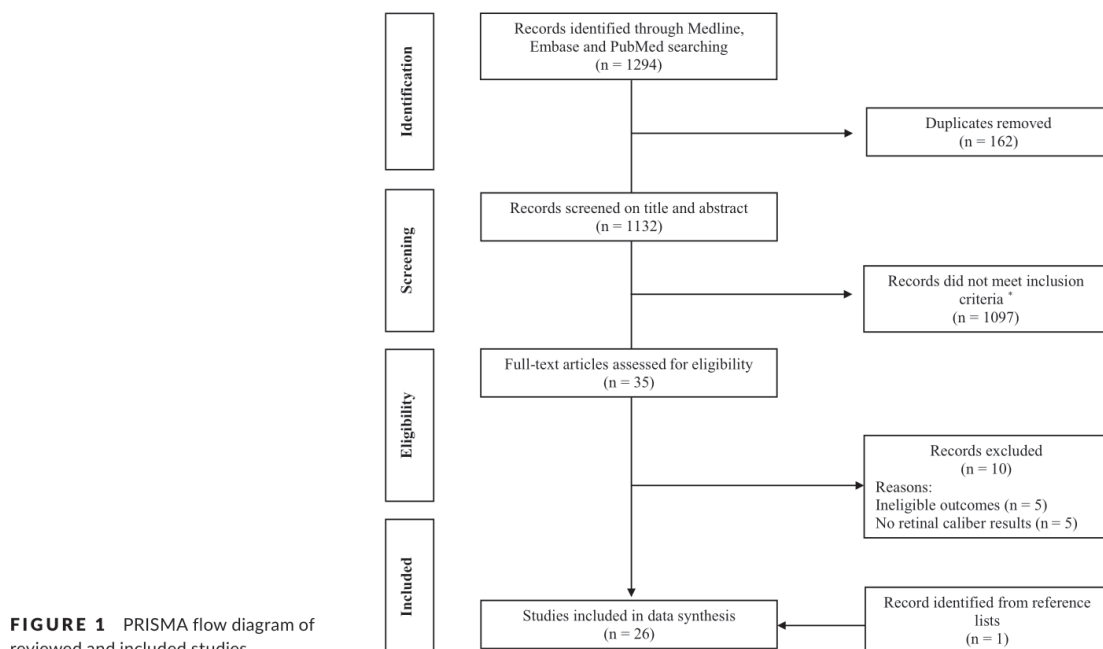


TABLE 1 Characteristics of observational studies included in the systematic review

Lead author; year	Country	Disease conditions	Sample size (women %)	Age (years) mean/median (range)	Exposure/s	Outcome/s	Study quality
General population							
Klein, R; 2000 ³¹	USA	–	8524 (56.4)	60 (51-72)	AVR	Carotid plaque	Medium
Wong, TY; 2003 ¹⁹	USA	–	1609 (61.1)	77 (69-97)	CIMT	AVR	High
Ikram, MK; 2004 ³²	Netherlands	–	5674 (59.0)	68 (ng)	CIMT, carotid plaque, aortic calcification	Retinal arteriolar and venular caliber, AVR	High
Liao, D; 2004 ³³	USA	–	8031 (56.0)	56 (45-64)	AVR	Arterial elasticity	High
van Hecke, MV; 2006 ³⁴	Netherlands	–	256 (48.0)	72 (60-85)	Retinal arteriolar and venular caliber, AVR	Flow-mediated vasodilatation, CIMT	Medium
Cheung, N; 2007a ³⁵	USA	–	2861 (52.5)	ns (45-84)	Arterial compliance	Retinal arteriolar and venular caliber	High
Cheung, N; 2007b ³⁶	USA	–	3425 (54.1)	62 (45-84)	Arterial distensibility	Retinal arteriolar and venular caliber	High
Liew, G; 2008 ³⁷	USA	–	8794 (54.6)	60 (45-64)	CIMT	Retinal arteriolar and venular caliber	Medium
Wong, TY; 2008 ³⁸	USA	–	6147 (52.3)	ns (45-84)	Retinal arteriolar and venular caliber	Coronary artery calcification	High
Nguyen, TT; 2010 ³⁹	USA	–	2851 (49.9)	63 (45-84)	Retinal arteriolar and venular caliber	Flow-mediated vasodilatation	High
Gishti, O; 2015 ¹⁷	Netherlands	–	4007 (ng)	6 (ng)	Retinal arteriolar and venular caliber	PWV	High
Garcia-Ortiz, L; 2015 ⁴⁰	Spain	–	229 (61.1)	56 (20-80)	Retinal arteriolar and venular caliber, AVR	CIMT, PWV, ankle-brachial index	Medium
Lin, F; 2015 ⁴¹	China	–	2169 (62.6)	52 (ng)	Retinal arteriolar caliber	PWV	High
Agladioglu, K; 2015 ²⁹	Turkey	–	43 (ng)	30 (20-40)	Retinal arteriolar and venular caliber	Internal carotid artery diameter	Low
Yang, JY; 2016 ⁴²	China	–	4004 (43.0)	60 (>40)	CIMT	Retinal arteriolar and venular caliber	Medium
Kawashima, K; 2018 ⁴³	Japan	–	6720 (68.5)	52.1 (30-74)	Retinal arteriolar and venular caliber	PWV	Medium
Cardiometabolic patient group							
Masaidi, M; 2009 ⁴⁴	Italy	Hypertension	386 (55.2)	56 (ng)	AVR	CIMT	Medium
De Silva, DA; 2012 ⁴⁵	Singapore	Acute stroke	145 (31.7)	62 (ng)	Retinal arteriolar and venular caliber, AVR	PWV	High
Garcia-Ortiz, L; 2012 ⁴⁶	Spain	Hypertension	205 (42.9)	56 (34-75)	AVR	CIMT, PWV	Medium
Lammert, A; 2012 ⁴⁷	Germany	Obesity	120 (72.5)	43 (ng)	AVR	CIMT	Medium

(Continued)

TABLE 1 Continued

Lead author; year	Country	Disease conditions	Sample size (women %)	Age (years) mean/median (range)	Exposure/s	Outcome/s	Study quality
Torres, FS; 2013 ²⁰	Brazil	Hypertension	173 (46.0)	58 (18-80)	CIMT	Retinal arteriolar and venular caliber	Medium
Gopinath, B; 2014 ⁴⁸	Australia	Coronary artery disease	991 (25.4)	60 (ng)	Retinal arteriolar and venular caliber	Coronary artery stenosis	Medium
Triantafyllou, A; 2014 ¹⁸	Greece	Hypertension	223 (36.3)	44 (ng)	Retinal arteriolar and venular caliber, AVR	PWV	Medium
Aissopou, EK; 2016 ⁴⁹	Greece	Hypertension	181 (44.8)	54 (ng)	Retinal arteriolar and venular caliber	PWV	Medium
Li, HY; 2016 ³⁰	China	Carotid artery stenosis	141 (41.1)	62 (ng)	Retinal arteriolar and venular caliber	Carotid artery stenosis	Low
Daien, V; 2017 ⁵⁰	France	Hypertension	88 (43.2)	54 (32-83)	PWV	Retinal arteriolar and venular caliber	Medium

Study quality was assessed using the modified Newcastle-Ottawa Quality Assessment Scale for observational studies,²⁵ which defined quality as high (10-9 stars), moderate (7-8 stars) or low (≤6 stars). Abbreviations: AVR, arteriole-to-venule ratio; CIMT, carotid intima-media thickness; PWV, pulse wave velocity; ng, not given; USA, United States of America.

measured the percentage of the variation across studies that is likely due to heterogeneity and cannot be explained by chance.²⁸ The level of heterogeneity was regarded as "low" (0%-25%), "moderate" (26-74%), or "high" (≥ 75%). We used the R software (version 3.4.2), "meta" package to analyze data.

3 | RESULTS

3.1 | Study selection and characteristics

Figure 1 shows the flow diagram of the review process, according to the PRISMA guidelines.²² We screened the abstracts and titles of 1132 non-duplicate studies, of which 35 studies were deemed potentially eligible for full-text screening, and 25 met our inclusion criteria. One further study was identified through handsearching of reference lists. In total, we extracted data from 26 studies.

Table 1 presents the characteristics of the included studies. With the exception of one recent longitudinal study, all were cross-sectional and most were conducted in Europe (11 studies) or the United States (eight studies). Of the 26 studies, 16 were performed in the general populations, most with large sample sizes (median sample size 3715), and 10 were in cardiometabolic patient populations (eg, hypertension, coronary heart disease, diabetes) with smaller sample sizes (median sample size 181). Only one study was conducted in children (median age six years), and most adult studies were conducted in middle-aged adults (44 to 77 years). Various intermediate large artery phenotypes of function and structure were included, most commonly PWV (nine studies) and CIMT (10 studies); others were arterial elasticity, flow-mediated vasodilatation, and carotid plaque. Despite their cross-sectional nature, most studies assessed the association with retinal microvascular caliber as the exposure and arterial function or structure as the outcome.

The risk of bias is summarized in Table 1. Study quality was generally deemed as medium (14 studies) or high (10 studies), with the exception of two studies.^{29,30}

3.2 | Synthesis of results

Direct comparison of studies was challenging due to the various measures used to assess large arterial intermediate phenotypes. We therefore conducted a narrative review that focused on the most commonly measured large arterial intermediate phenotypes, PWV and CIMT (Table 2), and then briefly examined all other intermediate phenotypes (Table S3). A meta-analysis also estimated the combined correlation coefficients for PWV and CIMT where possible (Figure 2-3, Table 3).

3.3 | Narrative review

3.3.1 | Associations of retinal microvascular caliber with functional intermediate phenotypes

Of the retinal vascular measures, retinal arteriolar caliber was most consistently associated with PWV in adults with cardiometabolic

TABLE 2 Study-specific associations of retinal microvascular caliber with pulse wave velocity and carotid intima-media thickness

			Retinal arteriolar caliber (↓ = worse)		Retinal venular caliber (↑ = worse)		Arteriole-to-venule ratio (↓ = worse)	
Lead author; year	Sample	Statistical methods	Estimate	P value	Estimate	P value	Estimate	P value
Pulse wave velocity (Children)								
Gishti, O.; 2015 ¹⁷	General	Linear regression (standardized β)	0.02 (−0.01, 0.05)	ng ^b	0.04(0.01, 0.07)	<0.05		
Pulse wave velocity (Adults)								
De Silva, DA; 2012 ⁴⁵	Patient	Pearson's correlation	−0.21	<0.01	−0.01	0.89	0.01	0.14
Triantafyllou, A; 2014 ¹⁸	Patient	Pearson's correlation	−0.21	0.002	−0.04	0.56	−0.12	0.08
Garcia-Ortiz, L; 2015 ⁴⁰	General	Pearson's correlation	−0.05	ng ^b	0.11	ng ^b	−0.18	<0.01
Aissopou, EK; 2016 ⁴⁹	Patient	Pearson's correlation	−0.23	<0.001	−0.06	<0.05		
Daïen, V; 2017 ⁵⁰	Patient	Linear regression (standardized β) ^d	−0.21 (−0.32, −0.11)	0.04	−0.22 (−0.33, −0.11)	0.04		
Garcia-Ortiz, L; 2012 ⁴⁶	Patient	ANCOVA					ng	0.88
Kawashima, K; 2018 ⁴³	General	ANCOVA	Ng	<0.001	ng	<0.001		
Lin, F; 2015 ⁴¹	General	Logistic regression (odds ratio) ^b	3.09 (2.01, 4.76)	<0.001				
Carotid intima-media thickness (all adults)								
Masaïdi, M; 2009 ⁴⁴	Patient	Pearson's correlation					0.12	0.02
Lammert, A; 2012 ⁴⁷	Patient	Pearson's correlation					−0.22	0.02
Torres, FS; 2013 ²⁰	Patient	Pearson's correlation	−0.12	0.13	0.13	0.09		
Garcia-Ortiz, L; 2015 ⁴⁰	General	Pearson's correlation	−0.06	ng ^a	0.12	ng ^a	−0.19	<0.01
Ikram, MK; 2004 ³²	General	Linear regression (standardized β) ^d	−0.06 (−0.10, −0.03)	<0.05	−0.01 (−0.04, 0.02)	ng ^a	−0.07 (−0.08, −0.03)	<0.05
van Hecke, MV; 2006 ³⁴	General	Linear regression (standardized β)	0.09 (−0.03, 0.21)	>0.05	0.14 (0.01, 0.25)	<0.05	−0.04 (−0.16, 0.09)	>0.05
Yang, JY; 2016 ⁴²	General	Linear regression (standardized β)	−0.06 (−0.10, −0.03)	0.001				
Wong, TY; 2003 ¹⁹	General	Logistic regression (odds ratio) ^c					0.7 (0.5, 1.0)	0.46
Garcia-Ortiz, L; 2012 ⁴⁶	Patient	ANCOVA					ng	0.46

Abbreviations: ANCOVA, analysis of covariance; General, general population; ng, not given; Patient, patient group; β, regression coefficient.

^aP value not given and stated as not significant.

^bOdds of elevated PWV (eg, highest quartile versus lowest quartile) by comparing the retinal caliber in highest quartile with the lowest quartile.

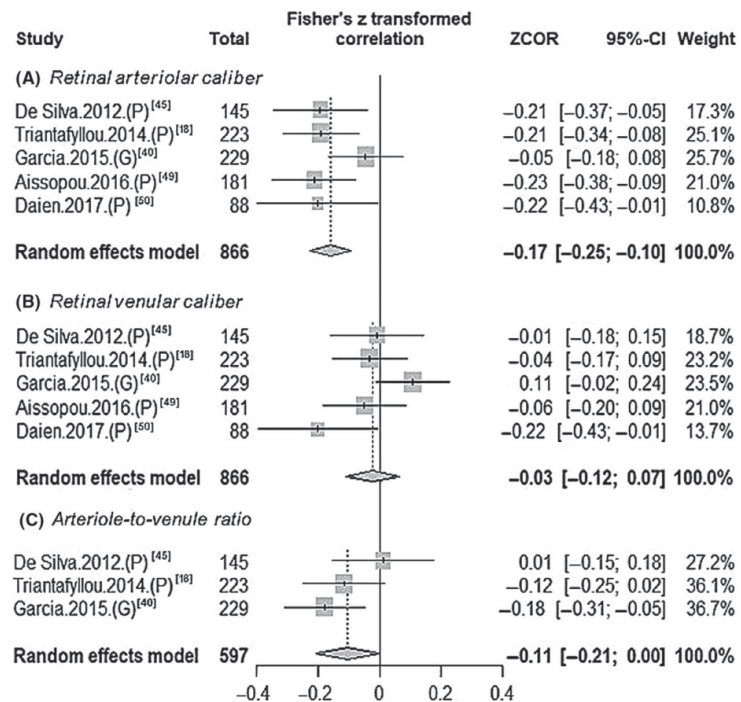
^cOdds of elevated CIMT (eg, highest quartile versus lowest quartile) by comparing the retinal caliber in highest quartile with the lowest quartile.

^dThe standardized β was transformed by the reviewer from the reported raw β to make the results comparable.

disorders. In these patients, narrower (worse) retinal arteriolar caliber was consistently and modestly correlated with higher (worse) PWV (correlation coefficient (*r*) range from −0.21 to −0.23, Table 2).^{18,45,49,50} This effect was only observed in one of three adult general population studies that compared extreme values using categorical variables.⁴¹ In children who were six years of age, there was little evidence of an association.¹⁷

Associations between retinal venular caliber and PWV were inconsistent. In children, there was little evidence that venular caliber was associated with PWV (*r* = 0.04).¹⁷ However, two of the three studies of hypertensive adult patients showed that narrower (usually considered to be better) retinal venular caliber was weakly associated with higher PWV (*r* = −0.06 to −0.22).^{49,50} Notably, this finding was in the opposite direction to that expected. Of the four studies

FIGURE 2 Forest plot for associations of retinal microvascular caliber with pulse wave velocity. The hypothesis was that arterioles would show inverse, and venules direct, associations. ZCOR, Fisher's z transformation of correlations; P, patient group; G, general population; CI, confidence interval



examining AVR, two European studies provided some evidence that lower (worse) AVRs were associated with higher PWV (in general population $r = -0.18$, $P < 0.01$ and in hypertensive patients $r = -0.12$, $P = 0.08$).^{18,40}

Of the six studies that examined other large arterial function measures (eg, arterial elasticity, arterial compliance), all were in the general population (Table S2). Results were largely consistent with the findings for PWV. For example, modest associations were predominantly observed between narrower retinal arteriolar caliber and worse functional intermediate phenotypes,^{35,36,39} while evidence for associations with retinal venular caliber and AVR were weak and less consistent.^{33,39,40}

3.3.2 | Associations of retinal microvascular caliber with structural intermediate phenotypes

Six studies in the general population reported on associations between retinal arteriolar caliber and CIMT, with inconsistent results. Two studies reported a weak association between narrower arteriolar caliber and higher CIMT (standardized $\beta = -0.06$; Table 2),^{32,42} while the other four studies did not show a significant association. However, in the only study with cardiometabolic patients, this association was not replicated.²⁰

Of the four studies examining retinal venular caliber and CIMT in the general population, only one study showed a weak association, with wider (worse) retinal venular caliber associated with higher (worse) CIMT (standardized $\beta = 0.14$).³⁴ There was weak evidence of

a similar association in the only patient study of hypertensive patients ($r = 0.13$).²⁰ Associations between AVR and CIMT were inconsistent in both general population and cardiometabolic patients.

Four studies reported data on retinal microvascular caliber and other structural intermediate phenotypes (eg, aortic calcification, coronary artery stenosis; Table S3).^{29,32,38,48} There was limited evidence of associations between retinal microvascular caliber and coronary artery stenosis,⁴⁸ but overall the findings from these studies were inconsistent.

3.4 | Meta-analysis

Figures 2–3 and Table 3 summarize meta-analysis results of retinal microvascular caliber associations with PWV and CIMT in adults. Overall, the level of heterogeneity (I^2) among pooled studies was low to moderate (Table 3). High heterogeneity was observed among pooled studies of venular caliber with CIMT and AVR with CIMT.

3.4.1 | Associations of retinal microvascular caliber with PWV

The random-effects meta-analysis of all five studies (general population 1, patients 4) showed that narrower retinal arteriolar caliber was modestly correlated with higher PWV across adults ($r = -0.17$, 95% CI -0.25 to -0.10 ; Figure 2A). Evidence of an association was stronger for patients (r ranging from -0.21 to -0.23) than the general population ($r = -0.05$, 95% CI -0.18 to 0.08 ; Table 3). The random-effects

TABLE 3 Meta-analysis of correlations of retinal microvascular caliber with pulse wave velocity and carotid intima-media thickness

Classification	Sample	No. of studies	Sample size	Estimate (r)	95% CI	P value ^b	I ² (%)
Pulse wave velocity							
Retinal arteriolar caliber	All	5	866	-0.17	-0.25 to -0.10	<0.0001	13.4
	General	1 ^a	229	-0.05	-0.18 to 0.08	N/A	N/A
	Patient	4	637	-0.22	-0.30 to -0.14	<0.0001	0
Retinal venular caliber	All	5	866	-0.03	-0.23 to 0.07	0.59	49.1
	General	1 ^a	229	0.11	-0.02 to 0.24	N/A	N/A
	Patient	4	637	-0.06	-0.14 to 0.02	0.12	0
Arteriole-to-venule ratio	All	3	597	-0.11	-0.21 to 0.00	0.05	37.4
	General	1 ^a	229	-0.18	-0.30 to -0.05	N/A	N/A
	Patient	2	368	-0.06	-0.19 to 0.06	0.32	28.7
Carotid intima-media thickness							
Retinal arteriolar caliber	All	5	9367	-0.05	-0.09 to -0.02	0.001	35.4
	General	4	9194	-0.05	-0.08 to -0.01	<0.01	46.0
	Patient	1 ^a	173	-0.12	-0.26 to 0.03	N/A	N/A
Retinal venular caliber	All	4	5414	0.08	-0.02 to 0.18	0.10	74.2
	General	3	5241	0.07	-0.04 to 0.19	0.22	77.5
	Patient	1 ^a	173	0.13	-0.02 to 0.27	N/A	N/A
Arteriole-to-venule ratio	All	5	5747	-0.07	-0.17 to 0.03	0.18	80.0
	General	3	5241	-0.09	-0.15 to -0.02	0.01	43.3
	Patient	2	506	-0.04	-0.38 to 0.29	0.81	90.4

Abbreviations: N/A, not applicable.

^aAs there was only one coefficient estimate no meta-analysis was performed.^bP value for the pooled correlation coefficients.

meta-analysis showed little evidence of an association between retinal venular caliber and PWV overall ($r = -0.03$, 95% CI -0.23 to 0.07 ; Figure 2B). Of the three studies with sufficient data to perform meta-analysis for AVR (general population 1, patients 2), lower AVR scores were associated with higher PWV ($r = -0.11$, 95% CI -0.21 to 0.00 ; Table 3).

3.4.2 | Associations of retinal microvascular caliber with CIMT

In the random-effects meta-analysis of five studies (general population 4, patients 1), narrower retinal arteriolar caliber was weakly associated with higher CIMT ($r = -0.05$, 95% CI -0.09 to -0.02 ; Figure 3A). There was little evidence of an association between retinal venular caliber and CIMT or AVR and CIMT (Table 3).

4 | DISCUSSION

4.1 | Main findings

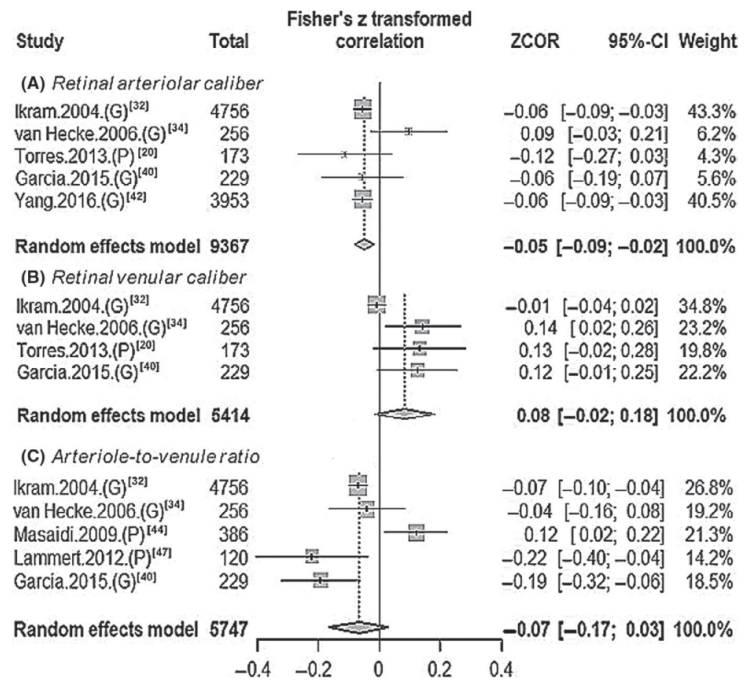
This systematic review and meta-analysis examined 26 studies to determine associations between retinal microvascular caliber and large arterial function and structure in both the general population and patients with cardiometabolic disease. There was little evidence of any associations for retinal venular caliber, but retinal arteriolar

caliber was associated with both large arterial function and structure. In the meta-analysis, the strongest associations were observed in the adult patient population for arteriolar caliber and vascular function (PWV; r ranging from -0.21 to -0.23). Although there was little evidence of a similar association in adults within the general population (PWV $r = -0.05$, 95% CI -0.18 to 0.08), the estimate was imprecise, reflecting the limited data of only a single small study on this topic. No associations were seen in children. A similar pattern was observed for vascular structure, although there was a dearth of studies in adult patients (one small study) and children (no studies) populations, precluding any conclusions in these groups. In the general population, retinal arteriolar caliber showed small associations with large arterial structure (CIMT $r = -0.05$, 95% CI -0.09 to -0.02).

4.2 | Strengths and limitations

Our study has several strengths. This is the first systematic review and meta-analysis to comprehensively describe and quantify associations between the retinal microvasculature and intermediate phenotypes of large arteries. Studies across a number of large arterial intermediate phenotypes observed similar findings, providing confidence in the results. We investigated both the function and structure of large arteries, as functional changes may precede structural changes in early atherosclerosis.⁵¹ In addition, our meta-analysis findings were consistent with those of our narrative review. We

FIGURE 3 Forest plot for associations of retinal microvascular caliber with intima-media thickness. The hypothesis was that arterioles would show inverse, and venules direct, associations. ZCOR, Fisher's z transformation of correlations; P, patient group; G, general population; CI, confidence interval



included studies reported in both English and Chinese to address potential language bias. The majority of included studies (92.3%) were of moderate or high quality, indicating that most had attempted to minimize bias. The most common source of bias was the selection of the sample (eg, unjustified sample size or no description of response rate, Table S2), which may limit the generalizability of our conclusions. Future studies warrant more rigorous study design to minimize selection bias.

We acknowledge a number of limitations, many of which were unavoidable. Given that almost all the included studies were cross-sectional, we were unable to explore the temporal directions of the observed associations. The only longitudinal study, which followed an adult population over five years, suggested that narrower retinal arteriolar caliber was an independent predictor of increased PWV.⁴³ Longitudinal replication in other cohorts is warranted. Participants were mainly middle- or older-aged adults, limiting conclusions that could be drawn for younger stages of the life course. The paucity of data from childhood, when atherosclerosis is first established and interventions may be more effective, remains a significant knowledge gap.^{3,52,53} In the meta-analysis, we pooled studies reporting unadjusted correlation coefficients and those with minimally adjusted standardized regression coefficients. This may have added bias to the results given that correlation coefficients are equal to unadjusted standardized regression coefficients, not minimally adjusted models, yet one would expect the results to be very similar. Given that we only considered associations based on unadjusted and minimally adjusted models, the combined results may be confounded

by factors have not been accounted for, such as demographics and CVD risk factors. This may have biased the results but was unavoidable given the different levels of adjustment in each study. We are not able to report funnel plots to visualize publication bias given the limited number of studies. Publication bias may exist, as there may be null findings that are unpublished studies.

4.3 | Interpretation of main findings

The limited data in children make conclusions in this population challenging. The one large study of 6-year-old children ($n = 4007$) did not suggest retinal microvascular caliber was associated with vascular function intermediate phenotypes¹⁷ and there were no data available for structural intermediate phenotypes. In adults, the narrative review and meta-analysis for retinal arteriolar caliber consistently showed small associations with arterial functional and structural intermediate phenotypes. These associations were most pronounced in the patient groups. This supports the concept that shared pathophysiologic processes may contribute progressively to shared small and large arterial dysfunction, and only later to damage in large arterial structure, as individuals' progress toward disease through the life course.

The small covariance we observed between retinal arteriolar caliber and large arterial intermediate phenotypes could also suggest that microvascular changes contribute to macrovascular pathology, which aggravates vascular damage.^{14,54,55} Evidence from animal experiments indicates that decreased vasa vasorum flow in the microcirculation leads to abnormal morphology of elastin and collagen

fibers of arterial walls, resulting in increased arterial stiffness.⁵⁶ This hypothesis is supported by a longitudinal population-based study indicating that narrower arteriolar caliber was independently associated with increased PWV five years later.⁴³ Similarly, damage to the structure and function of large arteries may itself result in adverse changes to the microcirculation.¹⁴

Little evidence on an association was observed between retinal venular caliber and large arterial intermediate phenotypes across both populations. One explanation why we did not observe an association could be that adverse changes of venules may have an adaptive response and emerge when CVD is overt.^{60,61} Our review included three studies performed in patients with different stages of hypertension^{18,49,50}; all studies showed consistent associations between arteriolar caliber and PWV, but results were inconsistent for venular caliber. In early-stage hypertensive patients, there was little evidence to suggest that retinal venular caliber was associated with PWV,¹⁸ while in never-treated hypertensive patients there was a moderate association in the unexpected direction (eg, smaller venular caliber and worse PWV).^{49,50} This indicates that retinal venules adapt to the hypertensive state with a decrease in caliber,^{49,50} but then there is a tipping point when cardiometabolic disease becomes overt, and this adaption breaks down, leading to an increase in venular caliber.^{60,61} Another explanation for the lack of association observed for venular caliber is different risk factors related to venular changes. Increased retinal venular caliber is associated with inflammatory markers, such as C-reactive protein and leukocyte count, smoking and increased body mass index.^{15,57-59} Previous studies have shown that retinal venular caliber is an independent predictor of CVD events and mortality.^{60,61} Thus, it is plausible that inflammation and these other factors are a mechanistic link between changes in retinal venular caliber and later CVD events.

Results regarding the associations of AVR and large arterial intermediate phenotypes were mixed.^{18,40} This may be because AVR fails to capture the different pathophysiological pathways of the retinal arterioles and venules, which is increasingly recognized.⁵⁷

4.4 | Implications

Our observations have a number of potential clinical implications. Firstly, the stronger association seen in cardiometabolic patients compared with the general population could suggest a synergistic effect on the variation of small and large vessels, where degradation is escalated once the disease begins. This is consistent with other studies showing that individuals with both type 2 diabetes and hypertension have an increased risk of CVD compared with those with either condition alone.⁶² Thus, it highlights the potential importance of the microcirculation in CVD pathogenesis and management, which has largely been overlooked.

Secondly, given the association between retinal arterial caliber and large arterial phenotypes was small, each phenotype may contribute to CVD primarily through independent causal pathways. Therefore, combining assessment of both may offer better prediction of CVD. For example, McGeechan et al. found that adding

both arteriolar and venular caliber to the Framingham risk score improved prediction of coronary heart disease in individuals without diabetes by 1.7%.⁶³ In persons with diabetes, Ho et al. showed that the prediction value for CVD was higher (3.0%) when retinal arteriolar, venular caliber, and diabetic retinopathy were added to other CVD risk factors.⁶⁴ However, it is unclear whether the inclusion of *both* large arterial phenotypes and retinal microvascular caliber adds to clinical risk prediction of CVD and which populations could benefit most. Future studies with clinical outcomes can explore this question by combining both measures from large arteries and microcirculation to inform the optimal use of these measures.

Thirdly, the lack of association seen in the only child study could highlight that early in life the micro- and macro-vasculature may operate under different determinants. Over time the micro- and macro-vasculature may gradually come to share pathophysiology and with physiologic dysregulation evident in mid-adulthood. Future studies warrant to examine the potential life course trends of this co-variation.

5 | CONCLUSIONS

Our systematic review and meta-analysis showed that retinal arteriolar but not venular caliber had small associations with large arterial function and modest associations with arterial structure, with stronger evidence in patients with cardiometabolic disease. This suggests that preclinical changes in large arteries and the microcirculation have some shared but mainly unique determinants.

PERSPECTIVE

The microcirculation is increasingly recognized for its role in the pathogenesis of CVD. We additionally showed that it shares a small amount of co-variation with preclinical cardiovascular phenotypes of large arterial function and structure. This indicates that the contribution of microcirculation to CVD is through some common pathways with the large vessels, but mainly through its own. Given that the small and large vessels do not appear to deteriorate in unison, it is important to understand their unique contribution to CVD across the life course. However, early life and longitudinal data with clinical outcomes are lacking. Such knowledge could inform whether the assessment of both the small and large vessels could improve risk prediction models and when in the life course this could offer the most value.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHORS' CONTRIBUTIONS

DB, KL, MW, TW and MH supervised the study. MW, DB, KL, TW, MH, and ML conceptualized and designed the study. ML, DB, KL and YX coordinated the data selection and extraction. ML, DB, KL, and MW involved in analysis and interpretation. ML drafted the manuscript. All authors contributed to critical revisions of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary online

Table 1S. Full search strategy developed in conjunction with institution librarian

Table 2S. Quality assessment for each included study

Table 3S. Cross-sectional associations of retinal vascular calibre with other types of vascular intermediate phenotypes

Table 1S. Full search strategy developed in conjunction with institution librarian

Medline search
1. Heart Rate/ or Aging/ or Aorta, Abdominal/ or Aorta/ or Cardiovascular Diseases/ or exp Hypertension/ or exp Carotid Artery, Common/ or Carotid Arteries/ or Coronary Artery Disease/ or Coronary Vessels/ or exp Carotid Artery Diseases/ or Atherosclerosis/ or Arteriosclerosis/
2. exp Retinal Vessels/ or exp Retinal Artery/ or exp Retinal Vein/ or retinal diseases/ or exp diabetic retinopathy/ or hypertensive retinopathy/
3. exp Ultrasonography/ or dg.fs. or exp Photography/ or Carotid Intima-Media Thickness/ or exp Pulse Wave Analysis/ or Blood Flow Velocity/ or tunica intima/ or exp tunica media/ or exp Vascular Stiffness/ or exp Elasticity/ or Blood Pressure/ or Vasodilation/ or Endothelium, Vascular/ or Calcinosi/s/ or Vascular Calcification/ or Ankle Brachial Index/
4. exp cohort studies/ or cross-sectional studies/ or follow-up studies/ or prospective studies/ or risk factors/ or Risk Assessment/ or case-control studies/ or Regression Analysis/ or Multivariate Analysis/ or Mass Screening/
5. 1 and 2 and 3 and 4
6. Exp animals/ not human*.sh.
7. 5 not 6
8. Limit to (English or Chinese)
EMBASE search
1. Cardiovascular Diseases/ or Cardiovascular risk/ or Common Carotid Artery/ or Carotid Artery/ or Coronary Artery Disease/ or Carotid Artery Diseases/ or Atherosclerosis/ or Arteriosclerosis/ or carotid atherosclerosis/ or coronary artery/
2. Retinal blood vessel/ or retina blood vessel occlusion/ or Retinal Artery/ or Retinal Vein/ or retinal diseases/ or diabetic retinopathy/ or hypertension retinopathy/ or eye photography
3. Arterial wall thickness/ or Pulse Wave/ or Blood Flow Velocity/ or artery intima/ or artery media/ or artery intima proliferation/ or arterial Stiffness/ or artery compliance/ or augmentation index/ or vasodilatation/ or vascular endothelium/ or tonometry/ or computer assisted tomography/ or coronary artery calcium score/ or coronary artery obstruction/ or artery calcification/ or calcification/ or abdominal aorta/ or artery occlusion/ or ankle brachial index/ or ultrasound/ or dg.fs. or carotid artery obstruction/
4. Cohort analysis/ or cross-sectional studies/ or observational study/ or prospective study/ or risk factor/ or longitudinal study/ or controlled studies/ or Regression Analysis/ or logistic regression analysis/ or risk assessment/ or epidemiology/ or priority journal or multiple regression or major clinical study/ or population research/
5. 1 and 2 and 3 and 4
6. Exp animal/ not human*.sh.
7. 5 not 6
8. Limit to (English or Chinese)
Pubmed search

-
1. "Cardiovascular Diseases" OR Arterioscleros* OR Atheroscleros* OR "Coronary Artery Disease" OR "Carotid Artery Diseases" OR "Carotid Artery Disease"
 2. "retinal vessel" OR "retinal vessels" OR "retinal artery" OR "retinal vein" OR Microvessel* OR "Hypertension retinopathy" OR "Hypertensive retinopathy" OR "Diabetic retinopathy" OR "retinal vascular calibre" OR "retinal vascular caliber" OR "retinal arteriolar calibre" OR "retinal arteriolar caliber" OR "retinal venular calibre" OR "retinal venular caliber" OR "retinal vascular changes" OR "retinal blood vessels" OR "retinal blood vessel" OR "retinal arteriolar narrowing" OR "retinal microcirculation" OR "retinal vasculature"
 3. distensibility OR "Artery compliance" OR "Tunica Intima" OR "Tunica Media" OR "common Carotid arteries" OR "common Carotid artery" OR "Carotid artery" OR "Carotid Arteries" OR "Intima-Media Thickness" OR "Pulse Wave Velocity" OR "Pulse Wave Analysis" OR "arterial wall thickness" OR "arterial thickness" OR "arterial stiffness" OR "flow-mediated vasodilation" OR FMD OR "calcium score" OR "arterial tonometry" OR "carotid ultrasound" OR "ENDOPAT"
 4. NOTNLM OR publisher[sb] OR inprocess[sb] OR pubmednotmedline[sb] OR indatareview[sb] OR pubstatusaheadofprint
 5. (animal* OR rat OR rats OR mice OR murine OR pig OR pigs OR cow OR cows OR bovine OR sheep) NOT (human OR humans OR newborn* OR neonat* OR infan* OR pre-schooler* OR preschooler* OR child OR children OR childhood OR adolescen* OR pediatric* OR paediatric* OR youth* OR teen* OR teens OR teenage* OR adult OR adults)
 6. (1 and 2 and 3 and 4) not 5
-

Table 2S. Quality assessment for each included study

Study ID [reference no.]	Selection of study population				Comparability of study groups	Adequacy of outcome assessment		Score	Study quality
	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7		
Klein, R; 2000[31]	1	1	1	2	0	2	0	7	Moderate
Wong, TY; 2003[19]	1	1	1	2	1	2	1	9	High
Ikram, MK; 2004[32]	1	1	1	2	2	2	1	10	High
Liao, D; 2004[33]	1	1	1	2	2	2	1	10	High
van Hecke, MV; 2006[34]	1	1	0	2	1	2	1	8	Moderate
Cheung, N; 2007a[35]	1	1	0	2	2	2	1	9	High
Cheung, N; 2007b[36]	1	1	1	2	2	2	1	10	High
Liew, G; 2008[37]	1	1	0	2	1	2	1	8	Moderate
Wong, TY; 2008[38]	1	1	0	2	2	2	1	10	High
Nguyen, TT; 2010[39]	1	1	1	2	2	2	1	10	High
Gishti, O; 2015[17]	1	1	1	2	2	2	1	10	High
Garcia-Ortiz, L; 2015[40]	0	1	0	2	2	2	1	8	Moderate
Lin, F; 2015[41]	1	1	0	2	2	2	1	9	High
Agladioglu, K; 2015[29]	0	0	0	2	0	2	1	5	Low
Yang, JY; 2016[42]	1	1	0	2	0	2	1	7	Moderate
Kawashima, K; 2018[43]	1	1	0	2	0	2	1	7	Moderate
Masaidi, M; 2009[44]	0	0	0	2	2	2	1	7	Moderate
De Silva, DA; 2012[45]	1	1	1	2	2	1	1	9	High
Garcia-Ortiz, L; 2012[46]	1	0	0	2	2	2	1	8	Moderate
Lammert, A; 2012[47]	1	0	0	2	1	2	1	7	Moderate
Torres, FS; 2013[20]	1	0	0	2	2	2	1	8	Moderate
Gopinath, B; 2014[48]	1	0	0	2	2	2	1	8	Moderate
Triantafyllou, A; 2014[18]	1	0	0	2	2	2	1	8	Moderate
Aissopou, EK; 2016[49]	1	0	0	2	2	2	1	8	Moderate
Li, HY; 2016[30]	0	0	0	2	0	2	0	4	Low
Daïen, V; 2017[50]	1	0	0	2	2	2	1	8	Moderate

Selection: (Maximum 5 stars)

Item 1: Representativeness of the sample

Item 2: Sample size

Item 3: Non-respondents

Item 4: Ascertainment of the exposure (Measurements)

Comparability: (Maximum 2 stars)

Item 5: The subjects in different outcome groups are comparable, based on the study design or analysis. Confounding factors are controlled.

Outcome: (Maximum 3 stars)

Item 6: Assessment of the outcome

Item 7: Statistical test

Table 3S. Cross-sectional associations of retinal vascular calibre with other types of vascular intermediate phenotypes

Lead author; year	Population	Vascular intermediate phenotypes	Statistical methods	Retinal arteriolar calibre (↓= worse)		Retinal venular calibre (↑ = worse)		Arteriole-to-venule ratio (↓ = worse)	
				Magnitude of association	<i>p</i> values	Magnitude of association	<i>p</i> values	Magnitude of association	<i>p</i> values
Vascular function intermediate phenotypes									
Liao, D; 2004[33]	General	Arterial elasticity	ANCOVA					ng	*
van Hecke, MV; 2006[34]	General	Flow-mediated vasodilatation	Linear regression model (standardized β)	0.02 (-0.16, 0.21)	ng	-0.04 (-0.16, 0.09)	ng	0.05 (-0.08 to 0.18)	ng
Cheung, N; 2007 ^a [35]	General	Arterial compliance ¹	Linear regression model (standardized β)	ng	**	ng	0.74		
Cheung, N; 2007 ^b [36]	General	Aortic distensibility ¹	Logistic regression model	2.48 (1.72, 3.60)	*	0.98 (0.68, 1.43)	ng		
Nguyen, TT; 2010[39]	General	Flow-mediated vasodilatation	Linear regression model (standardized β)	-0.13 (-0.25, 0.02)	*	0.14 (-0.36, -0.13)	***		
Garcia-Ortiz, L; 2015[40]	General	Ankle-brachial index	Pearson’s correlation	-0.07	ng	-0.07	ng	0.005	ng
Vascular structure intermediate phenotypes									
Klein, R; 2000[31]	General	Carotid plaque	ANCOVA					ns	**
Ikram, MK; 2004[32]	General	Aorta calcification ^a	Linear regression model (standardized β)	0.03 (-0.3, 0.4)	ng	0.4 (0.1, 0.8)	ng	-0.001 (-0.002, 0.001)	ng
		Carotid plaque score ^a		-0.2 (-0.5, 0.1)	ng	0.4 (0.1, 0.8)	*	-0.002 (-0.003, -0.001)	*
Wong, TY; 2008[19]	General	Coronary artery calcification ^a	Linear regression model (standardized β)	ns	0.42	ns	0.30		
Gopinath, B; 2014[48]	Patient	Presence of stenotic lesion	Logistic regression model (women)	2.22 (1.09, 4.53)	*	1.77 (0.88, 3.55)	ng		
			Logistic regression model (men)	1.45 (0.99, 2.15)	ng	1.54 (1.03, 2.29)	*		
Agladioglu, K; 2015[29]	General	Internal carotid artery diameter	Pearson’s correlation	-0.11	0.48	0.12	0.44		
Li, HY; 2016[30]	Patient	Carotid artery stenosis	Chi-square test	6.92	0.08	4.16	0.25	1.50	0.71

Asterisks (*) indicate significant associations at the following significance values: <0.05, *; <0.01, **; <0.001, ***.

¹. Retinal microvascular calibre was regarded as the outcome variable in the analysis.

Abbreviations: Patient, patient group; General, general population; ng, not given; ANCOVA, analysis of covariance; β, regression coefficient.

Chapter 5. Materials and methods

This thesis was conducted within the Longitudinal Study of Australian Children (LSAC) and its recent biological and physical wave, the Child Health CheckPoint (CheckPoint) study. The doctoral candidate's unique contribution to this dataset was generating the retinal microvascular parameters from retinal images and completing the reliability tests.

This chapter introduces LSAC's study design, recruitment and assessment methods, and retention rates from waves 1 to 6 (e.g., up until the CheckPoint wave). This is followed by outlining CheckPoint's recruitment and assessment methods, and data collection and data generation protocols for measures germane to the thesis aims (e.g., retinal microvascular parameters, preclinical large arterial phenotypes, obesity-related measures, blood pressure and biomarkers). This chapter also summarises other relevant data collection protocols for measures that the thesis draws on as covariates and methods of deriving new variables (e.g., carotid arterial elasticity and anthropometric measures). Finally, the cohort profile with characteristics of participated samples and an overview of statistical analysis plans are presented.

5.1 Study design

The study design outlined in this section is for both LSAC and CheckPoint. Full methods for both LSAC and CheckPoint have been published elsewhere.^{207, 208}

5.1.1 The Longitudinal Study of Australian Children

LSAC is the largest and only nationally-representative children's longitudinal study in Australia.²⁰⁹ It is conducted by the Australian Government Department of Social Services, in partnership with the Australian Institute of Family Studies and the Australian Bureau of Statistics. LSAC is designed to create a modern Australian population-based childhood cohort study since born, to continuously examine the impact of Australia's unique social and cultural environment on the millennium generation.²⁰⁹ The study aims to provide a strong evidence base for policy development and service delivery based on the understandings of a broad range of issues relating to child development. The design of LSAC included random sample selection, biennial data collection, family (primary caregiver and child) respondents,

linkage to other health care, education and census datasets, and open access for researchers. The adult respondents are the primary caregivers of the children, usually their biological parents, which refers to as ‘adults’ in this thesis.

5.1.1.1 Sampling strategy and recruitment

In 2004, LSAC recruited two childhood cohorts: 1) the Birth (‘B’) cohort with 5,107 infants aged 3 to 19 months and 2) the Kindergarten (‘K’) cohort with 4,983 children aged 4 to 5 years.²⁰⁹ A two-stage random sampling strategy was applied. First, 10% of all Australia postcodes were randomly selected, stratified by state and urban/rural locations. Then, researchers selected in-age children within those postcodes from Australia’s universal health care system, the Medicare database.²¹¹ Medicare contains 98% of Australian children’s information by their first birthday. Very remote postcodes and postcodes with less than 20 children (n= 874 postcodes, 3.2% of the population) were excluded. Data collection is ongoing and has been occurring in biennial waves of home visits from 2004.

5.1.1.2 LSAC Birth cohort

The B cohort has pregnancy and birth data by linking with Medicare, with data collection spanning children’s entire postnatal lives. In addition, the child and adult paired data collection design enables the understanding of families’ influences on child development and health. Table 5-1 summarises age and number of participated children in biennial waves of the B cohort from wave 1 to 6. Three-quarters of children retained at wave 6 data collection.

Despite LSAC’s rich and comprehensive measures of the child’s psychosocial and demographic exposures, it lacked information on physical health measures. The exception was anthropometric measures that were collected from wave 2 onwards and blood pressure which was measured at wave 6. The addition of physiological measures and biomarkers could help to understand the early life mechanism underlying children’s health and wellbeing. To address this gap and enrich the ongoing LSAC B cohort, in 2015-16, our team undertook the Child Health CheckPoint, an inter-generational physical health and biomarkers wave.

Table 5-1 LSAC B Cohort recruitment and retention from wave 1 to 6

Birth cohort	Wave 1 2004	Wave 2 2006	Wave 3 2008	Wave 4 2010	Wave 5 2012	Wave 6 2014
Age range (yrs)	0-1	2-3	4-5	6-7	8-9	10-11
Number of participants	5,107	4,606	4,386	4,240	4,077	3,764
Retention rate	100%	90%	86%	82%	80%	75%

5.1.2 The Child Health CheckPoint

The CheckPoint was conducted as a cross-sectional data collection wave between LSAC's waves 6 and 7. CheckPoint aimed to provide data on the distribution of cardiovascular, respiratory and other health problems, risk factors for future disease and biomarkers in Australian parent-child dyads. It creates a database of children's and adults' health both as outcomes of all previous LSAC waves and predictors of LSAC's future waves (Figure 5-1).

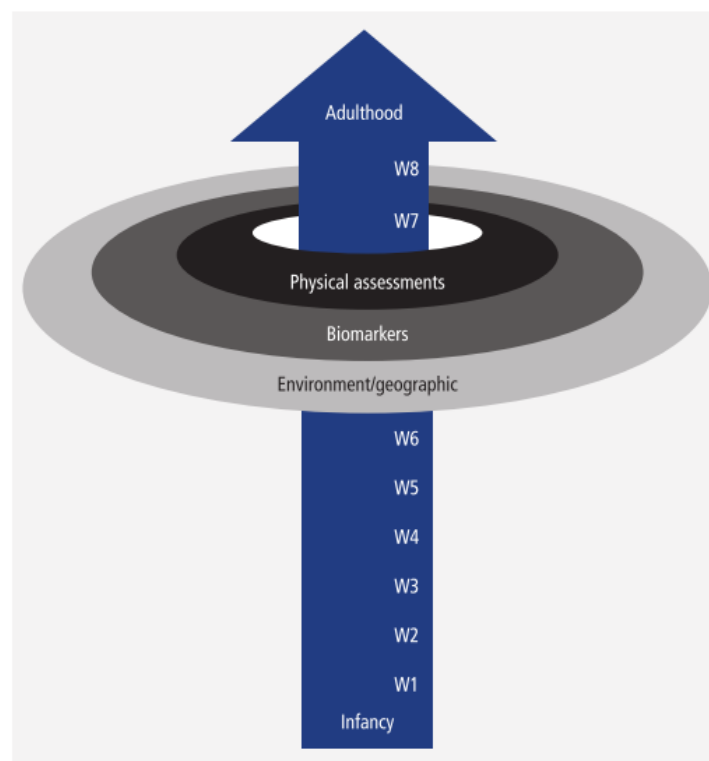


Figure 5-1 Child Health CheckPoint within the LSAC Birth cohort

Originally published by our research team in ref.²¹²; reproduced with permissions.

5.2 Ethical approval and consent

LSAC data collection was approved by the Australian Institute of Family Studies Ethics Committee. Correspondingly, CheckPoint data collection was approved by the Australian Institute of Family Studies Ethics Committee (14-26), as well as The Royal Children's Hospital Melbourne Human Research Ethics Committee (33225D).

Adults provided written informed consent for themselves and their children at each LSAC wave and the CheckPoint. In CheckPoint, optional consent was requested for the collection, storage and non-genetic analysis of biospecimens; genetic analyses of biospecimens; sharing images and samples with other researchers; access to the child's birth data and dried newborn heel-prick blood samples that are stored indefinitely by most Australian states. Participants were aware that no health, genetic or other information would be returned to them, beyond a summary of physical health measurements provided at the end of the visit.

5.3 Procedures

5.3.1 Participant recruitment

All B cohort families who took part in LSAC wave 6 data collection (2014) were eligible for CheckPoint (n= 3,764 families). During the wave 6 home visit, interviewers from Australian Bureau of Statistics introduced the upcoming CheckPoint study to families and asked them to provide written consent for their details to be passed onto the CheckPoint team for recruitment purposes. A total of 3,513 families (93% of wave 6 families and 69% of the original cohort) agreed to be contacted by the CheckPoint team. Then, the Australian Bureau of Statistics passed the consenting families' contact details to the CheckPoint team, who was based at the Murdoch Children's Research Institute, Melbourne, Australia.

The CheckPoint team mailed families a pre-notification postcard, followed by an invitation letter and the Information and Consent booklet, and then made a recruitment and scheduling call. Families were primarily offered an Assessment Centre ("Main" or "Mini", see details in 5.3.3) appointment. Families living in regional and remote areas were offered contributions towards travel and accommodation costs to maximise participation. A Home Visit appointment was

offered to those who were not able to attend an Assessment Centre. The geographical distribution of the sample mirrored the Australian population. Overall, CheckPoint visited over 30 cities and towns over a 12-month period (Figure 5-2).

- 18 cities, 16 venues
- Total km travelled = 16,896

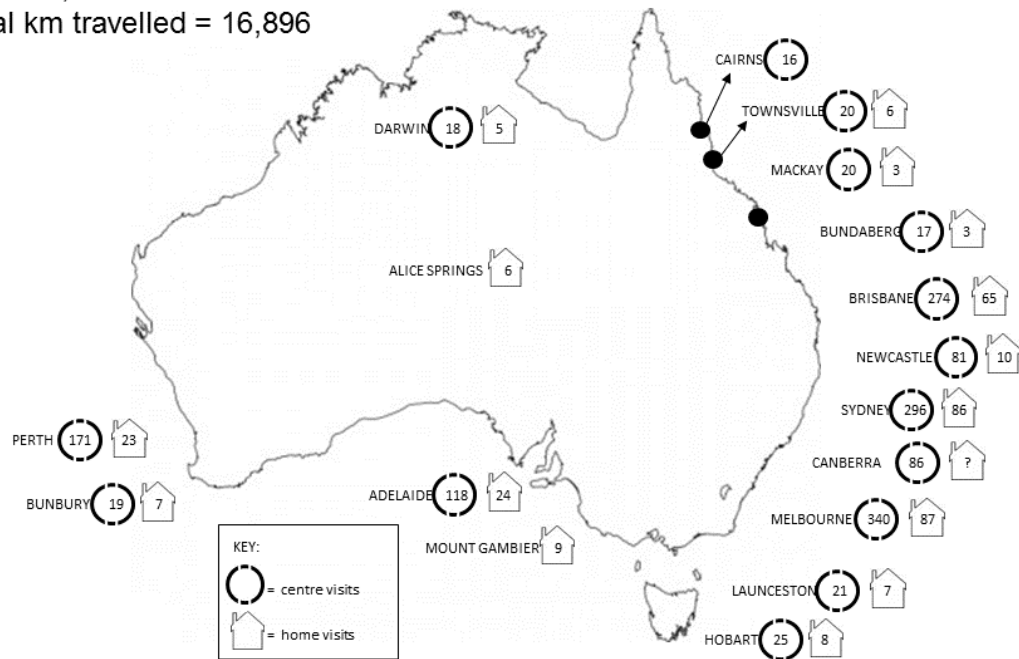


Figure 5-2 CheckPoint data collection around Australia

5.3.2 CheckPoint participants

The CheckPoint invited the study child and one primary caregiver to participate in the comprehensive health assessments. Families can choose whom to attend, and whether or not this is a biological parent, which was in keeping with the methodology of main LSAC waves. In practice, this ‘attending parent’ was usually the mother (See Table 5-7 in Section 5.6.1.1). Of the 3,513 LSAC wave 6 families that agreed to be contacted by CheckPoint, 84% (n= 3,152) provided valid contact details; 1,875 of these families took part in the CheckPoint. However, one family withdrew consent after assessment, making the number of families with data 1,874. Figure 5-3 shows the flowchart of participation from LSAC wave 6 to CheckPoint.

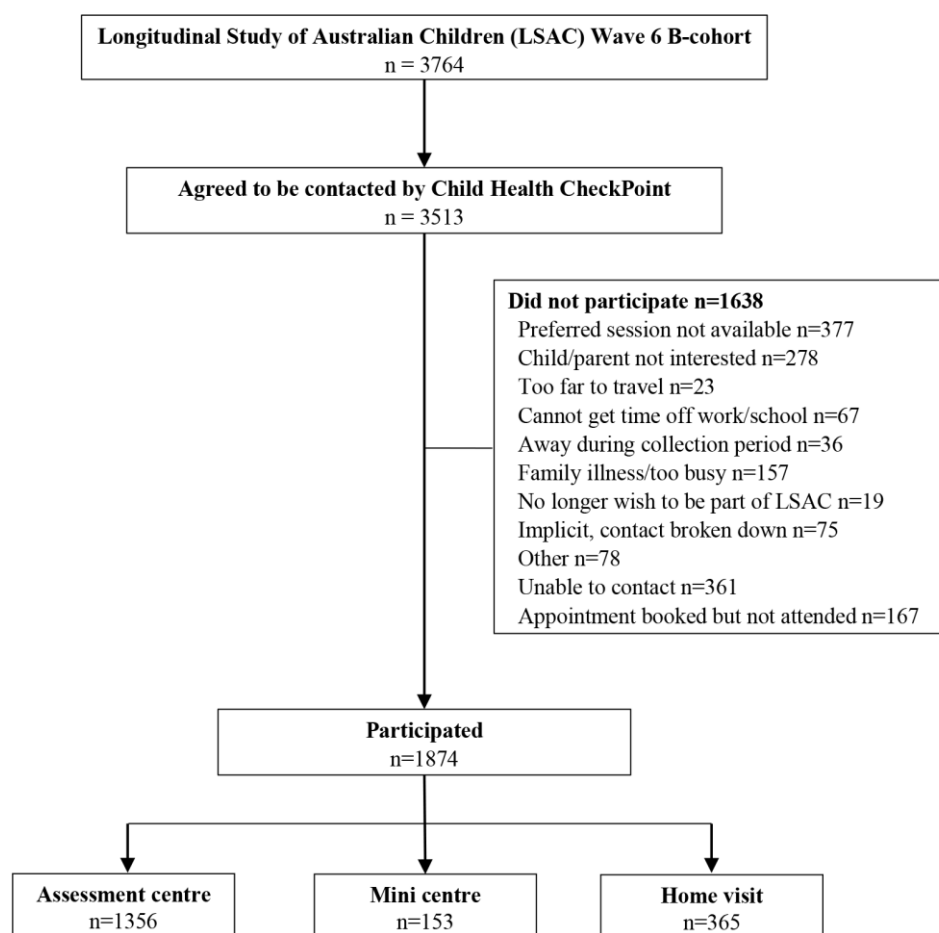


Figure 5-3 Participants flow from LSAC wave 6 to CheckPoint

5.3.3 Assessment visit types

5.3.3.1 Assessment Centres

CheckPoint set up the ‘pop-up’ Main and Mini Assessment Centres in 16 cities and towns. The Main Assessment Centres were set up in seven major Australian cities (Melbourne, Canberra, Sydney, Newcastle, Brisbane, Adelaide and Perth). On each operating day, up to 24 families took part, arriving every 15 to 30 minutes for a 3.5-hour visit. Children and adults rotated through 17 stations assessing their heart and lung health, fitness, strength, vision, diet, anthropometry and more. At the end of 2 to 8 weeks of data collection, the Main Assessment Centre was packed up and transported by road to the next city. Overall, most families (n= 1,356, 72%) attended the Main Assessment Centres.

Mini Assessment Centres were operated in regional cities where there was necessary infrastructure, and at least 40 eligible families were within the radius. The ‘pop-up’ centres were operated for up to a week between data collection periods in Main Assessment Centres. Participants that visited the Mini Assessment Centres were offered most of the assessments same as at the Main Assessment Centres for 2.5 hours, except those requiring large equipment that was unable to be checked in as personal luggage on commercial flights (e.g., pQCT scanner, retinal fundus camera).

5.3.3.2 Home Visits

A 1.5-hour Home Visit was offered to those who were unable to attend a Main or Mini Assessment Centre. They occurred concurrently in regions where Main and Mini Assessment Centres took place and in other regional towns. Home Visit included a subset of measures that could be conducted at home by a trained research assistant using portable equipment.

5.3.4 Data collection

At Assessment Centres, every child and adult rotated separately through numerous 15-minute assessment stations (except that child *Lung Fun* was 30 minutes), see Figure 5-4. Most stations were conducted one-on-one, but in some, the study child and adult both presented, and two children could present at any one time for some stations. The sequence of the stations was designed carefully to minimise systematic errors. For example, the use of bronchodilator administration at *Lung Fun* followed cardiovascular measures, and the snack station at *Food Stop* was scheduled after saliva and semi-fasting blood collection but before exercise.

The measures relevant to this thesis were collected at the *Measure Up* station that included anthropometric measures (weight, height and waist circumference); *Heart Lab* that included measures of preclinical large arterial function and structure; *Young Bloods* that collected blood samples; and *See Here* where retinal images were collected. In Section 5.4, I outline details of how we measure and generate the data.



Figure 5-4 CheckPoint Main Assessment Centre assessment stations

5.3.5 Data storage and transfer

In CheckPoint, we save, backup and transfer all participant data use a secure web application: Research Electronic Data Capture (REDCap, Vanderbilt University, US). For direct measures, such as PWV and CIMT, data were generated from specific platforms and transferred to REDCap by our data manager. For other measures (e.g., anthropometry), the staff member transcribed data from relevant instruments into REDCap. Biospecimen barcode registration and tracking are also via REDCap. In addition, we used the FileZilla software with encrypted passwords to transfer retinal images from Melbourne to China for scoring.

5.4 Measures

Table 5-2 below summarises the assessment centre types, stations and equipment for each key measure. As this thesis also draws on data from the LSAC waves 1 to 6, relevant LSAC data are also shown in Table 5-2.

Table 5-2 LSAC and CheckPoint measures

Construct & Measure	LSAC						CheckPoint				Equipment/instrument								
	W1		W2		W3		W4		W5			W6		Main Mini		Station			
	C	A	C	A	C	A	C	A	C	A		C	A	C	A				
Microvascular parameters																			
Retinal vascular calibre														•	•	 See Here	Fundus camera EOS 60D SLR (Tokyo, Japan) and Integrative Vessel Analysis (IVAN, University of Wisconsin, Madison, US).		
Retinal geometric parameters														•		 See Here	Fundus camera EOS 60D SLR (Tokyo, Japan) and Singapore I Vessel Assessment software (National University of Singapore, Singapore).		
Anthropometry																			
Height			•	•	•	•	•	•	•	•	•	•	•	•	•	•	 Measure Up	LSAC: Invicta stadiometer or laser stadiometer CheckPoint: Portable rigid stadiometer (Invicta IP0955, Leicester, UK).	
Weight			•	•	•	•	•	•	•	•	•	•	•	•	•	•	 Measure Up	LSAC: Salter Australia glass bathroom scales (150 kg x 50 g), HoMedics digital BMI bathroom scales (180 kg x 100 g) or Tanita body fat scales were used. CheckPoint: 4-limb segmental (InBody230, Biospace, Seoul, Korea) or 2-limb (Tanita BC-351, Kewdale, Australia) body composition scales.	
Waist circumference				•		•		•		•		•		•		•	 Measure Up	Steel anthropometric measuring tape (Lufkin Executive Diameter W606PM, Maryland, US).	
Large arterial phenotypes																			
Pulse wave velocity, blood pressure														•	•	•	•	 Heart Lab	Sphygmocor XCEL (AtCor Medical Pty Ltd., West Ryde, NSW, Australia)
Carotid intima-media thickness														•	•	•	•	 Heart Lab	Portable ultrasound (GE Health Vivid-I BT06 with 10MHz linear array probe, Little Chalfont, UK) with electrocardiogram and Carotid Analyzer (Medical Imaging Applications, Coralville, IA, US).
Biospecimen																			
Venous blood														•	•	•	•	 Young Bloods	S-Monovette vacutainers: 2.7ml potassium EDTA (05.1167.001), 9 ml K3 EDTA (02.1066.001), 7.5ml Lithium Heparin liquid (01.1608.001), 9ml Serum Gel with Clotting Activator (02.1388.001), Sarstedt, Australia

Abbreviations: LSAC, Longitudinal Study of Australian Children; W, study wave; C, child; A, attending adult; Main, Main Assessment Centre; Mini, Mini Assessment Centre.

5.4.1 Retinal microvascular parameters

Both retinal vascular calibre and geometric parameters are analysed in this project. I was responsible for and heavily involved in the image scoring process. As described in the literature review, retinal vascular calibre is the most commonly measured retinal microvascular parameter in population-based studies. The scoring of both child and adult retinal vascular calibre was conducted either at the Zhongshan Ophthalmic Centre in Guangdong, China (77%) or the Centre for Eye Research Australia in Melbourne, Australia (23%). As the Guangdong grading team were Chinese, I was responsible for all liaison regarding the scoring requirements, reliability tests, sending images and feedbacks between groups and helping translate and solve queries. After nearly six months of work, my efforts resulted in the complete retinal vascular calibre dataset (2,624 images).

Retinal geometric parameters were not generated for adults due to time and funding limitations. The scoring of retinal geometric parameters was conducted either by thesis author at Murdoch Children's Research Institute (83%) or graders at the Centre for Eye Research Australia (17%). Before starting the scoring, I completed a 4-month training program at the Centre for Eye Research Australia. Then, I performed image scoring 3 to 4 hours per day for 10 months and completed the retinal geometric parameter dataset (1,283 images). I was also responsible for the reliability statistics and finalising documentation for the eventual public release of the data.

5.4.1.1 Retinal imaging

At *See Here* station, a trained research assistant explained the procedure of taking retinal photos to each participant; the room was then darkened once the participant sat in front of a fundus camera. The participant rested their chin and forehead on the headrest of the fundus camera and focused on the screen. With the instructions from the operator, the participants saw the screen for taking retinal photos with their right and left eye in turn. At least four digital photographs (one for each eye centring on the optic disc and macula) were taken using the standard protocol from the Centre for Eye Research Australia. Detailed information about retinal image collection and

preparation is provided in the *See Here* data collection Standard Operating Procedure (SOP) in Appendix A.

CheckPoint retinal images were not taken at Mini Assessment Centres or Home Visit because the large, delicate camera was not portable. Few families refused to have the retinal image measure (Child $n=3$, adult $n=2$), and some families did not have this measure due to equipment failure or time constraints (Child $n=46$, adult $n=37$). Thus, around 70% of CheckPoint participants (1,307 children and 1,317 adults) had retinal images available.

5.4.1.2 Retinal vascular calibre

Correlations of retinal vascular calibre between right and left eyes have been shown to be high ($r > 0.71$).^{213, 214} Thus, in our study, right eye images were selected as the first choice for scoring. This also allowed harmonisation with preclinical large arterial phenotypes obtained in CheckPoint, such as PWV and CIMT, which also assessed the right-sided circulation. Left eye images were scored only when right eye images were deemed ungradable. Issues preventing grading of images included poor focus with potentially blurring vessel edges, dark images which increase the difficulty for graders to visually validate the vessel trace, and confounding pathology (e.g., bright reflections) which can obscure the vessels. In total, all 2,624 images were graded, and most images were scored from right eyes (87% and 92% for children and adults, respectively).

One of four experienced graders (two from China and two from Australia) individually scored each image blinded to participant ID using the semi-automated software program IVAN (University of Wisconsin, Madison, US; Figure 5-5). The software can automatically identify retinal vessels as arterioles or venules from a specific grading area (0.5 to 1 disc diameter from the margin of the optic disc). Graders need to make corrections if the software misclassifies arterioles and venules. The software will then trace a segment of each vessel from the grading area automatically. After ensuring the vessel segments are traced properly, diameters of all the selected segments were measured by IVAN. The scoring procedure of each image took 3-5 minutes to complete (the scoring protocol can be seen in Appendix B).

For each participant, the average retinal vascular calibre of the six largest arterioles or venules were calculated according to the Big-Six (revised Knudston-Parr) formula.²¹¹ The software provides information about retinal arteriolar and venular calibre, and the arteriole-to-venule ratio can then be calculated (e.g., venular calibre divided by the arteriolar calibre). However, this ratio fails to capture the different pathophysiological pathways of the retinal arterioles and venules,¹⁸⁹ so it was not included in this thesis. As outlined in the literature review, narrowing arteriolar calibre and widening venular calibre denote worse retinal vascular changes.^{80, 105}

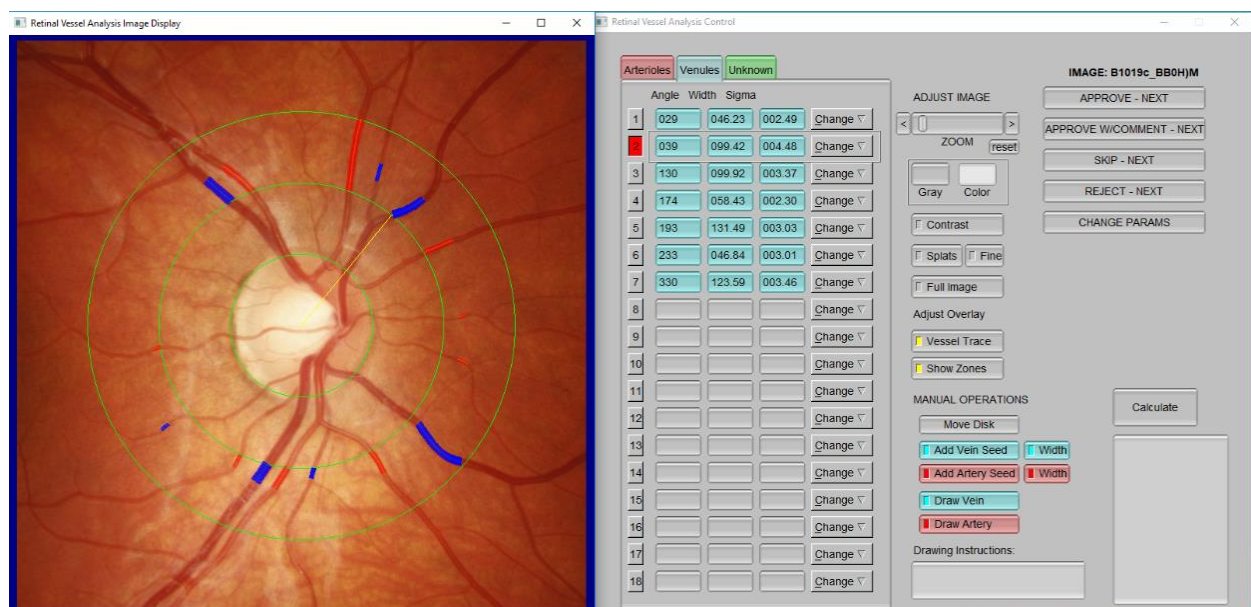


Figure 5-5 The interface of image scoring software IVAN

Arterioles are highlighted in red and venules in blue. The measured area of retinal vascular calibre is within 0.5 to 1.0 disc diameters away from the disc margin.

IVAN inter-grader reliability was assessed by three of the four graders analysing a subset of 50 randomly chosen images, while the intra-grader reliability was assessed by the same three graders each re-grading 25 of the 50 randomly chosen images. (one of the graders' reliability [Ms Lauren Hodgson] was missing. Since she had a rich experience of IVAN scoring and had high reliability records from previous work, her scoring (15%) is considered reliable and included in the dataset.) Two-way mixed-effects intraclass correlation coefficients were calculated for the reliability analysis. Between graders, the interclass correlations were high at 0.79 (95% CI 0.47 to 0.91), 0.92 (95% CI 0.77 to 0.96) for retinal arteriolar and venular

calibre, respectively. Within-grader, the intraclass correlations were also very high across the three graders, 0.90 to 0.99 (95% CIs 0.76 to 0.99) for arteriolar calibre and 0.92 to 0.98 (95% CIs 0.82 to 0.99) for venular calibre.

5.4.1.3 Retinal geometric parameters

Retinal geometric parameters (fractal dimension, tortuosity and branching angle) were generated for children using the semi-automated SIVA software (version 3.0; Figure 5-6).⁸² In total, 1,283 images were graded from children, and 93.4% of images were scored from right eyes.

Three graders masked to participant ID conducted the scoring (two based at the Centre for Eye Research Australia and the thesis author based at Murdoch Children's Research Institute). Similar to IVAN, the SIVA software can automatically detect and trace the optic disc, but it sets a wider grading grid on the retinal image, which is between 0.5 and 2.0 disc diameters away from the disc margin, and automatically trace vessel path. Graders need to check if the software accurately classifies vessels as either arterioles or venules, with manual vessel tracking required to ensure completed of each tracing. The average scoring procedure for each image took about 20-40 minutes. (The scoring protocol can be seen in Appendix B).

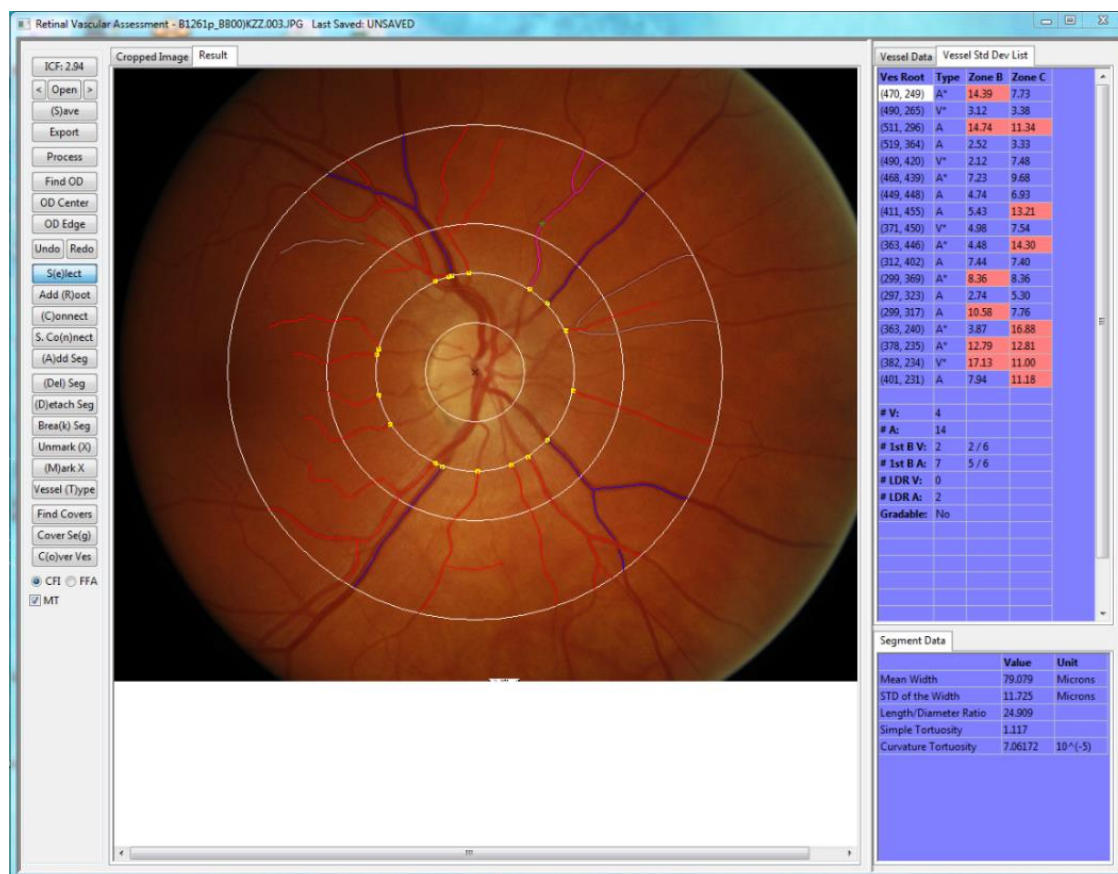


Figure 5-6 Interface of image scoring software SIVA

Arterioles are highlighted in red and venules in blue. The measured area of retinal geometric parameters is demarcated by white circles 0.5 to 2.0 disc diameters away from the disc margin.

Retinal fractal dimension is calculated using the ‘box-counting method’, which divides each photograph into a series of squares of various side lengths. Fractal dimension is defined as the gradient of logarithms of the number of boxes and the size of those boxes (Figure 5-7(a)). Tortuosity is calculated by the actual length of vessel divided by the Euclidean distance between the first and last points of that vessel (e.g., the length of the straight line connecting the two points, Figure 5-7(b)). Branching angle is defined as the first angle subtended between two daughter vessels at each vascular bifurcation (Figure 5-7(c)).

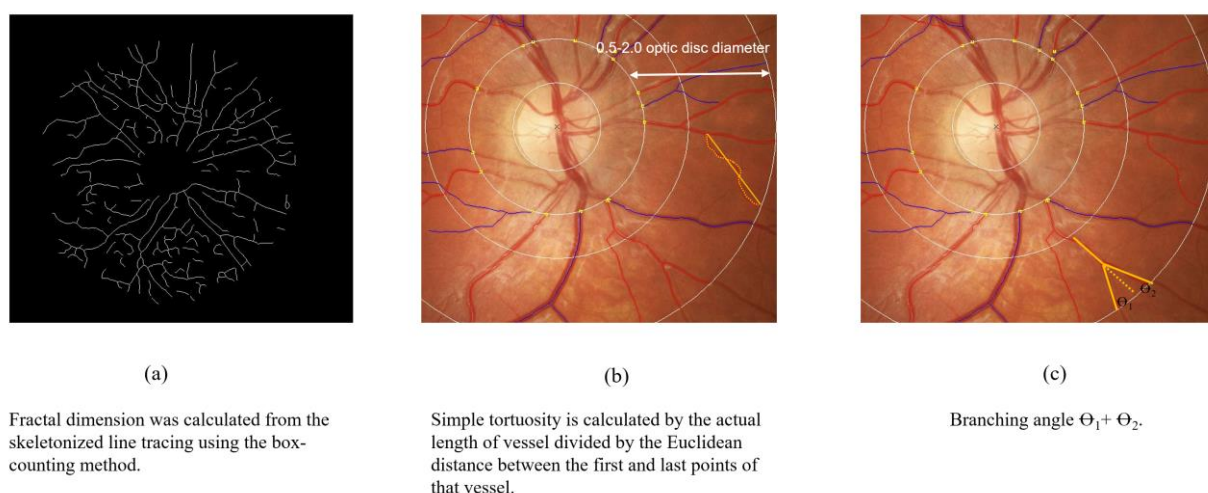


Figure 5-7 Retinal geometric parameters assessed via SIVA software

The inter- and intra-grader reliability were assessed by three graders analysing a subset of 30 and 46 randomly chosen images, respectively. Two-way mixed-effects intraclass correlation coefficients were calculated. Between graders, the interclass correlations for retinal arteriolar and venular fractal dimension were 0.60 (95% CI 0.42 to 0.76) and 0.59 (95% CI 0.41 to 0.75); for arteriolar and venular tortuosity were 0.86 (95% CI 0.77 to 0.92) and 0.78 (95% CI 0.66 to 0.87); and for arteriolar and venular branching angle were 0.61 (95% CI 0.44 to 0.76) and 0.19 (95% CI 0.06 to 0.48). The within grader interclass correlations for retinal arteriolar and venular fractal dimension were 0.67 (95% CI 0.40 to 0.82) and 0.65 (95% CI 0.37 to 0.81); for arteriolar and venular tortuosity were 0.93 (95% CI 0.88 to 0.96) and 0.75 (95% CI 0.55 to 0.86); and for arteriolar and venular branching angle were 0.81 (95% CI 0.66 to 0.90) and 0.49 (95% CI 0.08 to 0.72). Because the reliability is relatively low for branching angle, this parameter is not used in the analysis.

5.4.2 Preclinical large arterial phenotypes

Preclinical large arterial phenotypes, including functional phenotypes PWV and structural phenotype CIMT, were collected at the *Heart Lab* station across Main and Mini Assessment Centres. Home Visit families had PWV measures, but not CIMT, as the equipment for PWV measure was portable and feasible within the 1.5-hour home assessment. Another large artery functional phenotype, carotid arterial

elasticity, is derived from the data collected at *Heart Lab* station, which is introduced later in Section 5.5.1.

Within the 15-min *Heart Lab* station, carotid ultrasound, PWV and blood pressure acquisition each took approximately five minutes. Ultrasound was performed first to provide sufficient supine participant time prior to PWV and blood pressure measurements.

5.4.2.1 Carotid intima-media thickness

Carotid artery images were obtained using a portable ultrasound machine and 10 megahertz (MHz) linear array probe (Vivid-I, GE Healthcare, Chicago, IL, US). All participants lay supine with their head turned 45° to the left to expose the right side of the neck. The ultrasound probe was applied to the right side of the neck at around 45° to the midline with concurrent 3-lead electrocardiogram (ECG) trace. The duration of the captured real-time B-mode ultrasound cine-loops was 10 cardiac cycles. One of the four operators acquired two images per participant with a third measurement taken if feasible. The procedure was conducted using standardised protocols developed in accordance with recommendations of the American Society of Echocardiography²¹³ and Mannheim Consensus statements.²¹⁶

CIMT scores were analysed using Carotid Analyzer (Medical Imaging Applications, Coralville, IA, US). All carotid artery images were reviewed by one technician to select optimal loops that met key criteria: comprising a clear far wall intima-media, clear lumen, straight vessel, presence of the carotid bulb and an ECG trace. Raters calibrated the images using ultrasound image markers. The best quality 5 to 7 cardiac cycle section of the loops were trimmed and extracted. These loops were further processed to Carotid Analyzer. CIMT was measured at the vessel region of the highest quality 5 to 10 mm from the carotid bulb. After algorithmic detection of the intima-media interface over the entire cine-loop, frames were manually adjusted as needed or rejected if the intima-media interface was unclear or blurred. Maximum CIMT values were used in analyses, which refer to the 3 to 5 frame average of the measured region. The higher score of CIMT represents a thicker carotid artery wall.

Inter- and intra-rater reliability was assessed by re-analysing a subset of 105 randomly selected images four times at the end of the scoring process. Images were

reassessed twice each by two raters in a balanced incomplete block design as not all raters assessed the complete subset. The intraobserver variability for the maximum CIMT values was 4.9% (95% CI 4.6 to 5.2), and the interobserver variability was 6.2% (95% CI 5.2 to 7.2). Within-rater intraclass correlations was 0.62 (95% CI 0.54 to 0.71) while the between-rater intraclass correlations was 0.59 (95% CI 0.49 to 0.68). Further details are described elsewhere.²¹⁷

5.4.2.2 Pulse wave velocity

The large arterial function was measured using the SphygmoCor XCEL device (AtCor Medical Pty, West Ryde, NSW, Australia) with the participants supine. The blood pressure cuffs were set up on the participant's right arm and right leg to measure the arterial waveforms. Over a 10-second period, the SphygmoCor software detected the time taken for the arterial waveform to propagate from the carotid to the femoral artery. Distance travelled by waveforms was measured with a tape from the carotid pulse to the suprasternal notch, suprasternal notch to right femoral pulse and femoral pulse to top of thigh cuff. Each section length was entered into the SphygmoCor software individually, and the software can then calculate the total distance automatically.²⁸ PWV was calculated automatically by the software as the ratio of the distance travelled by the pulse wave and the time delay between the waveforms (m/s). Data were collected one to three times for each participant, and the mean was calculated. A quicker PWV indicates higher levels of arterial stiffness.

To assess inter-rater reliability, 112 recorded waves from a random sample of 40 participants (20 children and 20 adults) were presented blindly to two analysts for review. The sample was stratified by an analyst, ensuring each analyst had originally assessed half the participants' waveforms. Analysts needed to rate the quality of waveforms as 1 'good', 2 'adequate' or 3 'poor'. The majority of the sample waveforms were assessed as 'good' and none were of 'poor' quality. The positive agreement between two analysts was high (0.99). The absolute agreement by analysts was observed for 110 (98%) of the 112 waveforms assessed.

5.4.2.3 Blood pressure

Blood pressure was also collected using the SphygmoCor XCEL device. The brachial systolic and diastolic blood pressure were recorded at the brachial artery

with either a standard adult cuff (for arm circumference 23-33cm) or large adult cuff (for arm circumference 31-40cm). For children 11 to 12 years, the use of ‘adult’ brachial cuffs was deemed appropriate as the arm size is sufficient.²¹⁸ Data were collected one to three times for each participant, and the mean was calculated.

5.4.3 Anthropometry

We drew on anthropometric measures from LSAC waves 1 to 6 and from the CheckPoint *Measure Up* station. Although the anthropometric data collection in LSAC and CheckPoint used different equipment, the measurement protocols were similar.

5.4.3.1 LSAC anthropometry

5.4.3.1.1 Height

Height (cm) was measured directly for children from age two years. For children, height was measured using an Invicta stadiometer from Modern Teaching Aids in waves 2 to 3, and using a laser stadiometer in waves 4 to 6. Two measurements were taken, and a third measurement was taken if the two measurements differed by 0.5 cm or more. The average of the two closest measurements was used. Adult’s height was self-reported at all waves.

5.4.3.1.2 Weight

From waves 2 to 6, the child’s weight (kg) was measured directly. In waves 2 and 3, a mixture of Salter Australia glass bathroom scales (150 kg * 50 g) and HoMedics digital BMI bathroom scales (180 kg * 100 g) were used. In waves 4 to 6, Tanita body fat scales were used. Weight was measured once to the nearest 50 g in bare feet and light clothing. Adult’s weight was self-reported at all waves.

5.4.3.1.3 Waist circumference

Waist circumference (cm) was only measured for children aged two years and older, but not for adults. Waist circumference was measured using a non-stretch dressmaker’s tape, positioning horizontally over the child’s navel. In all waves, two measurements were taken, and a third measurement was taken if the first two differed by 0.5 cm or more. The average of the two closest measures was used.

5.4.3.2 CheckPoint anthropometry

In CheckPoint, children and adults had the exactly same anthropometric assessments in the *Measure Up* station.

5.4.3.2.1 Height

The Invicta stadiometer (IP0955) was used to measure participant's height (cm). In light clothing without shoes or socks, participants stood straight with their heels, back and shoulders against the stadiometer and their head in the Frankfort plane. Two measurements were taken. If the two measurements differed by 0.5 cm or more, a third measurement was taken. The average height was calculated as the mean of all height measurements.

5.4.3.2.2 Weight

Bioelectrical impedance analysis (BIA) scales (InBody230 four-limb segmental body composition analyser) were used to measure weight (kg). Participants wore light clothing with no shoes or socks. Staff entered participant ID, age, sex and mean height into the BIA scales. Weight was then measured once (to the nearest 0.1 kg) with the participant standing on the scale footplates holding two horizontal handles.

5.4.3.2.3 Waist circumference

Waist circumference (cm) was measured on the skin (to the nearest 0.1 cm) at the narrowest point between the 10th rib and the top of the iliac crest using a steel anthropometric measuring tape (Lufkin Executive Diameter, W606PM). If no narrowing was present, the measurement was taken at the midpoint between these two landmarks. If two measurements differed by 1 cm or more, a third measurement was taken. The average waist circumference was calculated as the mean of all measurements.

5.4.4 Biomarkers

5.4.4.1 Venous blood

Venous blood samples were collected from both children and adults in CheckPoint's *Young Bloods* station at Main and most Mini Assessment Centres, but not at Home Visits due to logistics. Participants were asked to have an early light breakfast, and

they reported when they had last eaten or had a drink. The median fasting time was 4.2 hours before phlebotomy. At a minimum, they fasted for the period during which they were completing other CheckPoint assessments (2.5 hours for children and 75 minutes for adults).

An experienced phlebotomist collected approximately 28 mL blood from the brachial vein of the non-dominant arm in a semi-reclining position. Participants who requested the option of topical anaesthetic cream had it applied to left and right antecubital fossas for 45 minutes before blood collection. Blood was collected in four vacutainers using a butterfly needle, so only a single venepuncture was required. Tubes were filled in the following order: 1) 2.7mL EDTA tube, 2) 9mL EDTA tube, 3) 9mL serum tube, 4) 7.5mL Lithium Heparin tube. EDTA and Heparin tubes were immediately inverted six times to ensure mixing with the anticoagulant.

Blood samples were processed onsite within an hour into various blood fractions (whole blood, plasma, buffy coat and serum into 0.5mL aliquots, blood clot into a 1.0mL FluidX screwcap tube) and frozen at -80 °C (Thermo Fisher Scientific, Waltham, US). All tubes were transferred to an on-site laboratory within four hours for storage. At the completion of each Assessment Centre, a single batch of all frozen samples was shipped on dry ice to the Melbourne Children's Bioresource Centre at the Murdoch Children's Research Institute for long term storage at -80 °C (whole blood, plasma, serum and blood clot) or vapour phase liquid nitrogen (buffy clot). A temperature data logger was included in each shipment to confirm optimal temperature throughout. Samples were tracked using QR code scanners and FreezerPro Enterprise software (RuRo, Maryland, US).

5.4.4.2 Biomarker analysis

A single 0.5 ml serum aliquot for each participant was shipped in a single batch on dry ice to Nightingale Health (Helsinki, Finland) for metabolomics analysis. Proton nuclear magnetic resonance (NMR) spectroscopy metabolomics (Nightingale Ltd, Vantaa, Finland) was performed on all child and adult serum blood samples. The Nightingale® NMR metabolomics platform quantified 288 metabolic biomarkers from 0.35 mL of serum using the 2017 version quantification of Nightingale's

algorithm.²¹⁹ Metabolomics data were linked to the participant by an external staff member using a linkage key.

The experimentation, quality control, quantification algorithm, and applications of the NMR platform have been described elsewhere.²¹⁹ The overall procedure can be seen in Figure 5-8. Briefly, samples are prepared and analysed using a Nightingale self-developed robotics-controlled and fully automated high throughput system. A spectrometer non-destructively measured the resonant frequencies of the isotopes within the sample, which were transformed into frequency domain spectra using Fourier transformation. Nightingale proprietary software converted the spectral information into absolute concentrations of metabolites, using advanced calculation algorithms based on Bayesian modelling. This thesis used Nightingale derived GlycA, LDL, HDL cholesterol and triglyceride.

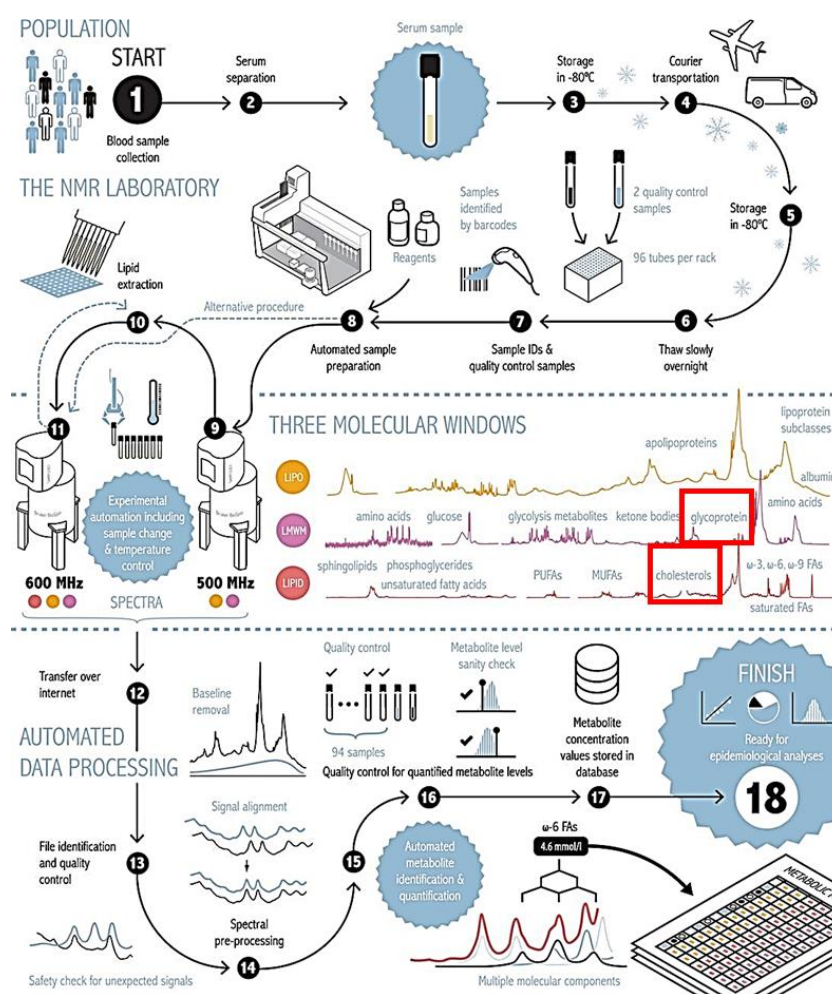


Figure 5-8 Process of high-throughput serum nuclear magnetic resonance metabolomics platform; figure adapted from ref.²²⁰

5.4.4.2.1 Glycoprotein acetyls

GlycA refers to an NMR signal, which reflects chronic cumulative inflammation. It reflects the number of N-acetyl groups bound to circulating glycoproteins.^{219, 220} Its signal arises from mobile carbohydrate side-chains of circulating acute-phase glycoproteins such as α 1-acid glycoprotein, α 1-antichymotrypsin, α 1-antitrypsin and haptoglobin.²²¹ GlycA has been shown to have greater stability and lower intra-individual variability than single acute-phase proteins.²²² In population-based studies, higher GlycA is a robust predictor of all-cause of mortality and CVD, even after adjusting for other inflammatory biomarkers.²²³⁻²²⁵ Thus, GlycA is regarded as a stable biomarker to track systemic inflammation and chronic inflammatory conditions.¹⁹⁵

GlycA was directly derived from proton NMR spectroscopy. Levels of GlycA were calculated using Nightingale 2017 quantification algorithms and were reported in mmol/L in this thesis.

5.4.4.2.2 Lipids

The metabolomics profiling also covers standard lipid measures, such as the lipid composition measures of lipoprotein subclass particles are included in the overall number of metabolic measures. Increasing evidence has shown that increased levels of triglycerides and LDL cholesterol and decreased levels of HDL cholesterol are important risk factors for microvascular disease and CVD.²²⁶ This thesis used proton NMR derived LDL, HDL cholesterol and triglyceride. Levels of these lipids were calculated using Nightingale 2017 quantification algorithms and were reported in mmol/L in this thesis.

5.4.5 Sociodemographic variables

Sociodemographic variables include age, sex, gestational age, birth weight, family socioeconomic position (SEP) and smoking, which data source is from LSAC.

5.4.5.1 Age and sex

Age was calculated to nearest week using the date of birth, either imported from Australia's Medicare database at the time of LSAC enrolment (child) or self-

reported (adult), and date of assessment. Sex was self-reported by adults at the time of LSAC enrolment and imported from Medicare for children at LSAC enrolment.

5.4.5.2 Gestational age and birth weight

The LSAC wave 1 interviewer copied gestational age (in weeks) from the Parent Health Record, which is completed by the birthing hospital for the majority of families. If the record was not available, interviewers asked the participated adults “After how many weeks of pregnancy was [child] born?”

Birth weight (kg) was also copied from the Parent Health Record. If the record was not available, interviewers asked adults “How much did child weigh at birth?”

5.4.5.3 Family socioeconomic position

LSAC constructed a composite measure for family SEP.²²⁷ The variable summarises parent-reported overall household income, current or most recent occupation of each parent and highest achieved educational qualification of each parent. From parent questionnaires, each component was calculated as follows: 1) the log of the sum of both parents (where available) income for combined household income, 2) numerical social ranking scores for each parental occupation that were developed for research purposes, and 3) highest educational qualification for each parent. Each component of the score was scaled (mean 0 and SD 1), and an unweighted average was calculated out of three items in a single-parent household (combined income, occupation and education of the single parent), or over five items in a dual-parent household (combined income, occupation and education of both parent). The unweighted average in each LSAC wave was then standardised within the wave to have a mean 0, and SD of 1.

In this thesis, LSAC wave 6 family SEP was used, approximately 12 months prior to the biophysical measurements in CheckPoint. As introduced above, this measure has the advantage of summarising several aspects of childhood socioeconomic position.

5.4.5.4 Smoking

In adults, the smoking metric was determined by their answer to the question “Are you currently smoking?” in the LSAC wave 6 questionnaire. In children, the lifetime passive smoke exposure was determined by the adult-reported questionnaire at each

LSAC wave. The score was positive if the adult reported a smoker(s) living in the home in any LSAC wave.

5.5 Derived variables

We derived several variables for the analysis, including carotid arterial elasticity and anthropometric variables (e.g., BMI, WHtR and growth trajectories).

5.5.1 Carotid arterial elasticity

The carotid arterial elasticity was derived from blood pressure measured by SphygmoCor XCEL device and carotid lumen diameter (LD) measured by the Carotid Analyzer (detailed above in Section 5.4.2). LD was calculated as the average media-media distance of each frame of the image loop (Figure 5-9). Each frame had an LD value, and the minimum and maximum LD were calculated as the single lowest value or highest value for LD throughout the image loop. Carotid arterial elasticity was calculated using LD and the different value of systolic and diastolic blood pressure (ΔP), according to previously published work from the Cardiovascular Risk in Young Finns Study:³²

$$\frac{\left(\frac{LD_{max} - LD_{min}}{LD_{min}}\right)}{\Delta P} \times 100\%$$

It measures the ability of the arteries to expand as a response to pulse pressure caused by cardiac contraction and relaxation.³¹ Lower arterial elasticity score is considered to represent lower arterial compliance.

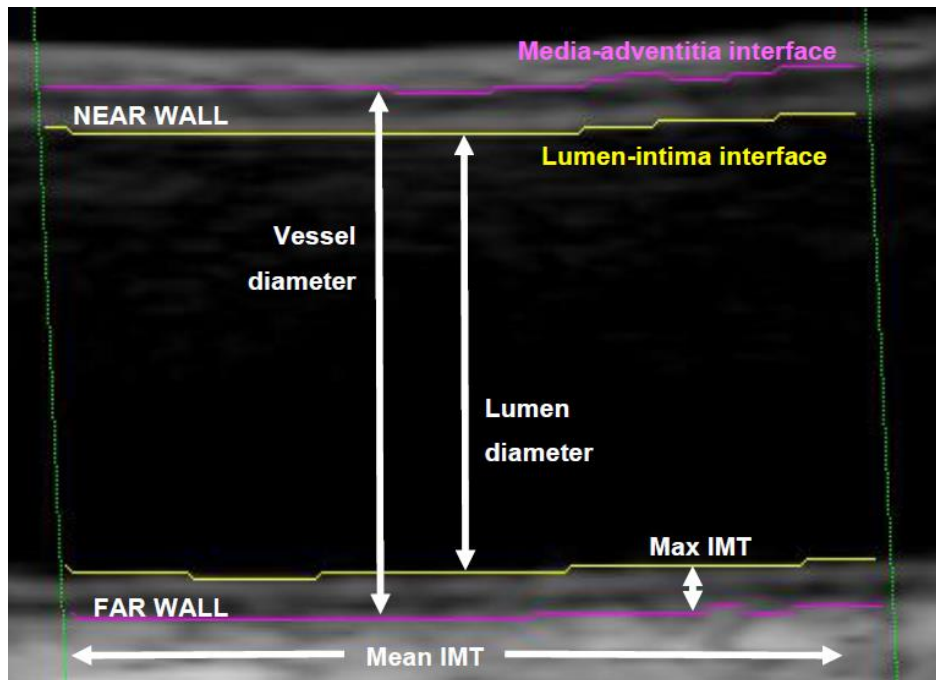


Figure 5-9 Labelled image of the carotid intima-media measurement

5.5.2 Body mass index

BMI (kg/m^2) was calculated as weight (kg) divided by height (m) squared. For children, biennial repeated BMI from LSAC and concurrent BMI from CheckPoint were transformed to age- and gender-adjusted BMI z-scores using the US Centres for Disease Control (CDC) growth reference charts.²²⁸ Concurrent BMI z-scores were further converted into their equivalent BMI percentiles. Then children were classified as underweight ($<5^{\text{th}}$ percentile), normal (5^{th} to $<85^{\text{th}}$ percentile), overweight (85^{th} to $<95^{\text{th}}$ percentile) and obese ($\geq 95^{\text{th}}$ percentile) according to the CDC recommendations.²²⁹ For adults, normal BMI was defined as BMI between 18.5 and 24.9 kg/m^2 , underweight as $<18.5 \text{ kg/m}^2$, overweight as between 25 and 29.9 kg/m^2 , obese as $\geq 30.0 \text{ kg/m}^2$, as suggested by the World Health Organization (WHO).²³⁰

5.5.3 Waist-to-height ratio

Child waist circumference was measured in LSAC from waves 2 to 6 and in CheckPoint, while adult waist circumference was only measured in CheckPoint. For children and adults, the WHtR was calculated as waist (cm)/height (cm) and

classified into two categories (<0.5 and ≥ 0.5), where a ratio ≥ 0.5 indicates central obesity.^{231, 232}

5.5.4 Growth trajectories

Evidence suggests that distinct growth trajectories in childhood are associated with different cardiovascular risk profiles later in life.^{169, 170} With the rich longitudinal data, we were able to generate decade-long BMI z-score and WHtR trajectories for children, and decade-long BMI trajectories for adults.

5.5.4.1 Body mass index trajectories

We identified BMI trajectories in children based on measures from LSAC waves 2 to 6 and in adults based on self-reported data gathered in LSAC waves 1 to 6. The ‘traj’ plug-in from Stata 15.0 was used for the group-based trajectory modelling.²³¹ Methods of how we generated the trajectories have been published by our research team.²³⁴ Only participants with BMI scores for at least four waves (children $n=3,900$, adults $n=3,702$) were included in the trajectories (Table 5-3).

Table 5-3 Number of participants with body mass index from LSAC waves 1 to 6

BMI score	Children	Adults
6 waves	-	2178
5 waves	3101	965
4 waves	799	559
3 waves	377	483
2 waves	217	284
1 wave	282	333
0 wave	331	305
Total	5107	5107

Note: Children’s data came from LSAC waves 2-6, so the maximum BMI points for children is five.

For trajectory modelling, BMI scores were modelled with censored normal distribution, which is designed for the analysis of repeatedly-measured continuous variables. To extract the most meaningful and distinct trajectories, I considered Bayesian information criterion (BIC) values, average posterior probabilities, the proportion of the sample in each trajectory and visual graphs of trajectories.²³⁵ Whether each trajectory was characterised by a linear, quadratic or cubic function

was also examined. To determine the best function, the significance of these three parameters (linear, quadratic, and cubic) was checked, and the nonsignificant ones were dropped. That is, linear, quadratic, and cubic parameters were included for each trajectory. The cubic parameter was dropped if it was nonsignificant, and a quadratic trajectory was then tested. It was dropped if it was nonsignificant and a linear trajectory was tested. The linear parameter was retained even if this was nonsignificant. After each iteration, I compared the results using the BIC criteria (see Table 5-4).²³⁶ The BIC is a method analogous to the adjusted R^2 , which balances model complexity and model fit. Higher numbers indicate a better model fitness.

Based on these criteria, trajectories were selected and named from visual inspection. A five-trajectory solution was selected for child BMI z-score, with 6.2% in the ‘low’, 31.3% ‘average’, 45.6% ‘always high’, 12.1% ‘always very high’ and 4.8% ‘low to high’ trajectories (Figure 5-10[a]). For adults, we selected a four-trajectory solution (51.0% ‘normal’, 32.8% ‘overweight’, 12.8% ‘obese’, and 3.4% ‘severely obese’; Figure 5-11[b]). Both child and adult BMI trajectories were relatively flat, with the exception of one child trajectory (‘low to high’), which was characterised by a steeply rising BMI z-score over time, while the ‘average’ and ‘high’ trajectories appeared to fall slightly.

Table 5-4 Criteria for selecting the number of trajectories

Number of latent classes	Trajectory shapes ^a	BIC ^b
Children body mass index z-scores (n= 3,900)		
2	3 3	-23776.79
2	2 3	-23772.79
2	1 3*	-23768.81
3	3 3 3	-22659.65
3	2 3 3	-22655.84
3	1 3 3*	-22652.35
4	3 3 3 3	-22093.58
4	3 2 3 3	-22091.11
4	3 1 3 3*	-22086.98
5	3 3 3 3 3	-21866.96
5	3 2 3 3 3	-21865.60
5	3 1 3 3 3	-21864.04
5	3 2 2 3 3	-21888.51
5	3 1 2 3 3	-21874.04
5	3 1 1 3 3*	-21871.10
6	3 3 3 3 3 3 ^c	-21612.19
Adults body mass index (n= 3,702)		
2	3 3	-57203.57
2	1 3*	-57201.05
3	3 3 3	-54114.56
3	2 3 2	-54110.39
3	1 3 1 *	-54106.58
4	3 3 3 3	-52716.47
4	2 3 3 2	-52708.95
4	1 3 3 1*	-52706.48
5	3 3 3 3 3 ^c	-52057.35
Children waist-to-height ratio (n= 3,893)		
2	3 3 *	34308.81
3	3 3 3 *	35157.48
4	3 3 3 3 ^c	35538.15

^a Trajectory shapes: 0= intercept; 1=linear; 2=quadratic; 3=cubic.

^b BIC, Bayesian information criterion; the model with the highest (least negative) value of BIC is preferred.

^c One or more of the groups had a small proportion of the observations.

* All parameters are significant (p<0.05).

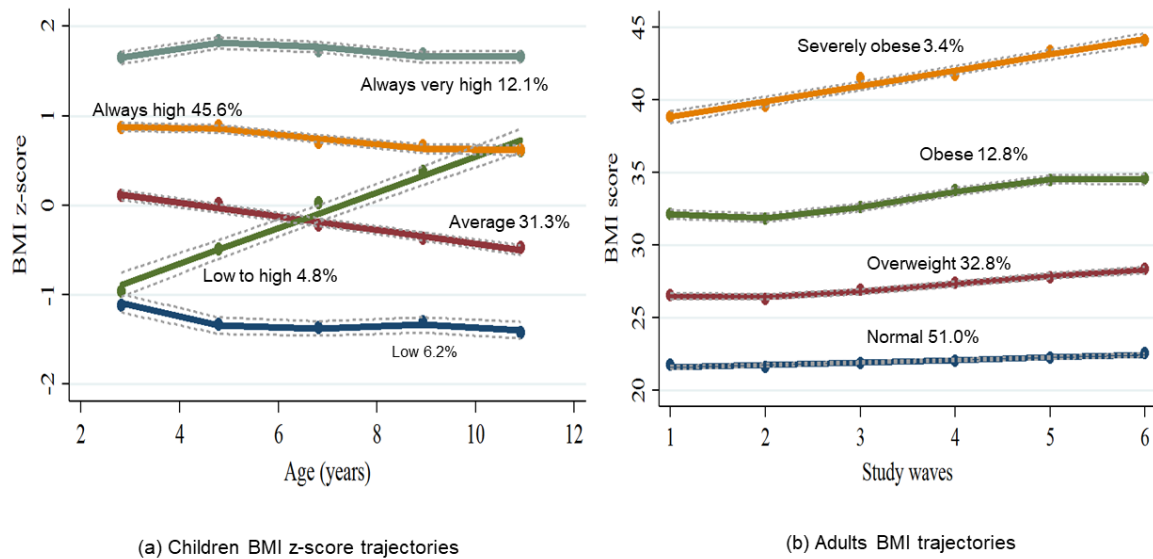


Figure 5-10 Child and adult body mass index trajectories

Finally, I calculated the posterior probabilities of individuals post hoc to estimate the probability that each case having been assigned to the trajectory that best matches his or her profile of BMI change. I then calculated the average posterior probabilities for each trajectory by averaging the posterior probabilities of individuals. The average posterior probability value for each trajectory was 0.80 or more for each group (Table 5-5), well above the recommended value of 0.70,²³⁵ indicating the model had good assignment accuracy.

Table 5-5 Average posterior probability value for trajectory groups

BMI trajectory group	Average posterior probability
Children	
Low (n=257)	0.91
Average (n=1141)	0.87
Always high (n=1666)	0.89
Always very high (n=627)	0.91
Low to high (n=245)	0.82
Adults	
Normal (n=1871)	0.97
Overweight (n=1202)	0.93
Obese (n=480)	0.97
Severely obese (n=149)	0.97
WHtR trajectory group (child only)	
Normal (n=2603)	0.94
High normal (n=1055)	0.88
Always high (n=235)	0.94

5.5.4.2 Waist-to-height ratio trajectories

The same procedure was used to generate WHtR trajectories for children. Briefly, WHtR scores were modelled with censored normal distribution, and we only included children with WHtR of at least four of the five waves for trajectory modelling (n= 3,893, Table 5-6). Similarly, Bayesian information criterion values (see Table 5-4 above), average posterior probabilities (Table 5-5 above) and the proportion of the sample in each trajectory were taken into account to extract the most meaningful and distinct trajectories.

Table 5-6 Number of children with waist-to-height ratio from LSAC waves 2 to 6

Waist-to-height ratio	Children
5 waves	3072
4 waves	821
3 waves	380
2 waves	219
1 wave	282
0 wave	333
Total	5107

For children's WHtR trajectories, a three-trajectory solution was selected and in line with the clinical cut-point of 0.5 named as 'normal' (72.3%), 'high normal' (23.9%) and 'always very high' (3.8%); see Figure 5-11.

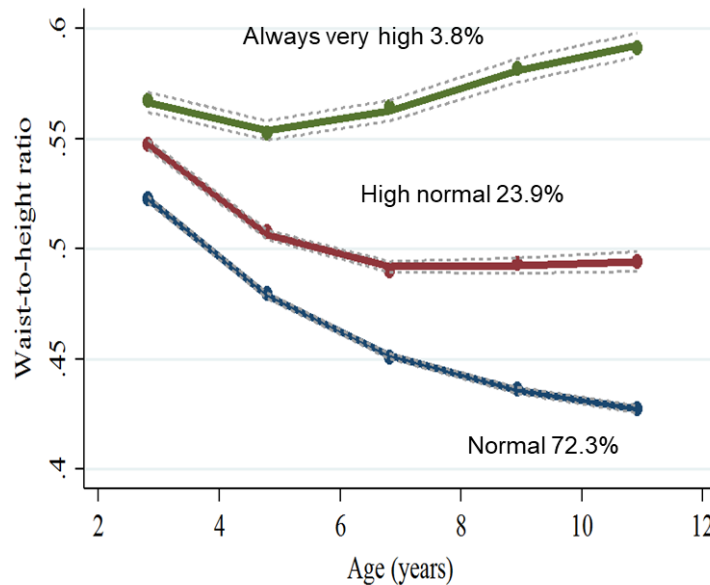


Figure 5-11 Child waist-to-height ratio trajectories

5.6 Cohort profile and statistical analysis overview

This section provides information on the sample characteristics of participants and analysis plan of the thesis. Statistical analyses were performed in Stata 15.0 software (StataCorp LP, TX, US) and R software 3.5.2 (R Foundation, Vienna, Austria). Some figures were annotated in Microsoft PowerPoint (Microsoft Corporation, WA, US).

5.6.1 Sample characteristics

5.6.1.1 Characteristics of CheckPoint responders and non-responders

Table 5-7 describes the baseline characteristics (collected at LSAC wave 1) of the CheckPoint sample, and the families who participated in LSAC wave 6 but not CheckPoint. We see that the age and sex of CheckPoint responders and non-responders were similar. CheckPoint attending adults were slightly older, and more likely to live in less disadvantaged areas than those who did not participate in CheckPoint.

Table 5-7 Baseline sample characteristics of CheckPoint responders and non-responders

Characteristic (% , unless indicated)	Baseline characteristics (2004) ¹	
	In CheckPoint n= 1,874 families	Not in CheckPoint n= 3,233 families
Child age in years, mean (SD)	0.8 (0.2)	0.8 (0.2)
Adult age in years, mean (SD) ²	32.1 (4.9)	30.4 (5.7)
Female child	49.0	48.9
Female adult	98.7	98.5
Child is the biological child of the adult	99.7	99.7
Australian state or territory of residence		
Aust. Capital Territory	2.9	1.6
Northern Territory	2.9	1.6
New South Wales	29.9	32.6
Queensland	20.0	20.1
South Australia	7.5	6.4
Tasmania	3.2	1.6
Victoria	22.2	25.8
Western Australia	11.8	9.7
Neighbourhood disadvantage ³ , mean (SD) and % in national quintiles	1018.6 (61.2)	1003.1 (58.9)
1 (least disadvantaged quintile)	29.0	18.9
2	20.3	19.8
3	19.3	21.6
4	19.8	21.7
5 (most disadvantaged quintile)	11.6	18.1

¹ Data collected in 2004 during LSAC wave 1; ² Adult data relates to the adult who participated in the CheckPoint visit; ³ Australia Bureau of Statistics 2011 SEIFA Index of Relative Socioeconomic Disadvantage.²³⁵

5.6.1.2 Characteristics of Checkpoint responders with retinal data

A limited number of images (19 children and 53 adults) did not meet the quality criteria for use in analyses. In total, 1,288 children and 1,264 adults had retinal microvascular parameters available in the dataset. Table 5-8 shows the sample characteristics of participants with and without valid retinal data in CheckPoint. Overall, participants with retinal data available were similar to those without data, except for some characteristics. For example, participants with retinal data are more likely from less advantaged families with less exposure to smoking, and children are less likely to be female (45% versus 51%). The factors examined in each aim of the thesis are similar between participants with or without retinal data.

Table 5-8 CheckPoint sample characteristics with and without retinal data

Characteristics ¹	Children				Adults			
	With retinal data		Without retinal data		With retinal data		Without retinal data	
	N	Mean (SD ²) or %	N	Mean (SD ²) or %	N	Mean (SD ²) or %	N	Mean (SD ²) or %
<i>Socio-demographics</i>								
Age (years)	1288	11.4 (0.5)	586	11.7 (0.5)	1264	43.8 (5.1)	610	43.6 (5.6)
Gender (% female)	1288	50.9	586	45.1	1264	87.6	610	90.0
Birth weight (kg)	1285	3.4 (0.6)	585	3.4 (0.6)				
Gestational length (weeks)	1279	39.2 (2.0)	586	38.9 (4.0)				
Socioeconomic position	1283	0.26 (0.97)	584	-0.02 (1.01)	1260	0.26 (0.97)	607	-0.01 (1.01)
<i>Traditional CVD risk factors</i>								
Smoking exposure	1287	20.7	586	27.8	1263	8.4	610	11.2
Body mass index z-score ³	1288	0.30 (0.96)	584	0.31 (1.04)				
Body mass index (kg/m ²)					1259	27.9 (6.2)	602	27.7 (6.1)
Systolic blood pressure (mmHg)	1213	108.2 (7.8)	564	107.8 (8.3)	1169	120.1 (12.8)	580	117.9 (12.0)
Diastolic blood pressure (mmHg)	1213	63.0 (5.5)	234	61.1 (6.1)	1169	73.5 (8.7)	580	71.8 (8.1)
Mean arterial blood pressure (mmHg)	1213	78.1 (5.7)	564	76.7 (6.0)	1169	89.1 (9.7)	580	87.2 (8.9)
<i>Preclinical large arterial phenotypes</i>								
Pulse wave velocity (m/s)	1242	4.4 (0.5)	564	4.5 (0.6)	1134	7.0 (1.1)	541	6.7 (1.1)
Arterial elasticity (%/10mmHg)	1151	4.8 (0.8)	193	4.7 (1.0)	1053	2.4 (0.1)	199	2.5 (0.7)
Carotid intima-media thickness (µm)	1273	580.0 (46.4)	212	589.0 (47.6)	1236	663.6 (97.0)	232	667.0 (99.4)
<i>Biomarkers (mmol/L)</i>								
Glycoprotein acetyls	1060	1.0 (0.1)	120	1.0 (0.1)	1158	1.0 (0.2)	167	1.0 (0.2)
Low-density lipoprotein cholesterol	1059	1.4 (0.3)	120	1.3 (0.3)	1157	1.7 (0.4)	167	1.6 (0.4)
High-density lipoprotein cholesterol	1059	1.4 (0.3)	120	1.4 (0.3)	1157	1.5 (0.4)	167	1.5 (0.3)
Triglyceride	1059	1.2 (0.5)	120	1.2 (0.6)	1157	1.5 (0.8)	167	0.5 (0.9)

¹. Most characteristics measured at the Child Health CheckPoint; birth weight, gestational age were collected at LSAC wave 1 in 2004, SEP was measured at LSAC Wave 6 in 2015, and smoking exposure was generated combining the questionnaire from LSAC waves 1 to 6; ². Standard deviation; ³. Body mass index was transformed to z-score with widely used Centres for Disease Control and Prevention (US)-growth charts.

5.6.2 Analytical sample

The analytic sample for each aim was defined as having relevant retinal microvascular parameters available (e.g., retinal vascular calibre or geometric parameters) and the other measures of interest (e.g., large arterial phenotypes, anthropometric derived variables or the inflammatory marker GlycA). The participant flow chart for each aim is presented in Chapters 6-9, respectively.

- Aim 1: 1,232 children and 1,125 adults had both retinal vascular calibre and PWV data for analysis. 1,262 children and 1,226 adults had both retinal vascular calibre and CIMT data for analysis. Fewer samples have both retinal vascular calibre and arterial elasticity data: 1,140 children and 1,047 adults comprised the analytic sample.
- Aim 2: 1,258 children and 1,229 adults had both anthropometric data across all LSAC waves and retinal vascular calibre data at CheckPoint, comprising the analytic sample across the six-time point. 1,255 children and 1,189 adults had both BMI/WHtR trajectories and retinal vascular calibre data comprising the longitudinal analytic sample.
- Aim 3: 1,054 children and 1,147 adults had BMI and/or WHtR, GlycA and retinal vascular calibre comprising the analytic sample.
- Aim 4: 1,126 children had data on all measures (traditional CVD risk factors, preclinical large arterial phenotypes and retinal geometric parameters) for analysis.

5.6.3 Normality

Normality of data occurs when the distribution of data is bell-shaped. I visually inspected continuous variables through histograms generated by Stata and followed on with formal tests to evaluate the skewness of each variable (see Table 5-9). In children, the skewness of most variables was positive, while the exception was retinal arteriolar calibre, arteriolar fractal dimension, CIMT and BMI. In adults, all variables' skewness was positive, except retinal arteriolar calibre, which was negative. Given that the skewness of most measures was small ($\text{skewness} < 2$), they were described by means and SDs and analysed by parametric statistics (e.g., linear regression). This is supported by the application of the central limit theorem, which

states that the average of a large number of independent random variables is approximately normally distributed around the true population mean.²³⁸

Table 5-9 Summary of the skewness of key variables

Variables	Skewness	
	Children	Adults
<i>Retinal microvascular parameters</i>		
Retinal arteriolar calibre (IVAN)	-0.14	-0.19
Retinal venular calibre (IVAN)	0.05	0.15
Arteriolar fractal dimension (SIVA)	-0.26	--
Venular fractal dimension (SIVA)	0.02	--
Simple arteriolar tortuosity (SIVA)	1.47	--
Simple venular tortuosity (SIVA)	1.29	--
<i>Traditional CVD risk factors</i>		
Body mass index	-0.21	1.16
Waist-to-height ratio	1.35	0.76
Systolic blood pressure	0.53	0.79
Diastolic blood pressure	0.25	0.65
<i>Preclinical large arterial phenotypes</i>		
Pulse wave velocity	0.90	0.68
Carotid arterial elasticity	0.32	0.71
Carotid intima-media thickness	-0.32	1.43
<i>Biomarkers</i>		
Glycoprotein acetyls	1.46	1.32
Low-density lipoprotein cholesterol	0.56	0.43
High-density lipoprotein cholesterol	0.04	0.28
Triglyceride	0.21	0.49

5.6.4 Analysis plan

In this thesis, I conducted all analyses separately for children and adults. Linear regression analyses were the main method used in this thesis for assessing the direction and strength of associations between variables. Exposure and outcome variables were selected to address the research question appropriately. Confounders were selected *a priori* according to hypothesised causal relationships constructed

with directed acyclic graphs (DAG). In addition, I also conducted a causal mediation analysis to assess whether inflammation mediates the association between obesity and retinal vascular calibre. The analyses for each aim are described in more detail in the published manuscripts that comprise results chapters 6 to 9.

Chapter 6. Associations of retinal vascular calibre with large arterial phenotypes (Aim 1)

6.1 Overview

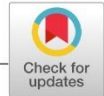
Chapter 6 addresses Aim 1, which examined the associations of retinal vascular calibre with preclinical large arterial phenotypes (measured by PWV, carotid arterial elasticity and CIMT) in children aged 11–12 years and mid-life adults. By conducting multivariable regressions adjusting for age, sex and SEP, narrower retinal arterioles and wider venules were shown to be associated with large arterial function as early as 11–12 years of age. Associations are larger in mid-life adults and also extend to arterial structure, although effect sizes remain small. The results are briefly discussed and are further elaborated upon in Chapter 10.

This paper is published in *Microcirculation*. The published paper and supplementary materials form this chapter.

- **Liu M**, Lycett K, Wong TY, Grobler A, Juonala M, He M, Dwyer T, Burgner D and Wake M. Associations of retinal microvascular calibre with large arterial function and structure: a population-based study of 11–12 year-olds and mid-life adults. *Microcirculation*. 2020:e12642.

6.2 Manuscript under review (Aim 1 results)

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ORIGINAL ARTICLE

Microcirculation WILEY

Associations of retinal microvascular caliber with large arterial function and structure: A population-based study of 11 to 12 year-olds and midlife adults

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Abstract

Objective: We examined associations between retinal microvascular and large arterial phenotypes to explore relationships between the micro- and macro-vasculature in childhood and midlife.

Methods: Participants were 1288 children (11-12 years, 50.9% female) and 1264 adults (mean age 44 years, 87.6% female) in a cross-sectional population-based study. Exposures were retinal arteriolar and venular caliber quantified from retinal images. Outcomes included arterial function (pulse wave velocity; carotid arterial elasticity) and structure (carotid intima-media thickness). Multivariable regression models were performed adjusting for age, sex, and family socioeconomic position.

Results: In children, one standard deviation wider arteriolar caliber was associated with slower pulse wave velocity (-0.15 SD, 95% CI -0.21 , -0.09) and higher elasticity (0.13 SD, 95% CI 0.06 , 0.20); per SD wider venular caliber was associated with faster pulse wave velocity (0.09 SD, 95% CI 0.03 , 0.15) and lower elasticity (-0.07 SD, 95% CI -0.13 , -0.01). The size of adult associations was approximately double. Wider arteriolar caliber was associated with smaller carotid intima-media thickness (-0.09 SD,

Abbreviations: BMI, Body mass index; CI, confidence interval; CIMT, carotid intima-media thickness; CVD, cardiovascular disease; LD, lumen diameter; LDL, low-density lipoprotein; LSAC, Longitudinal Study of Australian Children; PWV, pulse wave velocity; R^2 , model R square; SD, standard deviation; SEP, socioeconomic position.

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95% CI $-0.16, -0.03$) in adults but not children. Venular caliber and carotid intima-media thickness showed little evidence of association.

Conclusions: Narrower retinal arterioles and wider venules are associated with large arterial function as early as mid-childhood. Associations strengthen by midlife and also extend to arterial structure, although effect sizes remain small.

KEYWORDS

arterial elasticity, carotid intima-media thickness, population-based study, pulse wave velocity, retinal arteriolar caliber, retinal venular caliber

1 | INTRODUCTION

Identifying individuals at increased risk for CVD early in life could help target interventions to reduce the global disease burden.^{1,2} Decades of epidemiological and clinical evidence show that large artery measures consistently predict atherosclerosis throughout the lifespan, and future CVD outcomes in adults.^{3,4} Commonly used indicators of poorer large artery cardiovascular phenotypes include faster PWV, reduced arterial elasticity, and increased CIMT.⁵⁻⁷

While generally receiving less public health attention, the microcirculation is also associated with CVD.^{8,9} The retina is a unique site from which the microcirculation can be imaged directly and non-invasively, with repeated measurement possible over time.¹⁰ Structural vascular changes in the retina—in particular, arteriolar narrowing and venular dilation—have been associated with systemic CVD, including coronary heart disease and heart failure.¹¹⁻¹³

The retinal blood vessels and large arteries are inherently interconnected elements of the vascular tree and are influenced by the same intrinsic and environmental features with systemic circulation.^{14,15} However, the extent to which microvascular and macrovascular phenotypes co-develop, and if so from what age and from what state of health, are unclear. We previously conducted a systematic review of 26 studies, including a meta-analysis of five of these studies, to examine the association between retinal vascular caliber and intermediate phenotypes of large arteries (ie, CIMT, PWV).¹⁶ Most studies involved adults in later life, with only one study in children.¹⁷ Retinal arteriolar caliber showed small associations with large arterial function (pooled estimates $r = -.17$) and arterial structure ($r = -.05$), with stronger evidence in cardiometabolic patients than in the general population. Thus, large arteries may share some pathophysiological processes with the microvasculature, at least by later life and in those already expressing disease endpoints.

However, there is a paucity of data from children and younger adults, and it remains unclear when in the life course interrelationships between the small and large vascular phenotypes emerge and how they change with age. If they already co-vary prior to emergent disease, then this would suggest that the relationship is not solely the result of dysregulation, potentially advancing understanding of CVD pathogenesis. With technology and artificial intelligence putting retinal imaging into clinical and population research, microvascular

status could potentially enter precision prediction models for future CVD and other diseases.¹⁸ Before this could take place, it is important to understand the small vessels' unique and shared contributions with more traditional large artery measures. Besides, most previous studies have only assessed one aspect of large arterial health (ie, functional or structural intermediate phenotypes), but not both in the same individuals.¹⁶ Assuming that large arterial dysfunction precedes structural changes, retinal microvascular associations with large artery function might emerge earlier than with large artery structure.¹⁹

The national, population-based Longitudinal Study of Australian Children (LSAC) provided an opportunity to address these evidence gaps in two generations taking part in a large cross-sectional wave explicitly focusing on phenotypic health measurement. We aimed to examine associations of retinal microvascular caliber with large arterial intermediate phenotypes (measured by PWV, carotid arterial elasticity and CIMT) in Australian children aged 11-12 years and midlife adults (the children's parents).

2 | MATERIALS AND METHODS

2.1 | Study design and participants

The Child Health CheckPoint (CheckPoint) is a cross-sectional population-derived wave nested within the national LSAC study.²⁰ Details of the initial study design and recruitment are outlined elsewhere.^{21,22} Briefly, LSAC recruited a nationally representative birth cohort (B cohort) of 5107 infants using a 2-stage clustered design and has followed them in biennial "waves" of data collection since 2004. Most families retained at wave 6 in 2014 (73.7%, $n = 3764$).

At the wave 6 visit, all participating families ($n = 3513$) were invited to consent to their contact details being shared with the CheckPoint team. CheckPoint took place between February 2015 and March 2016, nested between LSAC waves 6 and 7 at child age 11-12 years. In total, 1874 children and their parents participated in this detailed cross-sectional biophysical assessment.²³ Most attended the CheckPoint Main Assessment Centre in seven large cities around Australia, where all measures were performed. A small minority of families who attended more limited assessments in regional

cities or home visits are not included in the analyses as the retinal photograph was not collected at these visits, and home visits did not include CIMT and arterial elasticity because the large and delicate equipment could not be readily transported.

The CheckPoint study was approved by the Royal Children's Hospital Melbourne Human Research Ethics Committee (33225D) and the Australian Institute of Family Studies Ethics Committee (14-26). The attending parents/caregivers provided written informed consent for themselves and their children to participate in the study.

2.2 | Retinal vascular caliber assessment

Two optic disk-centered digital photographs from each eye were taken by a fundus camera (EOS 60D SLR) in a darkened room. Details of scoring procedures are described elsewhere.²⁴ Briefly, right eye images were selected as the first choice for scoring. Each image was graded by one of four experienced graders (two from the Zhongshan Ophthalmic Centre, China and two from the Centre for Eye Research Australia, Australia) masked to participant age and sex using the software program Integrative Vessel Analysis (IVAN, University of Wisconsin, Madison, USA). Retinal vessels were identified as arterioles or venules from a specific area (one-half to one-disk diameter from the margin of the optic disk). Each grader selected a segment of each vessel within this area for measurement. Diameters of all the selected segments were measured automatically by the IVAN software. Summary estimates of the average retinal vascular caliber were calculated combining measurements of the six largest arterioles or venules according to the revised Knudtson-Parr formula.²⁵ Reproducibility of measurement by IVAN was high, with inter- and intra-grader intraclass correlation coefficients ranging of 0.76–0.99.²⁴

2.3 | Large arterial intermediate phenotypes

Details of each measure are described elsewhere.^{26,27} Carotid-femoral PWV measures arterial function and was assessed using the Sphygmocor XCEL (AtCor Medical Pty Ltd.) with participants in the supine position. Briefly, the arterial waveforms of the right carotid and femoral artery were measured by blood pressure cuffs on the arm and leg. Data were collected one to three times for each participant, and the mean was calculated. PWV was calculated as the ratio of the distance travelled by the pulse wave and the time delay between the waveforms, as expressed in meters per second (m/s). The agreement of PWV waveforms between two analysts was high (0.99).²⁶ A quicker pulse wave indicates higher levels of arterial stiffness.

CIMT measures large arterial structure, an indicator of subclinical atherosclerosis.²⁸ It was assessed using standardized protocols via B-mode ultrasound (GE Healthcare Vivid-I BT06 with 10 MHz linear array probe).^{29,30} To image the right carotid artery, the participants lay in the supine position with the neck slightly extended

and the head turned 45° toward the left. Measurements were made at the distal 10 mm of the right common carotid artery, and images were taken across five sites within a minimum of three longitudinal loops of five to ten cardiac cycles. CIMT is defined as the distance from the lumen-intima interface to the media-adventitia interface. Images were analyzed using semi-automated edge-detection software (Carotid Analyser, Medical Imaging Applications) to calculate CIMT far wall mean, where higher scores represent a thicker carotid artery wall. The inter- and intra-grader intraclass correlation coefficients of CIMT analyses were moderate, ranging of 0.59–0.71.²⁶

Carotid arterial elasticity (%/mm Hg) measures the ability of the arteries to expand as a response to pulse pressure caused by cardiac contraction and relaxation.³¹ We calculated elasticity from carotid artery images using intima-intima LD and the different value of systolic blood pressure and diastolic blood pressure (ΔP), according to previously published work from the Cardiovascular Risk in Young Finns Study³²:

$$\frac{\left(\frac{LD_{max} - LD_{min}}{LD_{min}}\right)}{\Delta P} \times 100\%$$

Lower arterial elasticity is considered to represent lower arterial compliance.

2.4 | Assessment of covariates

We considered several covariates when examining associations between retinal microvascular caliber and large arterial phenotypes. The technical details of these measures are summarized in Table 1. For example, age, sex, and family SEP were considered as covariates, given that both large arterial and microvascular phenotypes have been shown to vary by age (worse with age), have distinct sex differences, and to be worse in those from poorer socioeconomic groups.^{12,26} BMI (z-score for children),³³ LDL cholesterol, smoking exposure (passive smoking for children and ever smoked for adults), and early life risk factors for children (birthweight and gestational age) were also included as covariates. These are commonly reported risk factors for CVD that have been reported to influence both large arterial and microvascular variations.^{12,34–36} Additionally, we considered mean arterial blood pressure as a covariate as it might lie on the causal pathway linking microvascular variations to large arterial changes.¹⁵

2.5 | Statistical analyses

Linear regression assumptions were tested when fitting models to assess associations of retinal vascular caliber with large arterial phenotypes in children and adults. We considered retinal arteriolar or venular caliber as the independent variable and large arterial intermediate phenotypes as the dependent variable,^{37,38} although the reverse would be equally plausible since they were measured cross-sectionally. It has

TABLE 1 Measures of covariates

Measure	Equipment	Brief protocol
Model 1		
Age	Parental questionnaire	Age was calculated to nearest week using date of birth, either imported from Medicare Australia's database at the time of LSAC enrollment (child) or self-reported (parent), and date of assessment
Sex	Parental questionnaire	Sex was self-reported in the CheckPoint questionnaire by children and parents, respectively
Family SEP	Parental questionnaire	SEP was measured in LSAC wave 6 (child age 10-11), approximately 12 mo prior to measurement of biophysical measures in CheckPoint. This SEP variable summarized parent-reported combined household income, current or most recent occupation of each parent and highest achieved educational qualification of each parent. Each component of the score was scaled, and an unweighted average was calculated over three values in a single parent household, or over five values in a dual parent household. The unweighted average variable at LSAC was then standardized within the wave to have a mean 0, and SD of 1
Model 2		
BMI	Height: Invicta Stadiometer Weight: 4-limb segmental body composition scales	Height (cm): Participants were measured in bare feet. Mean of two measurements used. If two measures differ by >0.5 cm, a third was taken and mean of all measurements was used Weight (kg): Bare feet and in light clothing, participants' weight were measured to the nearest 50 g BMI was calculated as kg/m ² . For children, an age- and sex-adjusted BMI z-score was calculated using the US Centres for Disease Control and Prevention growth reference charts ³³
LDL cholesterol	Proton nuclear magnetic resonance (NMR)	Venous blood samples of 30 mL were collected from each participant at the main and mini assessment centers. Participants were at a minimum semi-fasted (ie, no food/drink for at least 2 h). A high-throughput NMR metabolomics platform was used to quantify the lipid and metabolite measures from stored EDTA plasma samples. The platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids
Smoking exposure	Parental questionnaire	At each LSAC wave, parents reported the number of smokers at home. We created a binary variable of "ever exposed to passive smoke" from this data; it was positive for the child if the answer to the smokers at home question in any LSAC wave (age 0-11) was greater than zero
Birth weight	Parental questionnaire	At LSAC Wave 1, the parent was asked "How much did the child weigh at birth?"
Gestational age	Parent Health Record Parental questionnaire	At LSAC Wave 1, the interviewer copied gestational age from the Parent Health Record recorded prospectively by the birthing hospital (the great majority) If the record was not available, interviewers asked parents the question "After how many weeks of pregnancy was [child's name] born?"
Sensitivity analysis		
Mean arterial blood pressure	SphygmoCor XCEL	Following 7 min in the supine position at rest, systolic and diastolic blood pressures were measured at the brachial artery up to three times, with mean values reported. Mean arterial blood pressure was calculated as 1/3 systolic and 2/3 diastolic blood pressure

been proposed that modeling arteriolar and venular caliber simultaneously may give less biased results.³⁹ We thus performed all analyses with both arteriolar and venular caliber in the same model.

We fitted two main models and ran one sensitivity analysis. Model 1 estimates were adjusted for age, sex, SEP, and jointly for

arteriolar or venular caliber. Model 2 estimates for children additionally adjusted for BMI z-score, LDL cholesterol, passive smoking, birthweight and gestational age; for adults, we further adjusted estimates for BMI, LDL cholesterol, and ever smoking. This fully adjusted model could inform whether the association is independent

of these common potential risk factors. We also conducted a sensitivity analysis further adjusting estimates for mean arterial blood pressure to examine whether it could lie on the causal pathway. For all analyses, we internally standardized exposures and outcomes (ie, mean of zero and a SD of 1) to present standardized regression coefficients. All analyses were conducted in Stata 14.2 (StataCorp LP, TX, USA).

3 | RESULTS

3.1 | Sample characteristics

Figure S1 presents the study flow from wave 1 of LSAC onwards. Of the 1874 parent-child dyads that took part in CheckPoint, 1288 children and 1264 adults had retinal caliber data available. The CheckPoint sample mean family SEP (0.26) was above the mean SEP of all families enrolled in LSAC at wave 1 (mean 0, SD 1), indicating more socioeconomic advantage in participating families. Participants with retinal data available were similar to those without these images, except for family SEP, which was around a quarter of a SD lower (ie, less advantaged) in those missing retinal data (Table 2). In addition, children without retinal images were slightly older (11.7 vs 11.4 years of age) and more likely to be male (51% vs 45%).

3.2 | Main results

Table 3 shows the associations of retinal vascular caliber with large arterial phenotypes in children and adults. In Model 1 (estimates adjusted for age, sex, SEP and jointly considering arteriolar and venular caliber), both worse arteriolar and venular caliber were found to consistently associate with adverse large arterial function. For example, in children, a one SD higher arteriolar caliber was associated with slower PWV (-0.15 SD, 95% CI -0.21 to -0.09) and higher arterial elasticity (0.14 SD, 95% CI 0.06 to 0.20), while a one SD higher venular caliber was associated with faster PWV (0.09 SD, 95% CI 0.03 to 0.15) and lower arterial elasticity (-0.07 SD, 95% CI -0.13 to -0.01). Similar patterns were mirrored in adults, but associations were about twice the size seen in children (Figure 1).

In Model 2, when estimates were additionally adjusted for common risk factors (ie BMI, LDL and smoking exposure), the associations of arteriolar and venular caliber with PWV were attenuated in both children and adults by 30%-40%. For example, in children per SD higher arteriolar caliber was associated with slower PWV (-0.09 SD, 95% CI -0.16 to -0.02), while higher venular caliber was associated with faster PWV (0.06 SD, 95% CI -0.01 to 0.12). The associations with arteriolar elasticity changed little after the further adjustment in Model 2. The model R^2 for the association of retinal

TABLE 2 Sample characteristics

Characteristics ^a	Children				Adults			
	With data on retinal image		Without data on retinal image		With data on retinal image		Without data on retinal image	
	N	Mean (SD ^b) or %	N	Mean (SD ^b) or %	N	Mean (SD ^b) or %	N	Mean (SD ^b) or %
Age (y)	1288	11.4 (0.5)	586	11.7 (0.5)	1264	43.8 (5.1)	610	43.6 (5.6)
Gender (% female)	1288	50.9	586	45.1	1264	87.6	610	90.0
Birthweight (kg)	1285	3.44 (0.55)	585	3.43 (0.62)				
Gestational age (wk)	1279	39.2 (2.0)	586	38.9 (4.0)				
SEP	1283	0.26 (0.97)	584	-0.02 (1.01)	1260	0.26 (0.97)	607	-0.01 (1.01)
Smoking exposure	1287	20.7	586	27.8	1263	8.4	610	11.2
BMI z-score ^c	1288	0.30 (0.96)	584	0.31 (1.04)				
BMI (kg/m ²)					1259	27.86 (6.15)	602	27.74 (6.11)
Mean arterial blood pressure (mm Hg)	1213	78.07 (5.66)	564	76.65 (6.03)	1169	89.05 (9.70)	580	87.20 (8.98)
Retinal arteriolar caliber (μm)	1288	159.1 (11.9)			1264	151.0 (14.0)		
Retinal venular caliber (μm)	1288	230.7 (16.6)			1264	218.9 (18.5)		
PWV (m/s)	1242	4.44 (0.54)	564	4.49 (0.63)	1134	6.96 (1.14)	541	6.66 (1.08)
Arterial elasticity (%/10 mm Hg)	1151	4.83 (0.87)	193	4.66 (1.00)	1053	2.44 (0.06)	199	2.50 (0.66)
CIMT (μm)	1273	580.03 (46.40)	212	589.01 (47.59)	1236	663.55 (97.01)	232	667.04 (99.37)

^aMost characteristics measured at the Child Health CheckPoint; birthweight and gestation were collected at LSAC wave 1 in 2004, SEP was measured at LSAC Wave 6 in 2015, and smoking exposure was generated combining the questionnaire from LSAC Wave 1-6.

^bSD.

^cBMI was transformed to z-score with widely used Centres for Disease Control and Prevention (US)-growth charts.

TABLE 3 Associations of retinal microvascular caliber with large arterial intermediate phenotypes

Retinal caliber (per SD increase)	PWV			Carotid arterial elasticity			CIMT		
	Standardized β (95% CI)	P-value	R ²	Standardized β (95% CI)	P-value	R ²	Standardized β (95% CI)	P-value	R ²
Children									
Model 1: adjusted for age, sex, SEP and jointly considering arteriolar and venular caliber									
Arteriolar	-0.15 (-0.21, -0.09)	<.001	7.3	0.13 (0.06, 0.20)	<.001	3.3	-0.03 (-0.10, 0.03)	.30	2.2
Venular	0.09 (0.03, 0.15)	<.01		-0.07 (-0.13, -0.01)	.03		-0.07 (-0.13, -0.01)	.03	
Model 2: Model 1 plus BMI z-score, LDL cholesterol, passive smoking, birth weight, and gestational age									
Arteriolar	-0.09 (-0.16, -0.02)	<.01	12.8	0.13 (0.06, 0.20)	<.01	6.1	-0.02 (-0.09, 0.05)	.51	3.6
Venular	0.06 (-0.01, 0.12)	.10		-0.08 (-0.14, -0.01)	.03		-0.08 (-0.15, -0.01)	.02	
Sensitivity analysis: Model 2 plus mean arterial blood pressure									
Arteriolar	-0.02 (-0.09, 0.04)	.51	20.1	0.08 (0.00, 0.15)	.04	10.0	-0.03 (-0.10, 0.04)	.42	3.3
Venular	0.02 (-0.04, 0.08)	.47		-0.05 (-0.12, 0.02)	.15		-0.07 (-0.14, 0.00)	.06	
Adults									
Model 1: adjusted for age, sex, SEP and jointly considering arteriolar and venular caliber									
Arteriolar	-0.31 (-0.37, -0.24)	<.001	16.3	0.31 (0.24, 0.37)	<.001	18.0	-0.09 (-0.16, -0.03)	<.01	16.8
Venular	0.12 (0.05, 0.18)	<.001		-0.14 (-0.21, -0.08)	<.001		0.05 (-0.01, 0.11)	.12	
Model 2: Model 1 plus BMI, LDL cholesterol and ever smoking									
Arteriolar	-0.20 (-0.27, -0.14)	<.001	26.7	0.24 (0.17, 0.30)	<.001	29.5	-0.05 (-0.12, 0.01)	.12	20.9
Venular	0.06 (-0.00, 0.13)	.06		-0.10 (-0.16, -0.04)	<.01		0.05 (-0.02, 0.11)	.17	
Sensitivity analysis: Model 2 plus mean arterial blood pressure									
Arteriolar	0.01 (-0.06, 0.07)	.82	43.7	0.12 (0.06, 0.19)	<.001	36.0	-0.01 (-0.08, 0.06)	.72	21.9
Venular	-0.02 (-0.08, 0.04)	.57		-0.06 (-0.12, -0.00)	.05		0.01 (-0.05, 0.08)	.72	

Note: All models included arteriolar and venular caliber, plus other covariates. SDs of arteriolar and venular caliber for children were 11.9 and 16.6 μ m and for adults were 14.0 and 18.5 μ m, respectively.

vascular caliber and functional phenotypes was about 6%-13% in children and about 30% in adults.

Results were mixed for associations of retinal microvascular caliber and large arterial structure. There was little evidence of an association between arteriolar caliber and CIMT in children, while in adults, per SD higher arteriolar caliber was associated with smaller CIMT scores (-0.09 SD, 95% CI -0.16 to -0.03, Model 1), and this association attenuated by 44% after common risk factor adjustment (-0.05 SD, 95% CI -0.12 to 0.01, Model 2). Unexpectedly, higher retinal venular caliber showed small associations with smaller CIMT in children (-0.08 SD, 95% CI -0.15 to -0.01), but there was little evidence of an association in adults. The proportion of variance explained in model 2 was about 4% for children and 20% for adults.

3.3 | Sensitivity analysis

Further adjustment for mean arterial blood pressure attenuated, but did not eliminate, associations of arteriolar and venular caliber with arterial elasticity in children and adults. However, it fully attenuated associations with PWV (Table 3). Adding mean arterial blood

pressure to the models did not change the lack of adjusted associations with CIMT.

4 | DISCUSSION

4.1 | Principal findings

In this study, we found that the microvasculature is associated with functional artery phenotypes (ie, PWV, arterial elasticity) from early in life, and this association strengthens with age. By midlife, there is evidence of an association between the microvasculature and large artery structural phenotypes (ie, greater CIMT). It is likely that these associations will increase with age and with the development of manifest cardiometabolic disease.¹⁶

4.2 | Strengths and limitations

Our study has several strengths. It adds important new knowledge to the only other study (to our knowledge) to consider the association of the microvascular and large arterial intermediate

*In the conclusion section of the Discussion, our principal findings are based on a cohort of two generations: children and mid-life adults.

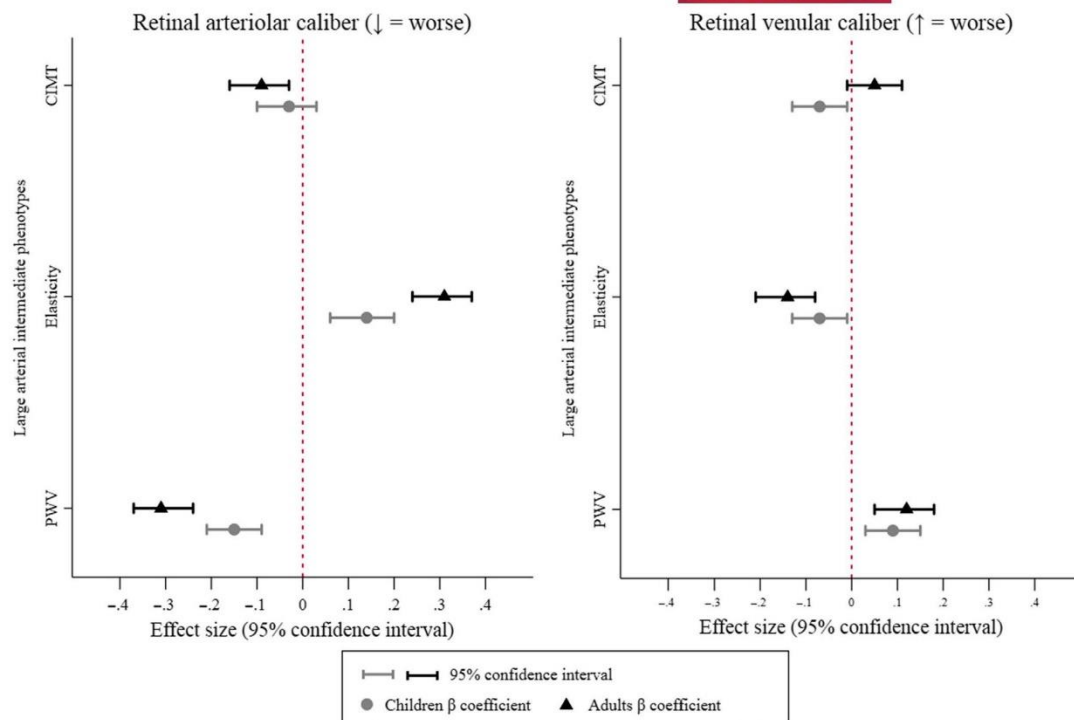


FIGURE 1 Associations of retinal microvascular caliber and large arterial intermediate phenotypes. Models included arteriolar and venular caliber simultaneously, adjusting for age, sex, and SEP (Model 1). Effect size, standardized regression coefficients β , representing per SD increase in arteriolar/venular caliber for a SD change in large arterial phenotypes

phenotypes in generally healthy children.¹⁷ Our study drew on a large, randomly selected national sample of Australian children and their parents, spanning diverse geographical areas across a very large country. All measures were objectively measured and processed under standard operating procedures, with moderate to high inter- and intra-rater reliability, and were conducted at the same time using the same equipment and processes for the two generations. This cross-generational design enabled us to efficiently infer temporal strengthening of associations with age, which would otherwise take decades to observe. Our well-phenotyped sample allowed us to consider the effects of important covariates. Unlike other studies, we were able to examine relationships of retinal microvascular caliber with both arterial function and structure simultaneously in the same individuals, across the two generations.

There are also limitations. Firstly, as our analytic sample was on average somewhat more advantaged than the national average, our findings may not generalize well to the most disadvantaged families (some of whom were nonetheless included). Secondly, limited data were available for adult men as most adults in the CheckPoint sample were mothers, but similar patterns of associations were nonetheless seen in adult women and men (Table S1). Thirdly, the cross-sectional

nature of the study precludes inference regarding the temporal sequence of the associations.

4.3 | Interpretation in light of other studies

To date, only one study has examined similar associations in healthy children.¹⁷ The Generation R Study found that in children (median age 6 years) each SD increase in retinal arteriolar and venular caliber was associated with 0.02 SD (95% CI -0.01 to 0.05) and 0.04 SD (95% CI 0.01 to 0.07) higher carotid-femoral PWV (SD 0.9 cm/s).¹⁷ In our study with children aged 11–12 years, we found a much stronger association with PWV and replicated this finding with another arterial function measure, arterial elasticity. In turn, we now show that the magnitude of associations between retinal vascular caliber and arterial functional phenotypes roughly doubles between mid-childhood and mid-adulthood. The explanatory power (ie, R^2 values) was small at age 11–12 years and much stronger in adults. These results are consistent with our meta-analysis of slightly older adults (mean age 54 years), which showed narrower retinal arteriolar caliber was associated with faster PWV ($r = -0.17$, 95% CI -0.25 to -0.10).¹⁶

We found little evidence that retinal arteriolar caliber was associated with CIMT in children, but a small association was observed in adults. In children of similar age (mean age 13.6 years) with type 1 diabetes, retinal vascular caliber has been associated with CIMT.⁴⁰ In our meta-analysis with older adults (mean age 63 years), narrower retinal arteriolar caliber was weakly associated with higher CIMT scores ($r = -.05$, 95% CI -0.09 to -0.02),¹⁶ and effect sizes were greater in patients with cardiometabolic disease. Taken together, both age and cardiometabolic disease conditions may contribute to the degree of co-variation between small and large artery structure.

Higher CIMT is associated with adverse changes in arterial structure, which has been shown to be linked with cardiovascular risk factors such as obesity and hypertension in children.⁴¹ The association we observed between wider (worse) venular caliber and lower (better) CIMT in children may be a chance finding, given its very small magnitude, unexpected direction and full attenuation on adjustment and in the sensitivity analysis.

4.4 | Implications

The potential association between the macro- or microcirculation and CVD risk are usually investigated separately. While it is plausible that their genetic determinants could be quite separate, biologically the two vascular systems are functionally related to the pulsatile transmission of pressure and flow.¹⁵ We have provided population-based evidence that the phenotypes of macro- and microcirculation co-vary from mid-childhood, and other studies suggest that this relationship is emerging from as young as 6 years onwards.¹⁷ The strength of the association increases with both age and with manifest cardiometabolic disease.¹⁶

Some of the observed associations between the macro- and microcirculation may arise from shared cardiometabolic risk factors.⁴² For example, we found that cardiovascular risk factors such as BMI, LDL cholesterol, and smoking exposures attenuated the association of retinal vascular caliber with functional and structural large artery phenotypes to a varying extent. However, these factors only contributed to a small amount of co-variations between small and large vascular phenotypes. It may be that small and large vessels are differentially susceptible to risk factors,⁸ or that they are mainly determined by distinct factors. In addition, the fully attenuated relationships between retinal microvascular caliber and PWV after adjusting for mean arterial blood pressure suggests the association may be mediated via blood pressure. This further emphasizes the likely importance of public health interventions to reduce blood pressure throughout life in order to protect all elements of the vascular tree.

There is strong evidence that microvascular variation plays an important role in the pathogenesis of CVD.⁹ The intermediate phenotype of large arteries represents a snapshot of preclinical cardiovascular health. In the current study, we found that phenotypic measures of microcirculation were associated with large arterial phenotypes, which in keeping with experiment models showing that

abnormality of small and large vessels often coexist in the pathogenesis of CVD.⁴³ Given the association between microvascular and large arterial phenotypes was relatively small, each phenotype may contribute to CVD through largely separate causal pathways. Further studies with clinical CVD outcomes would determine whether combining phenotypes of the microvasculature and large arteries improves risk prediction and inform the optimal use of these measures.

5 | CONCLUSION

Retinal microvascular caliber is associated with large arterial function by mid-childhood, with stronger (though still small) associations observed in midlife. Cumulative exposure to shared risk factors may underlie the age-dependent co-variation between small and large arterial phenotypes. Longitudinal studies with clinical outcomes would inform the optimal use of these parameters in early cardiovascular risk management.

PERSPECTIVES

Variations in the micro- and macro-vasculature underlie CVD pathogenesis, but how they may influence each other at different life stages is not well understood. We showed a small amount of co-variation between the two in childhood, which doubled in strength by midlife. This suggests it is important to understand the unique contribution of both the micro- and macro-vasculature to CVD across the life course, and whether the assessment of both the small and large vessels could improve risk prediction for CVD.

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CONFLICTS OF INTEREST

The authors report no relationships that could be construed as a conflict of interest.

AUTHOR CONTRIBUTIONS

MW conceived and led the CheckPoint study with the CheckPoint team and was LSAC's Health Design Leader. KL was the primary student supervisor, along with MW, DB, TW, MH, and oversaw all aspects of the study and the manuscript preparation. ML performed data analysis and wrote the main paper. All authors provided expert

advice and critical review of this manuscript, and had final approval of the submitted and published version of this paper.

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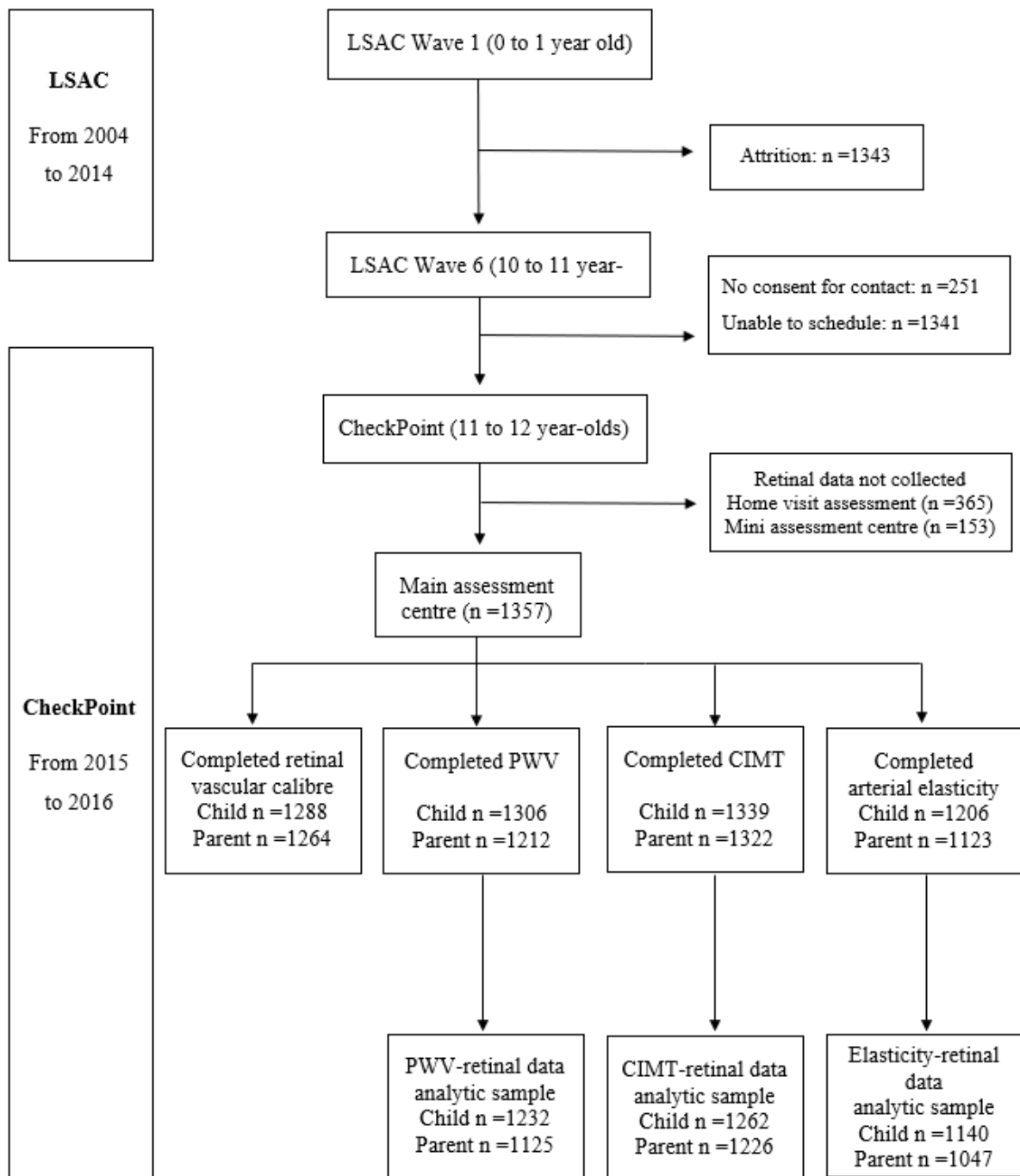
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary figure. Participant flowchart



Supplementary table. Associations between retinal microvascular calibre and large arterial intermediate phenotypes in adults stratified by gender; standardized regression coefficients for each standard deviation higher in retinal vascular calibre scores.

Retinal vascular calibre (Per SD increase)	Pulse wave velocity (SD: 1.1 m/s)		Carotid arterial elasticity (SD: 0.6%/10mmHg)		Carotid intima-media thickness (SD: 97.4 μ m)	
	Standardized β (95% CI)	<i>p</i>	Standardized β (95% CI)	<i>p</i>	Standardized β (95% CI)	<i>p</i>
Male						
Arteriolar	-0.32 (-0.51, -0.14)	<0.01	0.20 (0.05, 0.35)	<0.05	-0.10 (-0.31, 0.12)	0.38
Venular	0.27 (0.08, 0.46)	<0.01	-0.09 (-0.26, 0.07)	0.27	0.05 (-0.17, 0.27)	0.65
Female						
Arteriolar	-0.31 (-0.38, -0.24)	<0.001	0.32 (0.25, 0.40)	<0.001	-0.09 (-0.16, 0.03)	<0.01
Venular	0.09 (0.02, 0.16)	0.01	-0.15 (-0.22, -0.08)	<0.001	0.05 (-0.02, 0.11)	0.14

SDs of retinal arteriolar and venular calibre for adults were 14.0 and 18.5 μ m, respectively.

Abbreviations: SD, standard deviation; CI, confidence interval.

Chapter 7. Associations of decade-long obesity-related measures with retinal vascular calibre (Aim 2)

7.1 Overview

Chapter 7 addresses Aim 2, which examined whether retinal vascular calibre is predicted by i) BMI and (in children only) WHtR at multiple time points and ii) BMI and WHtR trajectories, all spanning the preceding decade in two generations – children aged 11–12 years and mid-life adults. I found that associations of decade-long high BMI trajectories and narrower retinal arteriolar calibre emerge in children, and are clearly evident by mid-life. Findings show that adiposity appears to exert its early adverse life course impacts on the microcirculation more via arteriolar than venular mechanisms. The results are briefly discussed and are further elaborated upon in Chapter 10.

This paper is published in the *International Journal of Obesity*. The published paper and supplementary materials form this chapter.

- **Liu M**, Lycett K, Wong TY, Kerr JA, He M, Juonala M, Olds T, Dwyer T, Burgner D and Wake M. Do body mass index and waist-to-height ratio over the preceding decade predict retinal microvasculature in 11–12 year-olds and mid-life adults? *International Journal of Obesity*. 2020; 44, 1712–1722.

7.2 Accepted manuscript (Aim 2 results)

International Journal of Obesity
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ARTICLE

Epidemiology and Population Health



Do body mass index and waist-to-height ratio over the preceding decade predict retinal microvasculature in 11–12 year olds and midlife adults?

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Abstract

Background/objectives Microvascular changes may contribute to obesity-associated cardiovascular disease. We examined whether body mass index (BMI) and waist-to-height ratio (WHtR) (1) at multiple earlier time points and (2) decade-long trajectories predicted retinal microvascular parameters in mid-childhood/adulthood.

Methods *Participants/design*: 1288 11–12 year olds (51% girls) and 1264 parents (87% mothers) in the population-based Child Health CheckPoint (CheckPoint) module within the Longitudinal Study of Australian Children (LSAC). *LSAC exposure measures*: biennial BMI z-score and WHtR for children at five time points from age 2–3 to 10–11 years and self-reported parent BMI at six time points from child age 0–1 years to 10–11 years. *CheckPoint outcome measures*: retinal arteriolar and venular caliber. *Analyses*: BMI/WHtR trajectories were identified by group-based trajectory modeling; linear regression models estimated associations between BMI/WHtR at each time point/trajectories and later retinal vascular caliber, adjusted for age, sex, and family socioeconomic status.

Results In time point analyses, higher child BMI/WHtR from age 4 to 5 years was associated with narrower arteriolar caliber at the age of 11–12 years, but not venular caliber. For example, each standard deviation higher in BMI z-score at 4–5 years was associated with narrower arteriolar caliber at 11–12 years (standardized mean difference (SMD): −0.05, 95% confidence interval (CI): −0.10 to 0.01); by 10–11 years, associations had doubled to −0.10 (95% CI: −0.16 to −0.05). In adults, these findings were similar, except the magnitude of BMI and arteriolar associations were similar across all time points (SMD: −0.11 to −0.13). In child and adult BMI trajectory analyses, less favorable trajectories predicted narrower arteriolar (p -trend < 0.05), but not venular (p -trend > 0.1), caliber. Compared with those in the average BMI trajectory, SMDs in arterial caliber for children and adults in the highest trajectory were −0.25 (95% CI: −0.44 to −0.07) and −0.42 (95% CI: −0.73 to −0.10), respectively. Venular caliber showed late associations with child WHtR, but not with BMI in children or adults.

Conclusions Associations of decade-long high BMI trajectories with narrowed retinal arteriolar caliber emerge in children, and are clearly evident by midlife. Adiposity appears to exert its early adverse life course impacts on the microcirculation more via arteriolar than venular mechanisms.

Introduction

Early childhood obesity is associated with adverse cardiovascular outcomes later in life [1, 2]. However, how early

obesity relates to a crucial component of the circulation system—the microcirculation has been largely overlooked. The microcirculation is implicated in obesity-associated cardiovascular disease (CVD) such as coronary artery disease [3, 4]. For example, in people with obesity, global microvascular dysfunction is a common pathway which predisposes to the development of coronary microvascular angina [3]. The microcirculation can be assessed by non-invasive retinal imaging and quantification of microvascular parameters, most frequently retinal arteriolar and venular caliber [5]. Understanding the relationship between obesity and the retinal microvasculature across the life course could

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SPRINGER NATURE

be informative, as variations in retinal vascular caliber are thought to mirror pathologic processes occurring in the systemic and coronary microcirculation [5, 6].

Most studies examining the association of body mass index (BMI) with the retinal microvasculature have used cross-sectional designs and mainly focused on adults [7]. In children, the only longitudinal study (the Singapore Cohort Study of Risk Factors for Myopia, $n = 421$) showed that 1 standard deviation (SD) higher BMI (3.03 kg/m^2) at the age of 7–9 years predicted 0.12 SD decreased arteriolar caliber ($p = 0.01$) and 0.13 SD increased venular caliber ($p < 0.01$) 5 years later. Inversely, arteriolar and venular caliber at baseline weakly predicted BMI at follow-up [8]. These results suggest that BMI is likely to be on the causal pathway, predicting changes to the retinal microvascular parameters, rather than the other way around.

The link between early or midlife BMI with future retinal microvascular pathology has not been clearly elucidated. Adverse BMI trajectories from adolescence to young adulthood are associated with an unfavorable cardiovascular profile (e.g., high blood pressure and insulin resistance) [9], but whether these findings extend to retinal microvascular parameters is unclear. An understanding of these relationships may be enlightening because distinct BMI trajectories impact differentially on the risk of cardio-metabolic disease later in life [9, 10]. For example, a 23-year longitudinal study found that compared with a normal BMI trajectory, a high-increasing childhood BMI trajectory was associated with poorer indicators of adult subclinical CVD [11]. In addition, most studies have focused on BMI, neglecting the possible impact of fat distribution on retinal microvasculature [12–14]. One of the few studies to examine fat mass and distribution is the Generation R study. Using dual-energy X-ray absorptiometry, higher total body and abdominal fat mass in 4145 6 year olds were associated with worse arteriolar but not venular caliber [13]. However, the cumulative effects of fat distribution patterns remain unclear since evidence is limited to cross-sectional studies. Waist girth (rather than direct body composition measurement) remains a common proxy for central adiposity, with waist-to-height ratio (WHtR) considered more predictive of CVD than BMI in adults [15]. Furthermore, if retinal vascular caliber changes reflect cumulative life course responses to systemic risk factors [16], and if adiposity tracks strongly through life, then associations should be larger in adults than in children. However, this is yet to be investigated. If there is a gradient in the risk with age, then this adds further weight to the importance of early obesity prevention.

The Child Health CheckPoint (CheckPoint) study nested within the Longitudinal Study of Australian Children (LSAC) provided an opportunity to examine these issues. In two generations—11–12 year olds and midlife adults (their parents)—we examined whether retinal vascular caliber is

predicted by (1) BMI and (in children only) WHtR at multiple time points and (2) BMI and WHtR trajectories, all spanning the preceding decade.

Materials and methods

Study design and participants

Details of the LSAC design and recruitment are outlined elsewhere [17, 18]. Briefly, in 2004, LSAC recruited a nationally representative birth cohort of 5107 infants (aged 0–1 year) and their parents using a two-stage clustered design and has since followed the children and their families biennially. The response rate to the initial invitation in 2004 was 57.2%, of which 73.7% ($n = 3764$) were retained to wave 6 in 2014 (e.g., when children were aged 10–11 years).

The CheckPoint study, LSAC's physical health and biomarkers module, was conducted between LSAC wave 6 (2014) and 7 (2016) [19]. At the wave 6 visit, interviewers invited all contactable families ($n = 3513$) to provide consent for their contact details to be shared with the CheckPoint team. In total, 1874 children (53.3%) aged 11–12 years took part in CheckPoint's cross-sectional biophysical assessment with one attending parent (detailed methods [20] and procedures [21] are published elsewhere).

Data collection was approved by the Australian Institute of Family Studies Ethics Committee (14–26) and the Royal Children's Hospital Melbourne Human Research Ethics Committee (33225D). Parents provided written informed consent for themselves and their children at each LSAC wave and the CheckPoint. In CheckPoint, parents also provided consent for sharing their and their child's images.

Procedures

Trained LSAC interviewers visited each family at home every 2 years from waves 1 to 6, during which they collected the anthropometric markers. Information from all waves was used.

The CheckPoint team booked an appointment for interested families from the same cohort between February 2015 and March 2016. The CheckPoint was a special one-off physical health assessment offered to the 11–12-year-old children and one of their parents. Most families attended a 3.5-h appointment comprising multiple measurement stations at CheckPoint's main assessment centers, which took place in the seven largest cities (mainly state capitals) around Australia. A small number of families ($n = 518$) who attended mini assessment centers in smaller regional cities (2.5-h appointments) or received a home visit (1.5-h) were not included in this study because the large and delicate equipment for retinal photography could not be readily transported to these centers.

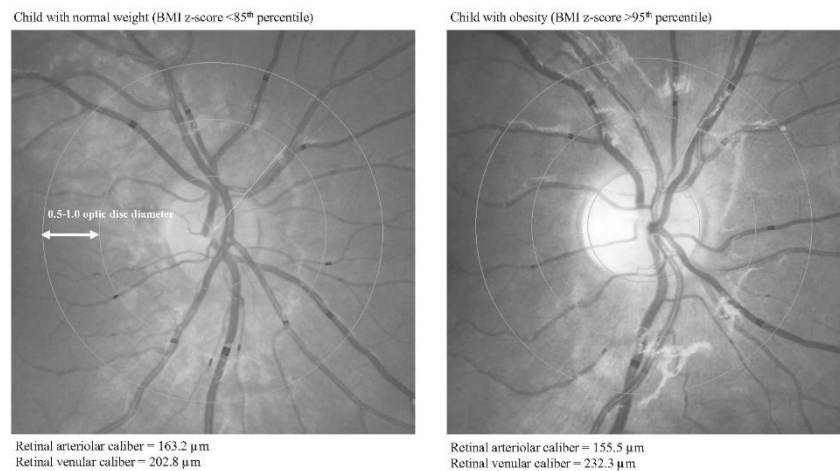


Fig. 1 Retinal images of a child with normal weight and a child with obesity on the grading platform of IVAN software. Blue (venule) and red (arteriole) marks are identified via IVAN software. For each participant, a segment of each vessel within the specific area (one-half to one disc diameter from the margin of the optic disc) was

selected by the grader for measurement; the IVAN software then estimates summary values for the average retinal vascular caliber according to the Big-Six (revised Knudston–Parr) formula, which combines measurements of the six largest arterioles or venules.

Measures

Exposures from LSAC

In children, height (to the nearest 0.1 cm) and weight (to the nearest 0.1 kg) were measured in light clothing and without shoes or socks. Two height measurements were taken, and if these measurements differed by 0.5 cm or more, a third measurement was taken; the average of the three measures was used. BMI was calculated as weight (kg)/height (m²), and then converted to age- and gender-specific z-scores using the US Centers for Disease Control growth reference charts [22]. Waist circumference (cm) was measured horizontally around the navel by lifting the shirt or jumper and lowering the belt or waistband in children. Mean of two waist measurements was used; if there was more than 0.5 cm difference on the first two, the mean of three was used. WHtR was calculated as waist (cm)/height (cm). In all waves, parents' height and weight were self-reported and BMI, but not WHtR, was calculated.

Outcomes from CheckPoint

During CheckPoint's 3.5-h visit, each child and parent took part separately in a 15-min retinal photograph assessment. Two optic disc centered digital photographs from each eye were taken by a fundus camera (EOS 60D SLR).

Right eye images were selected as the first choice for scoring. When right eye images were deemed ungradable,

left-eye images were used given the high correlation that has previously been reported between the two [23]. Details of retinal image grading are described elsewhere [24]. Briefly, four experienced graders scored each of the images using the Integrative Vessel Analysis (IVAN, University of Wisconsin, Wisconsin, USA) software program by masking the participant characteristics. Figure 1 shows the grading platform of IVAN. Retinal vessels were identified by the software as arterioles or venules from a specific area (one-half to one disc diameter from the margin of the optic disc). A segment of each vessel within this area was selected by the grader for measurement. Diameters of all the selected segments were measured by the IVAN software. For each participant, summary estimates of the average retinal vascular caliber were calculated by the software according to the Big-Six (revised Knudston–Parr) formula [25], which combines measurements of the six largest arterioles or venules. Inter-grader reliability correlation coefficients were $r = 0.79$ for retinal arteriolar and $r = 0.92$ for venular caliber. Intra-grader reliability ranged from $r = 0.90$ to 0.99 for retinal arteriolar and $r = 0.92$ to 0.98 for venular caliber.

Covariates

Age, sex, and family socioeconomic position (SEP) were selected as *a priori* potential confounders as they have been shown to associate with both BMI and retinal vascular caliber [26]. Age at CheckPoint was calculated to nearest week using date of birth, either imported from Medicare

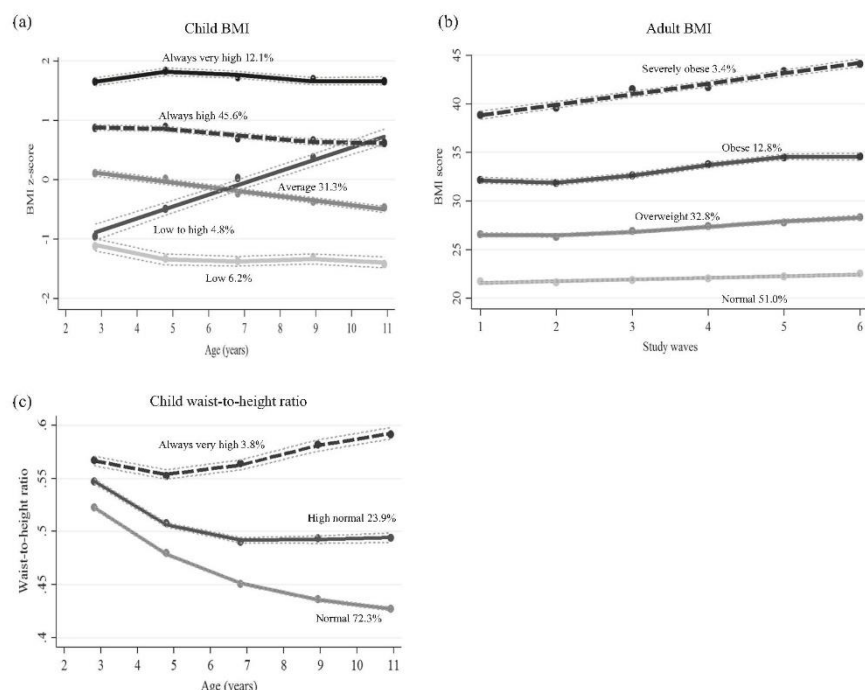


Fig. 2 Trajectories of body mass index and waist-to-height ratio in children and adults. **a** Child body mass index z-score. **b** Adult body mass index. **c** Child waist-to-height ratio.

Australia's database at the time of LSAC enrollment (child) or self-reported (parent), and date of assessment. Children's sex was from LSAC record, which was originally exported from the Medicare Australia database. Parents self-reported their sex in the CheckPoint questionnaire. SEP components were measured by questionnaires at LSAC wave 6, which summarized parent-reported combined household income, current or most recent occupation of each parent, and highest achieved educational qualification of each parent [27]. Each component of the score was scaled, and an unweighted average was calculated over three values in a single-parent household, or over five values in a dual-parent household. The unweighted average variable at LSAC was then standardized within the wave to have a mean 0, and SD of 1, with higher scores indicating better SEP.

Statistical analysis

We tested linear regression assumptions when fitting models. To visualize our findings, we internally constructed standardized scores ($(\text{observed value} - \text{mean})/\text{SD}$) for retinal vascular caliber, BMI for adults, and WHtR for children, and used the existing BMI z-scores for children. Thus, regression

coefficients represent the standardized mean difference (SMD) for a 1 SD higher score in the exposure (or 1 unit higher BMI z-score), and are reported with 95% confidence interval (CI) and *p* values. Multivariable linear regression models were performed for both aims with estimates adjusted for age, sex, and SEP. We did not adjust for glucose, lipids, or blood pressure since these would most likely reflect effect modification rather than confounding of any relationship between BMI/WHtR and retinal vascular caliber. All analyses were performed in Stata 14.0 (StataCorp LP, TX, USA), with children and adults considered separately.

Aim 1

Linear regression models were performed to assess whether BMI/WHtR at each of the preceding time point predicted retinal arteriolar and/or venular caliber at the CheckPoint assessment.

Aim 2

We identified BMI z-score/WHtR trajectories in children based on measures taken during LSAC waves 2–6 and the

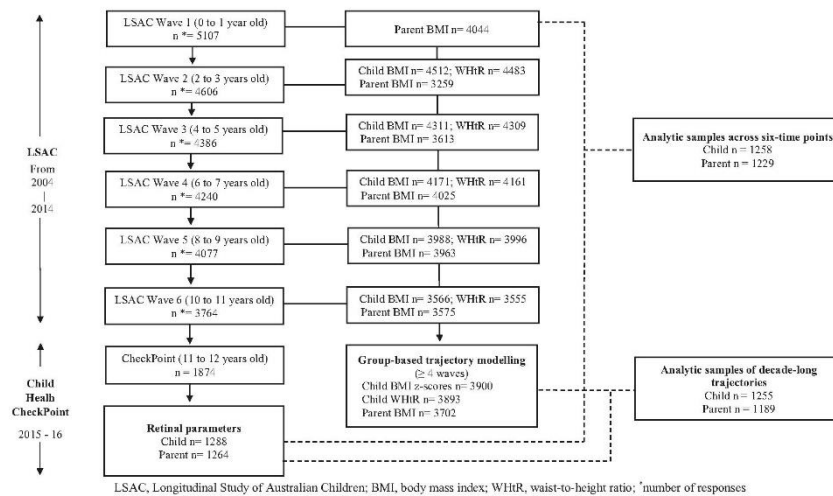


Fig. 3 Flowchart of Longitudinal Study of Australian Children and Child Health CheckPoint. LSAC Longitudinal Study of Australian Children, BMI body mass index, WHtR waist-to-height ratio. *Number of responses.

BMI trajectories for adults based on self-reported data gathered in LSAC waves 1–6. The “traj” plug-in from Stata 14.0 was used for the group-based trajectory modeling [28]. Methods of how we generated the trajectories have been published by our research team [29]. Briefly, BMI or WHtR scores were modeled with censored normal distribution, which is designed for the repeatedly measured continuous variables. For trajectory modeling, we included participants who had a BMI or WHtR value for at least four waves. In order to extract the most meaningful and distinct trajectories, Bayesian information criterion values, average posterior probabilities, and the proportion of the sample in each trajectory were taken into account (eTables 1 and 2) [30].

Based on these criteria, trajectories were selected and named from visual inspection. A five-trajectory solution was selected for child BMI z-score, with 6.2% in the “low,” 31.3% “average,” 45.6% always high,” 12.1% “always very high,” and 4.8% “low to high” trajectories (Fig. 2a). For adults, we selected a four-trajectory solution (51.0% “normal,” 32.8% “overweight,” 12.8% “obese,” and 3.4% “severely obese”; Fig. 2b). Adult BMI trajectories were quite flat, but one child trajectory (low to high) was characterized by a steeply rising BMI z-score over time, while the “average” and “high” trajectories appeared to fall slightly. For children’s WHtR trajectories, a three-trajectory solution was selected and, in line with the clinical cut-point of 0.5 [12], named as “normal” (72.3%), “high normal” (23.9%), and “always very high” (3.8%); Fig. 2c.

Multivariable linear regression models were performed to examine whether longitudinal BMI/WHtR trajectories predicted retinal vascular caliber in children and adults.

Sensitivity analysis

Previous studies reported that lower birth weight predicted poor retinal vascular caliber [31, 32], so we conducted a sensitivity analysis further adjusting birth weight (kg) for Aim 1 in children.

Results

Sample characteristics

Figure 3 shows the study flow from wave 1 of LSAC onward. Of the 1874 CheckPoint families, 1288 11–12 year olds and 1264 adults (mean age 44 years (SD: 5.1)) had retinal vascular caliber data available (Table 1). Around half of children (50.9%) were girls, while most adults (86.6%) were mothers. Families included in our analysis were slightly more advantaged (mean SEP: 0.3, SD: 1.0) than all families in LSAC wave 6 (mean: 0.0, SD: 1.0).

Aim 1: BMI and (children only) WHtR across the preceding multiple time points predict retinal vascular caliber

In children, higher BMI and WHtR from 4 to 5 years modestly predicted adverse retinal arteriolar, but not venular, caliber at the age of 11–12 years, and the associations strengthened with age (Table 2). At 4–5 years of age, per unit higher BMI z-score was associated with slight

Table 1 Characteristics of analytic samples (e.g., participants with retinal images in CheckPoint).

Characteristics	Children Means (SD ^a) or %	Adults Means (SD ^a) or %
Demographics		
Age (years)	11.4 (0.5)	43.8 (5.1)
Gender (% female)	50.9	86.6
Birth weight (kg)	3.45 (0.55)	
Family socioeconomic position (wave 6)	0.3 (0.1)	0.3 (0.1)
Exposures collected in LSAC		
BMI (z-score ^b for children; kg/m ² for adults)		
Wave 1 (child 0–1 year)	–	25.2 (4.8)
Wave 2 (child 2–3 years)	0.51 (1.06)	25.0 (4.7)
Wave 3 (child 4–5 years)	0.51 (1.07)	26.0 (5.0)
Wave 4 (child 6–7 years)	0.33 (0.93)	26.0 (5.3)
Wave 5 (child 8–9 years)	0.27 (1.02)	26.5 (5.8)
Wave 6 (child 10–11 years)	0.24 (0.97)	26.9 (6.0)
Waist-to-height ratio		
Wave 1 (child 0–1 year)	–	
Wave 2 (child 2–3 years)	0.53 (0.04)	
Wave 3 (child 4–5 years)	0.49 (0.03)	
Wave 4 (child 6–7 years)	0.46 (0.04)	
Wave 5 (child 8–9 years)	0.45 (0.05)	
Wave 6 (child 10–11 years)	0.45 (0.05)	
Outcomes collected in CheckPoint		
Retinal arteriolar caliber (μm)	159.1 (11.9)	151.0 (14.0)
Retinal venular caliber (μm)	230.7 (16.6)	218.9 (18.5)

CheckPoint, Longitudinal Study of Australian Children (LSAC)'s biophysical assessment module.

^aStandard deviation.^bBody mass index was transformed to z-score with Centers for Disease Control and Prevention (US)-growth charts.

narrowing of arteriolar caliber (SMD: -0.05 , 95% CI: -0.10 to 0.01). By the age of 10–11 years, the effect size of BMI on arteriolar caliber had doubled (SMD: -0.10 , 95% CI: -0.16 to -0.05). In adults, the magnitude of associations was similar across the six time points (SMD: -0.11 to -0.13). In children, the association of WHtR with arteriolar caliber changed little with age (at 10–11 years SMD: -0.08 , 95% CI: -0.14 to -0.01).

In comparison, the association between BMI and venular caliber was weak and did not vary with age in children or adults. However, an association between WHtR and venular caliber in children emerged from 8 to 9 years onward. Overall across each wave, the explanatory power of adult BMI for both arteriolar and venular caliber was larger than in children (Partial R^2 children from 2–3 to 10–11 years: 0.9–1.8%, adults from mean age 33–44 years: 2.0–2.6%).

Aim 2: decade-long BMI and (children only) WHtR trajectories predict retinal vascular caliber

In children, less favorable BMI trajectories were associated with narrower arteriolar caliber, with similar effects seen for

higher WHtR trajectories (p for trend <0.05 , Fig. 4). Compared with children following the “average” BMI trajectory, those following the “always very high” trajectory had arteriolar caliber that was -0.25 SMD (95% CI: -0.44 to -0.07) narrower. Compared with children following “normal” WHtR trajectory, those following a “high normal” and “always very high” trajectory had narrower arteriolar calibers of 0.14 (95% CI: -0.27 to -0.01) and 0.25 (95% CI: -0.54 to 0.03) SMD, respectively. Similarly, adults following an “overweight,” “obese,” or “severely obese” trajectory had narrower arteriolar caliber compared with those following the “normal” BMI trajectory, with the strongest effect seen for those who followed the “severely obese” trajectory (SMD: -0.42 , 95% CI: -0.73 to -0.10).

In contrast, we found little evidence for a gradient of higher BMI/WHtR trajectories with wider (e.g., poorer) venular caliber in either children or adults (p for trend >0.05). Nonetheless, point estimates for children were in the expected direction (Fig. 4), with trajectories characterized by “low to high,” “always very high” BMI, and “always very high” WHtR showing venular caliber that was wider by 0.13 (95% CI: -0.13 to 0.40), 0.19 (95% CI: 0.00 – 0.37),

Table 2 Associations of body mass index and waist-to-height ratio at multiple time points over the past decade with retinal vascular caliber in children and adults; model estimates adjusting for age, sex, and socioeconomic position

Adiposity marker by study wave	Children age from 2–3 to 11–12 years			Adults mean age from 33 to 44 years		
	Retinal arteriolar caliber		Retinal venular caliber		Retinal arteriolar caliber	
	Standardized mean difference (95% CI)	p	Standardized mean difference (95% CI)	p	Standardized mean difference (95% CI)	p
Body mass index (z-score ^a for children)						
Wave 1 (child 0–1 year)						0.69
Wave 2 (child 2–3 years)	−0.03 (−0.09, 0.02)	0.21	0.02 (−0.03, 0.08)	0.35	−0.11 (−0.17, −0.05)	<0.001
Wave 3 (child 4–5 years)	−0.05 (−0.10, 0.01)	0.07	0.02 (−0.03, 0.07)	0.42	−0.12 (−0.18, −0.05)	<0.001
Wave 4 (child 6–7 years)	−0.07 (−0.13, −0.02)	0.01	0.03 (−0.03, 0.09)	0.31	−0.11 (−0.17, −0.04)	0.001
Wave 5 (child 8–9 years)	−0.06 (−0.11, −0.01)	0.03	0.05 (−0.01, 0.10)	0.09	−0.13 (−0.19, −0.07)	<0.001
Wave 6 (child 10–11 years)	−0.10 (−0.16, −0.05)	<0.001	0.04 (−0.02, 0.09)	0.22	−0.13 (−0.19, −0.07)	<0.001
Waist-to-height ratio						
Wave 1 (child 0–1 year)						0.14
Wave 2 (child 2–3 years)	−0.03 (−0.09, 0.03)	0.34	0.02 (−0.03, 0.08)	0.42		
Wave 3 (child 4–5 years)	−0.10 (−0.16, −0.04)	<0.01	0.02 (−0.04, 0.07)	0.60		
Wave 4 (child 6–7 years)	−0.07 (−0.13, −0.01)	0.02	0.03 (−0.03, 0.09)	0.39		
Wave 5 (child 8–9 years)	−0.07 (−0.13, −0.01)	0.02	0.07 (0.01, 0.13)	0.03		
Wave 6 (child 10–11 years)	−0.08 (−0.14, −0.01)	0.02	0.08 (0.02, 0.14)	0.01		

The SDs for retinal arteriolar and venular caliber are 11.92 and 16.56 μm for children and 14.01 and 18.53 μm for adults, respectively.

^aBody mass index was transformed to z-score with widely used Centers for Disease Control and Prevention (US)-growth charts.

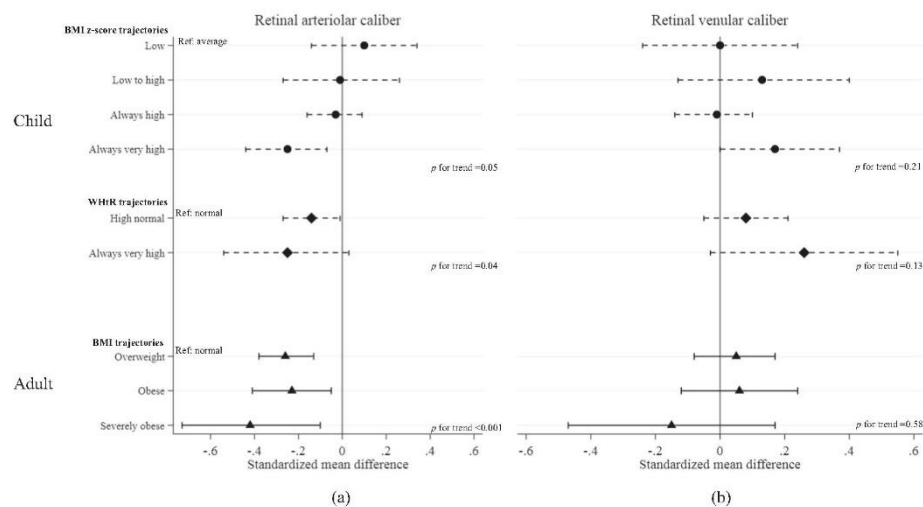


Fig. 4 Associations of decade-long body mass index and waist-to-height ratio trajectories with retinal vascular caliber. (a) Retinal arteriolar caliber, (b) Retinal venular caliber. Symbols in circles, diamonds, and triangles represent adjusted standardized mean difference of outcomes according to the body mass index z-score and waist-to-height

ratio trajectories in children and body mass index trajectories in adults, respectively. Horizontal bars indicate 95% confidence intervals of standardized mean difference; dash and solid bars represent results from children and adults, respectively. Ref: reference group.

and 0.26 (95% CI: -0.03 to 0.55) SMD, respectively, compared with “normal” trajectories.

Sensitivity analysis

When Aim 1 analyses were repeated including birth weight as a confounder, associations were largely unchanged (eTable 3).

Discussion

Principal findings

We found that higher BMI and WHtR from 4 to 5 years of age onwards predicted adverse retinal arteriolar caliber by the age of 11–12 years. Similar associations were seen in midlife adults’ BMI but with higher explanatory power. There was less convincing evidence that BMI over the preceding time points was associated with venular caliber in either children or adults, but there was evidence that WHtR from 8 to 9 years was associated with venular caliber in children. We observed a gradient of suboptimal decade-long higher BMI and (child only) WHtR trajectories predicting poorer retinal arteriolar, but not venular, caliber in children; again, we saw larger effects in adults. Only the least favorable BMI and WHtR trajectories were associated with adverse venular caliber in children.

Strengths and limitations

Strengths of our study include the large, population-based, cross-generational cohort with BMI and (children only) WHtR measured biennially across the preceding decade. The outcome measurements for children and adults were taken at the same time, with the same equipment, using the same protocols. Furthermore, the average posterior probability value for each trajectory was 0.82–0.97 for each group (see eTable 2), well above the recommended minimum value of 0.70 [33], indicating the models had good assignment accuracy.

Some limitations also warrant consideration. First, parent height and weight were self-reported and limited data were available from adult males ($n = 169$), as mothers typically accompanied their children to the CheckPoint assessment center. Nevertheless, evidence suggests self-reported BMI in longitudinal studies is acceptable for epidemiologic research, and the value correlates very highly with actual measurements in adults [34]. However, our estimates may lack precision in men given that our adult sample comprised 87% mothers. Second, retinal microvascular parameters were only collected at one-time point, limiting our ability to precisely pinpoint when the association first emerges. Future studies with repeated measures of both BMI and retinal vascular caliber are needed to establish exactly when these associations emerge. We recognize that both selection bias and attrition limit the population representativeness of our cohort. However, the sample covered a wide social and

geographic range which means that the risk factor associations are likely to be generalizable [35]. Lastly, WHtR is a proxy measure for central body fat. Replications are warranted in studies with longitudinal body fat measures.

Interpretation in light of other studies

We showed that the adverse microvascular variation at 11–12 years of age could be predicted from BMI as early as 4–5 years of age. This finding is consistent with the literature suggesting that the association between BMI and adverse retinal vascular variations may commence early in life. The youngest population-based sample among which this relationship has previously been examined were children aged 55.5 months (SD 10.3) taking part in the cross-sectional Sydney Pediatric Eye Disease Study [36]. In this small community sample ($n = 379$), each unit higher BMI was cross-sectionally associated with 1.06 μm narrower arteriolar caliber ($p = 0.01$) and 1.12 μm wider venular caliber ($p = 0.02$) [36]. Taken together with our findings, early BMI from 4 to 5 years may not only associate with cross-sectional, but also predict future retinal microvascular parameters. Our findings are also consistent with the Singapore Cohort Study of Risk Factors for Myopia, which included children of the same age with similar size of associations for arteriolar caliber, but not venular caliber [8].

In addition, by using two generations of participants with identical outcome measures, we can speculate that increasing adiposity may have cumulative effects on retinal microvascular parameters from childhood to mid-adulthood. Although the effects were similar in children and adults, the explanatory power (e.g., R^2) was higher in adults than in children. Furthermore, we found that consistently suboptimal decade-long BMI and/or WHtR trajectories were associated with adverse retinal vascular caliber. This supports our hypothesis that high BMI/WHtR has cumulative effects on retinal vascular caliber. The only other study that has examined the effect of BMI trajectories on retinal vascular caliber was from our research team [37]. In a small cohort ($n = 187$), we did not see the association of children's BMI trajectories (ten time points from 2 weeks to 14 years) and retinal vascular caliber [37]. However, the small size of the study and the fact that 90% of children were of normal-weight BMI may have limited the power to detect small associations [37].

Retinal arteriolar and venular caliber had different patterns of association with BMI and WHtR. The association of BMI with narrower arteriolar caliber in children and adults is in line with previous studies and a recent meta-analysis [14, 38]. We found little evidence of associations with retinal venular caliber for adults but did see some evidence in children. For instance, we found that WHtR, an index of central fat distribution, was related to venular caliber in children from 8 to 9 years; the “low to high,” “always very high” BMI and WHtR

trajectories among children were associated with wider venular caliber. These observations indicate that venular associations may appear later and be more closely related to central adiposity. Previous research has demonstrated mixed evidence regarding the relationship between BMI and WHtR with retinal venular caliber among children and adults [13, 39].

Implications

Mounting evidence suggests that obesity has adverse effects on both preclinical and clinical cardiovascular health [1, 2]. Our study suggests that greater BMI and WHtR predict adverse retinal microvascular parameters, a recognized early marker of later CVD [38]. Adverse microvascular parameters are predicted by BMI from the age of 4 years onwards and strengthen across the life course. Even though effects were relatively modest, at the population level they may have clinical implications. Data from 16 community-based studies estimated that the natural change in arteriolar caliber, without considering BMI, was estimated to be $-0.02 \mu\text{m}$ per decade [24]. Taken together with our current findings, high or rising BMI appears to accelerate adverse changes in microvascular parameters. For example, if a child's BMI z -score increased by 2 SD units (i.e., moved from normal into the obese range) at the age of 6–7 years, we estimate that his or her arteriolar caliber would be 1.7 μm narrower than the average. The Cardiovascular Risk in Young Finns Study reported that the improved ideal cardiovascular health from childhood to adulthood was significantly associated with wider arteriolar caliber in adulthood [40]. Thus, our estimated effect of BMI on arteriolar caliber may translate into substantial effects on future cardiovascular health. Our findings emphasize the importance of tackling obesity from early childhood, where its adverse effects are more likely to be reversible [41, 42].

How increasing levels of adiposity may contribute to microvascular variations is still unclear. Some studies suggest that variation of arteriolar and venular caliber may be determined by different risk factors [43–45]. For example, elevated blood pressure has been found to have stronger associations with arteriolar narrowing [26, 46], while inflammation markers have been more consistently associated with venular dilatation [13]. Further research is needed to elucidate the potential mechanisms by which adiposity adversely affects microvascular parameters. Prompt intervention in these pathways may prevent future microvascular disease and CVD.

Conclusion

Higher BMI and WHtR from 4 to 5 years, and less favorable decade-long trajectories, consistently predicted poorer retinal arteriolar caliber at 11–12 years. Similar results were observed in midlife adults with stronger explanatory effects.

There was little evidence of relationships with venular caliber, which may appear later and have closer relationships with central adiposity. Our findings suggest that greater adiposity may be a driver of poor microvascular parameters across the life course, but the underlying mechanisms of this relationship warrant further investigation to guide interventions.

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Supplementary online:

eTable 1. Criteria for selecting the number of trajectories

eTable 2. Average posterior probability value for trajectory groups

eTable 3. Associations of body mass index and waist-to-height ratio at multiple time points over the past decade with retinal vascular calibre in children; model estimates adjusting for age, sex, family socioeconomic position and birth weight

eTable 1. Criteria for selecting the number of trajectories

Number of latent classes	Trajectory shapes ^a	BIC ^b
Children body mass index z-scores (n=3900)		
2	3 3	-23776.79
2	2 3	-23772.79
2	1 3*	-23768.81
3	3 3 3	-22659.65
3	2 3 3	-22655.84
3	1 3 3*	-22652.35
4	3 3 3 3	-22093.58
4	3 2 3 3	-22091.11
4	3 1 3 3*	-22086.98
5	3 3 3 3 3	-21866.96
5	3 2 3 3 3	-21865.60
5	3 1 3 3 3	-21864.04
5	3 2 2 3 3	-21888.51
5	3 1 2 3 3	-21874.04
5	3 1 1 3 3*	-21871.10
6	3 3 3 3 3 3 ^c	-21612.19
Children waist-to-height ratio (n=3893)		
2	3 3 *	34308.81
3	3 3 3 *	35157.48
4	3 3 3 3 ^c	35538.15
Adults body mass index (n=3702)		
2	3 3	-57203.57
2	1 3*	-57201.05
3	3 3 3	-54114.56
3	2 3 2	-54110.39
3	1 3 1 *	-54106.58
4	3 3 3 3	-52716.47
4	2 3 3 2	-52708.95
4	1 3 3 1*	-52706.48
5	3 3 3 3 3 ^c	-52057.35

^a Trajectory shapes: 0= intercept; 1=linear; 2=quadratic; 3=cubic.

We examined the significance ($p < 0.05$) of all three parameters (linear, quadratic, and cubic) and dropped the ones that were nonsignificant. That is, we started by including linear, quadratic, and cubic parameters for each trajectory. If the cubic parameter was not significant, we dropped it and tested for a quadratic trajectory. We dropped the quadratic trajectory if it was not significant and tested for a linear trajectory. We retained the linear parameter even if this was not significant. After each change we compared the results using the BIC criteria.

^b BIC, Bayesian information criterion; the model with the highest (least negative) value of BIC is preferred.

^c One or more of the groups had a very small proportion of the observations.

* All parameters are significant ($p < 0.05$).

eTable 2. Average posterior probability value for trajectory groups

BMI trajectory group	Average posterior probability
Children	
Low (n=257)	0.91
Average (n=1141)	0.87
Always high (n=1666)	0.89
Always very high (n=627)	0.91
Low to high (n=245)	0.82
Adults	
Normal (n=1871)	0.97
Overweight (n=1202)	0.93
Obese (n=480)	0.97
Severely obese (n=149)	0.97
WHtR trajectory group (children)	
Normal (n=2603)	0.94
High normal (n=1055)	0.88
Always high (n=235)	0.94

eTable 3. Associations of body mass index and waist-to-height ratio at multiple time points over the past decade with retinal vascular calibre in children; model estimates adjusting for age, sex, family socioeconomic position and birth weight

Adiposity marker by study wave	Children age from 2–3 to 11–12 years			
	Retinal arteriolar calibre		Retinal venular calibre	
	Standardized β (95% CI)	<i>p</i>	Standardized β (95% CI)	<i>p</i>
Body mass index z-score				
Wave 1 (child 0–1y)				
Wave 2 (child 2–3ys)	-0.04 (-0.09, 0.01)	0.13	0.02 (-0.03, 0.07)	0.42
Wave 3 (child 4–5ys)	-0.06 (-0.11, -0.00)	0.04	0.02 (-0.03, 0.07)	0.45
Wave 4 (child 6–7ys)	-0.08 (-0.14, -0.02)	0.01	0.03 (-0.03, 0.09)	0.33
Wave 5 (child 8–9ys)	-0.07 (-0.12, -0.01)	0.02	0.05 (-0.01, 0.10)	0.09
Wave 6 (child 10–11ys)	-0.11 (-0.16, -0.05)	<0.001	0.04 (-0.02, 0.09)	0.20
Waist-to-height ratio				
Wave 1 (child 0–1y)				
Wave 2 (child 2–3ys)	-0.03 (-0.09, 0.03)	0.31	0.02 (-0.04, 0.08)	0.47
Wave 3 (child 4–5ys)	-0.10 (-0.16, -0.05)	<0.001	0.02 (-0.04, 0.07)	0.58
Wave 4 (child 6–7ys)	-0.07 (-0.13, -0.01)	0.02	0.03 (-0.03, 0.09)	0.38
Wave 5 (child 8–9ys)	-0.07 (-0.13, -0.01)	0.02	0.07 (0.01, 0.13)	0.02
Wave 6 (child 10–11ys)	-0.08 (-0.14, -0.02)	0.01	0.08 (0.02, 0.15)	0.01

a. Body mass index was transformed to z-score with widely used Centres for Disease Control and Prevention (US)-growth charts. The SDs for retinal arteriolar and venular calibre are 11.92 and 16.56 μm respectively.

Chapter 8. Associations of inflammation and retinal vascular calibre (Aim 3)

8.1 Overview

Chapter 8 addresses Aim 3, which examined whether the inflammatory biomarker GlycA is associated with retinal vascular calibre and whether GlycA mediates the association between obesity and retinal vascular calibre in children aged 11–12 years and mid-life adults. I found that inflammation mediated the associations between obesity and retinal venules, but not arterioles from mid-childhood, with higher mediation effects observed in adults. The results are briefly discussed and are further elaborated upon in Chapter 10.

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OPEN Inflammation mediates the relationship between obesity and retinal vascular calibre in 11-12 year-olds children and mid-life adults

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Obesity predicts adverse microvasculature from childhood, potentially via inflammatory pathways. We investigated whether inflammation mediates associations between obesity and microvascular parameters. In 1054 children (mean age 11 years) and 1147 adults (44 years) from a cross-sectional study, we measured BMI (z-scores for children) and WHtR, Glycoprotein acetyls (GlycA), an inflammatory marker, and retinal arteriolar and venular calibre. Causal mediation analysis methods decomposed a “total effect” into “direct” and “indirect” components via a mediator, considering continuous and categorical measures and adjusting for potential confounders. Compared to normal-weight BMI children, those with overweight or obesity had narrower arteriolar calibre (total effects -0.21 to -0.12 standard deviation (SD)); direct (not mediated via GlycA) effects were similar. Children with overweight or obesity had 0.25 to 0.35 SD wider venular calibre, of which 19 to 25% was mediated via GlycA. In adults, those with obesity had 0.07 SD greater venular calibre, which was completely mediated by GlycA (indirect effect: 0.07 SD, 95% CI -0.01 to 0.16). Similar findings were obtained with other obesity measures. Inflammation mediated associations between obesity and retinal venules, but not arterioles from mid-childhood, with higher mediation effects observed in adults. Interventions targeting inflammatory pathways may help mitigate adverse impacts of obesity on the microvasculature.

Obesity is a leading risk factor for cardiovascular disease (CVD), with adverse effects on the vasculature, including the microcirculation^{1,2}. Obesity is associated with adverse microvascular parameters from early childhood, but underlying mechanisms remain unclear^{3,4}. Inflammation is suggested to play a key role⁵.

Retinal microvasculature, which has been shown to mirror changes in other vascular beds, is a surrogate marker for systemic microvasculature⁶. Investigation of the retinal microvasculature is now feasible in population-based cohort studies by retinal imaging technique⁷, which is well-tolerated and reproducible from early childhood onwards⁸. Population-based studies have shown that increasing body mass index (BMI) and waist-to-height ratio (WHtR), two commonly used measures related to obesity, are associated with narrower retinal arterioles and wider venules in both children and adults^{9–11}.

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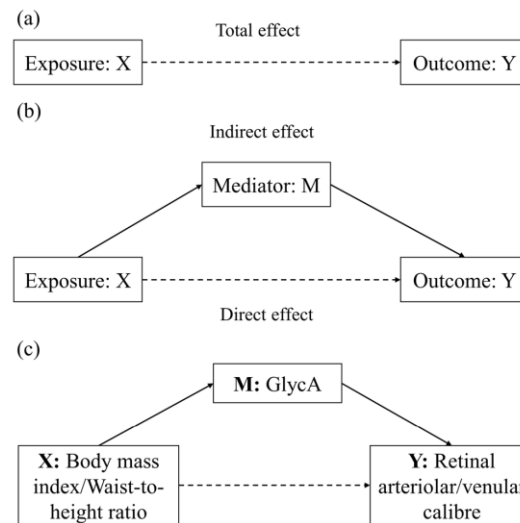


Figure 1. Causal mediation framework. (a) The effect of exposure X on outcome Y without taking into account mediator M is known as the “total effect”. (b) The effect of X on Y not via M is known as the “direct effect”, and the effect through M is the “indirect effect”. (c) X, M, Y are body mass index /waist-to-height ratio, glycoprotein acetyls (GlycA) and retinal arteriolar/venular calibre in the current study. Confounders included in the analysis were not shown in the figure.

Inflammatory markers, such as high sensitivity C-reactive protein (hsCRP) and white blood cell count, have been associated with adverse retinal microvascular variation in adults, most consistently with venular rather than arteriolar calibre^{3,12,13}. In children, acute phase reactants, such as hsCRP, are less informative about chronic inflammation. A novel biomarker, glycoprotein acetyls (GlycA), is suggested to be more indicative of chronic and cumulative inflammation in children and adults^{14,15}. Few studies of the relationship between inflammation and the microvasculature have considered the role of obesity, a key driver of chronic inflammation^{12,13}.

The question of whether the association between obesity and retinal microvasculature is explained by inflammatory pathways can be addressed within a causal mediation framework (Fig. 1a,b). Theoretically, this framework enables estimation of the extent to which the total causal effect of an exposure on an outcome occurs via an intermediate variable (“mediator”), by decomposing the total effect into an “indirect” effect through the mediator and a “direct” effect through other pathways.

While the “total (causal) effect” of obesity on any outcome is ill-defined, it is relevant to examine the extent to which obesity-related disparities in retinal microvascular parameters would be reduced, if the distribution of inflammatory markers in those with obesity were reduced to levels observed in the non-obese¹⁶, thereby representing potential “interventional effects”^{17,18}. Using this approach, we examined whether the inflammatory biomarker GlycA mediates the association between measures relating to obesity (BMI and WHtR) and retinal microvasculature (Fig. 1c), in 11–12 year olds and in mid-life adults. The child and adult sample can provide information regarding associations across the life course. We hypothesise that the association between obesity and retinal vascular calibre and the mediation effect of inflammation will be stronger in adults than in children.

Results

Sample characteristics. The study flow from wave 1 of LSAC onwards is shown in the Supplementary Figure. Of the 1874 participating families, 1054 children (mean age 11.4 years) and 1147 adults (their parents, mean age 43.8 years) had data on all measures. Table 1 shows the characteristics of the analytic sample. Over a quarter of children were in the overweight (14.4%) and obese (8.6%) categories, and 8.6% had WHtR ≥ 0.5 . In adults, 62.2% were in the overweight and obese categories, and 53.9% had WHtR ≥ 0.5 .

Interrelationships between key variables. Table 2 shows results from multivariable linear regression models, adjusted for age, sex and SEP. BMI and WHtR were moderately associated with GlycA, with stronger evidence for associations in adults than children. For example, in children and adults, one SD greater in BMI were associated with 0.39 SD (95% CI 0.33 to 0.45) and 0.47 SD (95% CI 0.42 to 0.52) higher GlycA scores, respectively.

Higher BMI and WHtR showed small associations with narrower arteriolar calibre, with effects slightly larger in adults than children. In contrast, the associations of BMI with venular calibre were weak for both children and adults (0.03 (95% CI -0.04 to 0.09), 0.02 (95% CI -0.04 to 0.07), respectively). Similar findings were observed for WHtR and venular calibre.

	Children (n = 1054)	Adults (n = 1147)
Characteristics	Means (SD ^a) or %	Means (SD ^a) or %
Age (years)	11.4 (0.5)	43.8 (5.1)
Sex (% female)	52.0	86.7
Socioeconomic position	0.3 (0.9)	0.3 (1.0)
Body mass index z-score ^b	0.3 (1.0)	
Body mass index (kg/m ²)		27.9 (6.1)
Current BMI status (%)		
Normal	77.0	37.8
Overweight	14.4	32.9
Obese	8.6	29.3
Waist-to-height ratio (WHtR)	0.4 (0.1)	0.5 (0.1)
Current WHtR status (%)		
<0.5	91.4	46.1
≥0.5	8.6	53.9
Retinal arteriolar calibre (μm)	159.0 (11.9)	151.1 (13.9)
Retinal venular calibre (μm)	230.9 (16.5)	219.0 (18.6)
Glycoprotein Acetyls (mmol/L)	1.0 (0.1)	1.0 (0.2)

Table 1. Sample characteristics. ^aStandard deviation; ^bBody mass index was transformed to z-scores using the Centres for Disease Control and Prevention (US) growth charts.

Models (the exposure and outcome)	Children		Adults	
	Effect size (95% CI)	p	Effect size (95% CI)	p
BMI/WHtR and proposed inflammatory mediator				
BMI and GlycA	0.39 (0.33, 0.45)	<0.001	0.47 (0.42, 0.52)	<0.001
WHtR and GlycA	0.44 (0.38, 0.49)	<0.001	0.53 (0.48, 0.57)	<0.001
BMI/WHtR and retinal vascular calibre outcomes				
BMI and arteriolar calibre	−0.12 (−0.19, −0.06)	<0.001	−0.16 (−0.21, −0.08)	<0.001
BMI and venular calibre	0.03 (−0.04, 0.09)	0.33	0.02 (−0.04, 0.07)	0.58
WHtR and arteriolar calibre	−0.10 (−0.16, −0.06)	<0.001	−0.16 (−0.22, −0.10)	<0.001
WHtR and venular calibre	0.05 (−0.01, 0.12)	0.08	0.03 (−0.03, 0.08)	0.36
Proposed inflammatory mediator and retinal vascular calibre outcomes				
GlycA and arteriolar calibre	−0.02 (−0.08, 0.04)	0.52	−0.09 (−0.15, −0.03)	<0.01
GlycA and venular calibre	0.09 (0.03, 0.15)	<0.01	0.06 (0.00, 0.12)	0.03
Proposed inflammatory mediator and retinal vascular calibre outcomes*				
GlycA and arteriolar calibre	0.03 (−0.03, 0.09)	0.41	−0.02 (−0.08, 0.05)	0.59
GlycA and venular calibre	0.09 (0.03, 0.16)	<0.01	0.07 (0.01, 0.14)	0.03

Table 2. Inter-relationships of key variables, assessed via multivariable linear regression models, per SD unit higher in the exposure (adjusted for age, sex and SEP). Abbreviations: 95%CI, 95% confidence interval; BMI, body mass index; WHtR, waist-to-height ratio; GlycA, glycoprotein acetyls. *The model estimates were further adjusted for body mass index as a confounding factor.

Higher GlycA had small associations with narrower arteriolar calibre in adults but not children. In adults, one SD higher GlycA was associated with −0.09 SD (95% CI −0.15 to −0.03) narrower arteriolar calibre. Further adjustment for BMI fully attenuated this association in adults. The association between higher GlycA and wider venular calibre in children was less evident in adults, and BMI did not appear to confound this association.

Does GlycA mediate the association of obesity with retinal vascular calibre? The results of the causal mediation analysis when exposures were considered as continuous variables are presented in Figs. 2 and 3. Although BMI had a small “total effect” on retinal arteriolar calibre (Fig. 2a,c), the direct effects, not mediated via GlycA, were similar to the total effects in children and adults. In contrast, BMI had a smaller effect on venular calibre for children and adults (Fig. 2b,d), with some evidence of mediation through GlycA in both groups. Direct effects, not involving GlycA, were in the opposite direction for children (−0.01 SD, 95% CI −0.08 to 0.06) and adults (−0.02 SD, 95% CI −0.01 to 0.05). The results were similar when the exposure was WHtR (Fig. 3).

When considering exposures as categorical variables, compared to those with normal weight, those with overweight or obesity - whether either measured by BMI or WHtR - had narrower arteriolar calibre, but again the direct effects not mediated via GlycA were similar to the total effects (Table 3).

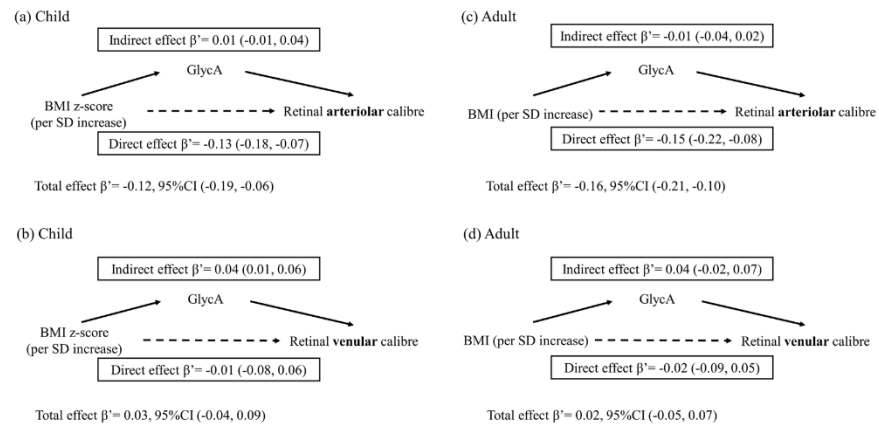


Figure 2. Mediation effects of GlycA on associations of body mass index (z-scores for children, standardised scores for adults) with standardised retinal venular and arteriolar calibre. Footnotes: β' represents effect estimates for direct, indirect, and total effect derived from causal mediation analysis. SDs of retinal arteriolar and venular calibre in children are 11.9 μm and 16.5 μm , and SDs of BMI, retinal arteriolar and venular calibre in adults are 6.1 kg/m^2 , 13.9 μm and 18.6 μm . Abbreviations: 95%CI, 95% confidence interval; SD, standard deviation; BMI, body mass index; GlycA, glycoprotein acetyls.

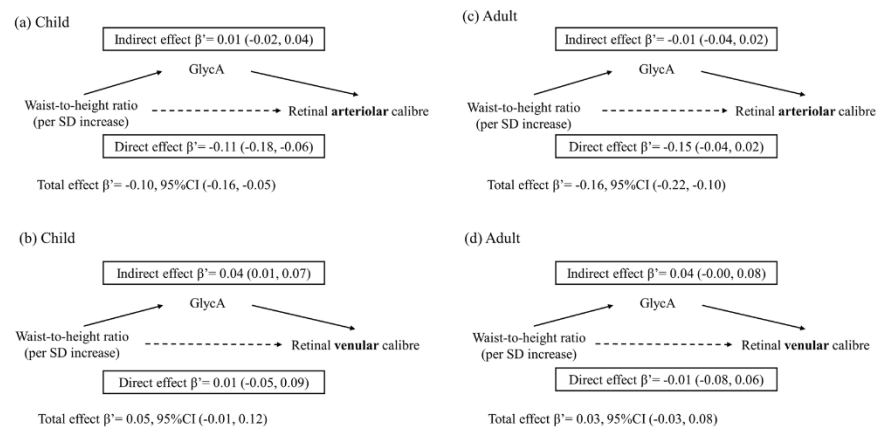


Figure 3. Mediation effects of GlycA on associations of standardised waist-to-height ratio with standardised retinal venular and arteriolar calibre. Footnotes: β' represents effect estimates for direct, indirect, and total effect derived from causal mediation analysis. SDs of waist-to-height ratio, retinal arteriolar and venular calibre in children are 0.1, 11.9 μm and 16.5 μm , and SDs of waist-to-height ratio, retinal arteriolar and venular calibre in adults are 0.1, 13.9 μm and 18.6 μm . Abbreviations: 95%CI, 95% confidence interval; SD, standard deviation; WHtR, waist-to-height ratio; GlycA, glycoprotein acetyls.

Children with overweight and obesity had 0.25 SD and 0.35 SD wider venular calibre (respectively) compared to those with normal-weight BMI, of which 19 to 25% was mediated via GlycA (indirect effect: overweight 0.05 SD, 95% CI 0.0 to 0.10; obese 0.09 SD, 95% CI 0.01 to 0.18). In adults, retinal venular calibre was similar for normal weight and overweight, but adults with obesity had 0.07 SD greater venular calibre and this effect was completely mediated by GlycA (indirect effect: 0.07 SD, 95% CI -0.01 to 0.16). Similar mediation effects were seen in children and adults with WHtR ≥ 0.5 compared with those WHtR < 0.5 , with 41% to 69% of the association with venular calibre mediated by GlycA, respectively.

Discussion

This is the first study to investigate whether the associations of obesity-related measures with microvascular parameters are mediated via inflammation. We showed that BMI and WHtR were associated both with GlycA and with adverse retinal vascular calibre (narrower arterioles and wider venules). Novel findings were that higher

Obesity category	Children				Adults			
	Retinal arteriolar calibre		Retinal venular calibre		Retinal arteriolar calibre		Retinal venular calibre	
	β' (95% CI)	<i>p</i>	β' (95% CI)	<i>p</i>	β' (95% CI)	<i>p</i>	β' (95% CI)	<i>p</i>
BMI: Overweight vs normal (ref)								
Indirect effect	0.00 (−0.05, 0.05)	0.93	0.05 (0.00, 0.10)	0.05	−0.01 (−0.04, 0.03)	0.71	0.03 (−0.00, 0.06)	0.08
Direct effect	−0.11 (−0.32, 0.10)	0.30	0.20 (0.01, 0.40)	0.04	−0.23 (−0.36, −0.08)	<0.01	−0.01 (−0.17, 0.13)	0.81
Total effect	−0.11 (−0.32, 0.10)	0.33	0.25 (0.06, 0.45)	0.01	−0.24 (−0.36, −0.10)	<0.01	0.02 (−0.13, 0.16)	0.85
Proportion mediated by GlycA	−0.01 (−1.98, 2.76)	0.96	0.19 (−0.00, 0.78)	0.06	0.03 (−0.13, 0.23)	0.71	1.92 (−5.12, 7.21)	0.88
BMI: Obese vs normal (ref)								
Indirect effect	0.00 (−0.08, 0.09)	0.88	0.09 (0.01, 0.18)	0.04	−0.01 (−0.04, 0.03)	0.71	0.07 (−0.01, 0.16)	0.08
Direct effect	−0.14 (−0.42, 0.14)	0.32	0.26 (−0.04, 0.55)	0.09	−0.23 (−0.37, −0.09)	<0.01	0.00 (−0.18, 0.16)	0.95
Total effect	−0.13 (−0.41, 0.13)	0.32	0.35 (0.07, 0.64)	0.02	−0.24 (−0.37, −0.10)	<0.01	0.07 (−0.08, 0.21)	0.34
Proportion mediated by GlycA	−0.02 (−2.40, 2.64)	0.92	0.25 (−0.00, 1.25)	0.06	0.03 (−0.13, 0.23)	0.71	1.00 (−6.86, 10.23)	0.39
WtHR: ≥ 0.5 vs <0.5 (ref)								
Indirect effect	−0.00 (−0.09, 0.08)	0.98	0.10 (0.02, 0.18)	0.01	−0.03 (−0.10, 0.03)	0.27	0.05 (−0.02, 0.12)	0.12
Direct effect	−0.18 (−0.41, 0.03)	0.11	0.13 (−0.08, 0.38)	0.22	−0.22 (−0.35, −0.09)	<0.001	0.02 (−0.12, 0.15)	0.76
Total effect	−0.18 (−0.41, 0.04)	0.10	0.23 (0.03, 0.46)	0.02	−0.25 (−0.37, −0.14)	<0.001	0.07 (−0.04, 0.19)	0.21
Proportion mediated by GlycA	0.01 (−1.29, 0.92)	0.98	0.41 (0.04, 1.99)	0.03	0.14 (−0.09, 0.46)	0.27	0.69 (−5.69, 7.24)	0.30

Table 3. Direct and indirect effects of overweight and obesity on retinal vascular calibre through GlycA; estimates adjusted for age, sex and SEP. β' represents effect estimates for direct, indirect, and total effect derived from causal mediation analysis, compared to the reference group. The proportion of the total effect mediated by GlycA was calculated as the ratio of the indirect effect to the total effect. Abbreviations: 95%CI, 95% confidence interval; BMI, body mass index; WtHR, waist-to-height ratio; GlycA, glycoprotein acetyls.

GlycA was associated with both wider venular calibre (in mid-childhood and mid-life adults) and narrower arteriolar calibre (in adults). Inflammation mediated associations between both measures related to obesity and retinal venular, but not arteriolar, calibre, with adults had higher mediation effects than children.

Our large, cross-generational cohort with standardised measurements using the same equipment and protocols may minimise potential measurement bias. The associations were investigated in children and adults, providing data from two ages in the life course. Similar results were observed for BMI and WtHR, which increases confidence in our findings, although these measures are highly correlated in children and adults (correlation coefficients 0.83 and 0.92 respectively). The use of GlycA, relatively novel in population-based studies¹⁸, may be advantageous given limitations of acute phase reactants in capturing chronic inflammation, especially in children¹⁵.

Potential limitations include the difficulty in interpreting mediated effects causally given the lack of a well-defined intervention on inflammation, so that our study can only suggest this as a potential intervention target. In addition, the cross-sectional and observational study design means there is potential for residual or unmeasured confounding. We assumed that there was no post-exposure confounding of mediator-outcome relationships that could arise through other potential mediating pathways. This assumption could be flawed; for example, higher LDL, another potential mediator, which may be increased in those with obesity and associate with narrower arteriolar and wider venular calibre directly²⁰, or via the LDL-inflammation association to indirectly associate with poor retinal microvascular parameters²¹. However, in our additional analyses, LDL appears to confound the association with venular calibre only slightly in adults (Supplementary Table). Our participants were marginally more advantaged socioeconomically than the original nationally representative LSAC sample, so our conclusions may be less relevant to disadvantaged populations. Our analysis and findings were based on an existing cohort. Pre-specified analyses from future prospective studies are warranted to replicate the findings.

Retinal arteriolar and venular calibre showed different patterns of association with BMI and WtHR, in keeping with current evidence that they may be influenced by different factors²². Our study supports previous findings that inflammation is more consistently associated with retinal venular than arteriolar calibre in children and adults. The Generation R Study of children aged 6 years found that each SD higher CRP was associated with 0.10SD (95% CI 0.06 to 0.14) wider venular calibre³. Similar findings are reported in mid-life and older adults and with other inflammatory markers, such as fibrinogen and white blood cells count^{23–27}. GlycA is a novel composite inflammatory marker with greater stability and lower intra-individual variability than single acute phase inflammatory proteins, such as hsCRP²⁸. In population studies, GlycA is a robust predictor of all-cause of mortality and CVD, even after adjusting for hsCRP^{29–31}. In adolescents, GlycA is associated with obesity and prediabetes³². To the best of our knowledge, this is the first study to demonstrate that GlycA is associated with retinal venular calibre in children and adults and the magnitude of association is similar to previous findings.

We extended prior studies by exploring whether inflammation mediated the associations of obesity and retinal microvascular parameters. We found that the association of obesity with venular calibre was mediated by inflammation in both age groups. Data from both population-based studies and animal experiments support this interpretation. Yau *et al.* reported that in adolescents, low-grade inflammation partially mediates the relationship between cardiovascular fitness and more favourable retinal venular, but not arteriolar parameters³³. In animal models, increased adiposity results in adipocyte dysfunction with increased pro-inflammatory cytokines and leukocyte activation, which may lead to the destruction of venular endothelium and consequently to wider retinal

of the average retinal vascular calibre were calculated combining measurements of the six largest arterioles or venules⁵⁵. Reproducibility of retinal vascular measurements has been reported, with inter- and intra-grader intraclass correlation coefficients of 0.76 to 0.99⁵⁴.

Inflammatory marker. At the assessment centre, semi-fasting (median 4.2 hours postprandial) peripheral blood was processed within four hours of collection at an on-site processing laboratory, with serum aliquots frozen at -80°C . Serum samples were shipped on dry ice for high-throughput proton NMR spectroscopy (Nightingale Ltd, Vantaa, Finland). Levels of GlycA were calculated using Nightingale 2017 quantification algorithms and were reported in mmol/L⁵⁶.

Covariates. Age, sex and family socioeconomic position (SEP) were considered as potential confounders of the exposure-outcome, exposure-mediator and mediator-outcome associations^{22,57,58}. SEP was measured in LSAC wave 6 (child age 10–11 years), approximately 12 months before the CheckPoint assessment. This SEP variable summarises parent-reported combined household income, current or most recent occupation of each parent, and highest educational qualification of each parent⁵⁹. Each component was scaled and an unweighted average was calculated and standardised within the wave to have a mean of 0 and standard deviation (SD) of 1. A higher score indicates more advantaged socioeconomic position.

Statistical methods. Statistical analyses were performed in R software (version 3.5.2). Data from children and adults were analysed separately, using the records with complete data on all the variables. Scores on all continuous exposure and outcome measures were internally standardised ($[\text{observed value} - \text{mean}] / \text{SD}$).

Firstly, we fitted linear regression models to explore associations of each exposure (BMI and WHtR as continuous variables) with the proposed mediator (GlycA) and with the outcome (retinal vascular calibre), as well as the association of the mediator with the outcome. Linear regression models were adjusted for age, sex and SEP. In addition, when examining associations between GlycA and retinal vascular calibre, models were additionally adjusted for BMI, as it is a potential confounder.

We then investigated the possible mediating role of GlycA using the causal mediation framework, while considering BMI and WHtR continuously and categorically (Fig. 1c), adjusted for age, sex and SEP⁶⁰. Specifically, we estimated the so-called “interventional” direct and indirect effects, which are estimable under relaxed assumptions compared to previous causal mediation approaches^{17,18}. The interventional indirect effect through M is interpreted as the change in the risk of the outcome Y in the exposed if we were to shift the distribution of M to that in the unexposed, and the interventional direct effect is the effect of X on Y that would remain after that shift. We used the “mediation” R package⁶¹, and specified the X, Y, and M in the model as continuous and categorical BMI/WHtR, retinal arteriolar/venular calibre and GlycA (Fig. 1c). This utilises a g-computation approach with linear regression for the outcome and mediator given their antecedent variables as building blocks. The direct and indirect effect estimates indicate the magnitude of change (in SDs) in the outcome variable per SD change in the continuous exposure variable or compared to the categorical reference group (i.e. normal-weight BMI and <0.5 WHtR category).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.W. conceived and led the CheckPoint study with D.P.B., R.S., K.L., J.A.K., T.Y.W. and M.J. as key CheckPoint investigators. K.L. was M.L.'s primary student supervisor, along with D.P.B., M.W., T.Y.W. and M.H. and oversaw all aspects of the study and the manuscript preparation. M.H. and M.L. contributed to the acquisition of data. M.L. conducted the data analysis with the guidance of M.M.B. M.L. drafted the manuscript. M.M.B., T.Y.W., M.J., J.A.K., M.W. and R.S. advised on statistical issues and interpretation. All authors critically reviewed the manuscript and had final approval of the submitted and published version of this paper.

Competing interests

The authors declare no competing interests.

Additional information

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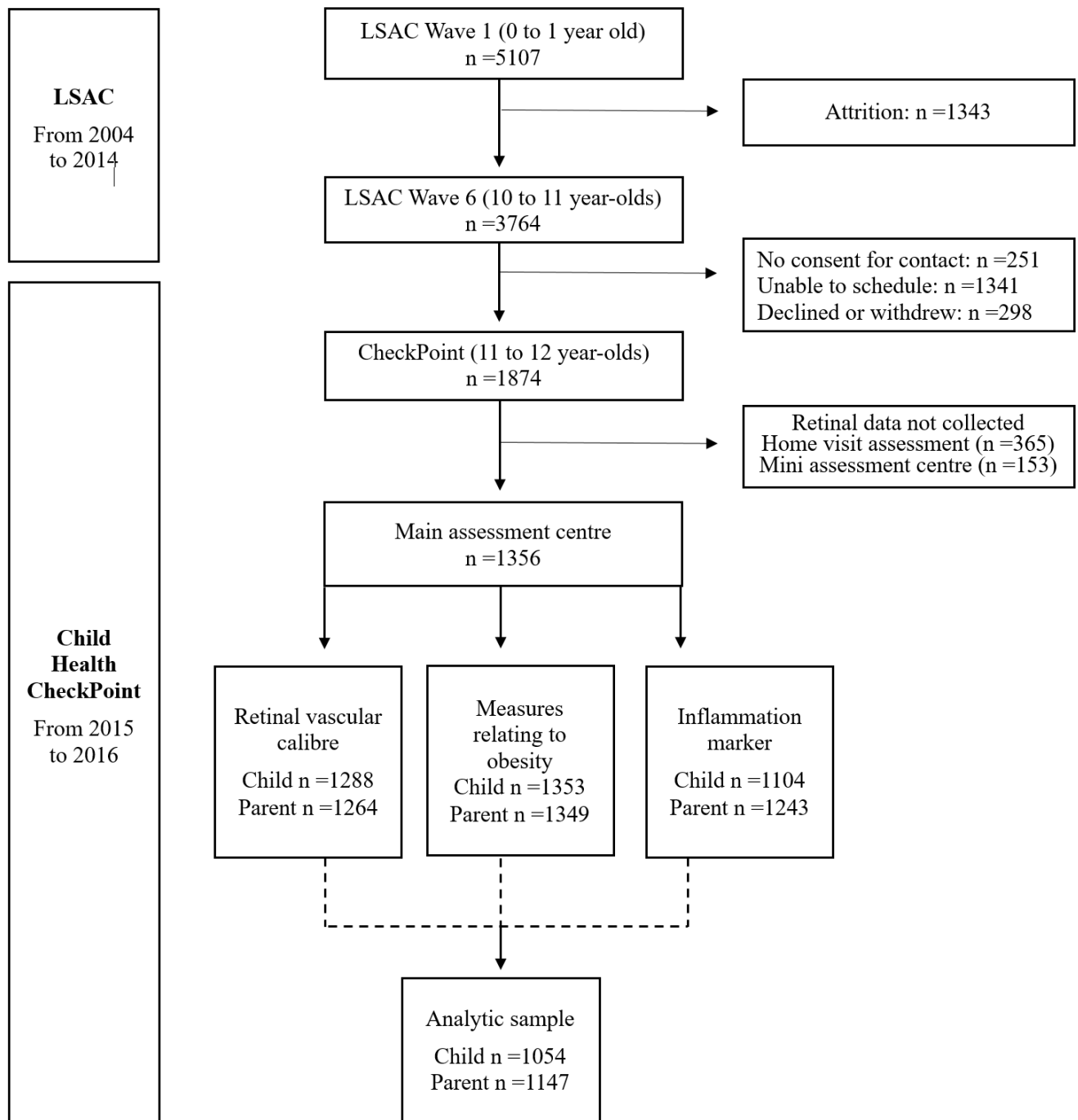
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Supplementary online:

Supplementary figure. Participant flowchart

Supplementary table. Associations of GlycA with retinal vascular calibre with adjustment for age, sex, SEP, BMI and LDL.

Supplementary figure. Participant flowchart



N: number of responses; LSAC, Longitudinal Study of Australian Children

Supplementary table. Associations of GlycA with retinal vascular calibre with adjustment for age, sex, SEP, BMI and LDL.

Outcome measures	Children		Adults	
	Effect size (95% CI)	<i>p</i>	Effect size (95% CI)	<i>p</i>
Retinal arteriolar calibre	0.004 (-0.06, 0.07)	0.29	-0.03 (-0.10, 0.05)	0.49
Retinal venular calibre	0.07 (0.01, 0.14)	0.03	0.03 (-0.04, 0.11)	0.37

Chapter 9. Associations of cardiovascular health and retinal vascular calibre (Aim 4)

9.1 Overview

Chapter 9 addresses Aim 4, which examined whether traditional CVD risk factors and preclinical large arterial phenotypes are associated with retinal geometric parameters in children aged 11–12 years. I found weak evidence of associations between some measures and retinal arteriolar, but not venular, geometry. However, there was no clear overall pattern across all factors considered. The results are briefly discussed and are further elaborated upon in Chapter 10.

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9.2 Published paper (Aim 4 results)

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Short Communication

Cardiovascular health and retinal microvascular geometry in Australian 11–12 year-olds

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ABSTRACT

Traditional retinal microvascular parameters (smaller arteriolar and greater venular caliber) are associated with cardiovascular risk factors, pre-clinical vascular phenotypes and clinical cardiovascular events in adults. Although novel retinal microvascular geometric parameters showed analogous associations in adults, less is known whether these parameters are associated with cardiovascular health from childhood. In a population-based cross-sectional study in children ($n = 1126$, mean age 11.4 years, 50.3% girls), we examined associations of cardiovascular risk factors and pre-clinical arterial phenotypes with retinal geometric parameters. Cardiovascular parameters included body mass index (BMI), an inflammatory marker (GlycA), low-density lipoprotein and high-density lipoprotein (HDL) cholesterol, systolic (SBP) and diastolic blood pressure, large artery functional (pulse wave velocity, PWV and carotid arterial elasticity) and structural (carotid intima-media thickness) phenotypes. Retinal geometric parameters (fractal dimension (D_f) and tortuosity) were quantified from retinal images. Multivariable regression models were performed and adjusted for potential confounders. Higher values for BMI, SBP and PWV showed weak associations with lower (i.e. worse) arteriolar but not venular D_f (standardized mean difference (SMD) ranging from -0.07 to -0.09 , 95% CIs -0.15 to -0.01). Higher HDL was associated with greater arteriolar D_f (SMD 0.07, 95% CI 0.01 to 0.13). Only higher SBP was associated with higher (i.e. worse) arteriolar but not venular tortuosity (SMD 0.09, 95% CI 0.02 to 0.16). In generally healthy children, some risk factors and pre-clinical arterial phenotypes show small associations with retinal geometric parameters. In childhood, emerging relationships between microvascular parameters and cardiometabolic risk may be better described by retinal vascular caliber than by geometric parameters.

1. Introduction

The retinal microcirculation can be imaged directly and non-invasively. In adults, retinal microvascular width (or caliber) has been widely studied (Struijker-Boudier et al., 2012), with meta-analyses showing that narrower arteriolar and wider venular caliber predict

cardiovascular diseases (CVD) including stroke and coronary heart disease (Ikram et al., 2013; McGeechan et al., 2009a; McGeechan et al., 2009b). However, assessment of retinal microvascular caliber overlooks structural variation and geometry across the overall retinal vasculature, which could be important.

The design of the retinal vascular tree follows the optimization

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principle, according to the validated theory of minimum work (Zamir, 1976). Thus, it is hypothesized that when the architecture of the vascular tree is compromised, the efficiency of metabolic transport is reduced, reflecting greater CVD risk and microcirculation damage (Zamir, 1976). Image scoring software such as the Singapore I Vessel Assessment (SIVA) software now allows assessment of retinal vascular geometry in large studies, beyond vessel caliber (Cheung et al., 2011; Liew et al., 2011). In adults, microvascular geometric parameters have been associated with CVD and risk factors, such as higher blood pressure (BP), and cholesterol levels (Campbell et al., 2018; Liew et al., 2011). These results have also been replicated using different scoring software (McGrory et al., 2018; Witt et al., 2006), but had mixed findings in the few children's studies (Pena et al., 2018; Tai et al., 2018), when these retinal parameters may be valuable potential early markers of CVD risk.

Using the national, population-based Child Health CheckPoint study nested within the Longitudinal Study of Australian Children (LSAC), we previously reported that retinal microvascular caliber was associated with body mass index (BMI), a chronic inflammation marker (Glycoprotein acetyls, GlycA), and phenotypes of large arterial function and structure in 11–12 year-olds (Liu, 2019; Liu, 2019). We now investigate whether retinal geometric parameters (considered here to be 'outcomes') show similar associations in the same cohort, and therefore whether they provide additional information about the early development of CVD risk beyond microvascular caliber.

2. Materials and methods

2.1. Study design and participants

In 2004, the LSAC study recruited a nationally representative sample of 5107 Australian infants who have since been followed biennially (retention 73.7% to wave 6 in 2014) (Ann and Johnstone, 2004). The Child Health CheckPoint, a cross-sectional data collection wave nested between LSAC's waves 6 and 7, was a one-off comprehensive biophysical assessment of 11- to 12-year old children and one attending parent. The study design and recruitment are described elsewhere (Clifford et al., 2019a) and the study flow is shown in the Supplementary Figure. Briefly, CheckPoint collected data on multiple health domains across Australia at assessment centers in major and regional cities. Retinal data were unavailable on 518 children participating in smaller cities or home visits as retinal images were not collected due to limitations in equipment mobility. Retinal geometric parameters are yet to be scored on parents.

2.2. Measures

Supplementary Table shows the equipment and procedures for cardiovascular risk (BMI, GlycA, systolic blood pressure (SBP), diastolic blood pressure (DBP), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, triglyceride, carotid intima-media thickness (cIMT), pulse wave velocity (PWV) and carotid arterial elasticity); retinal geometry (retinal fractal dimension (D_f), simple tortuosity; and a priori identified potential confounding variables. Collection procedures for each measure have been described previously (Clifford et al., 2019b; Dascalu et al., 2019; Ellul et al., 2019; Liu et al., 2019c). BMI was converted into age- and gender-specific z-scores using the US CDC growth reference charts (Kuczmarski et al., 2000).

Fig. 1 shows SIVA interface with two novel retinal microvascular geometric parameters. Tortuosity is an index of how much the retinal vessels deviate from a straight line of the same vessel segment; a lower tortuosity index represents straighter (i.e. better) vessels (Cheung et al., 2011). D_f summarizes the complexity of the branching of arterioles/venules (Liew et al., 2011), where larger values represent a more complex (i.e. better) branching pattern.

2.3. Statistical analysis

Multivariable linear regression models were performed with estimates adjusted for age, sex and socio-economic position (SEP). We considered retinal geometric parameters as the dependent variables, although for the large artery measures the reverse would be equally plausible since they were measured cross-sectionally. To visualize our findings, we internally constructed standardized scores ($[\text{observed value} - \text{mean}] / \text{SD}$) for all measures except BMI z-score; thus regression coefficients represent the standardized mean difference (SMD). Analyses were performed in Stata 15.0 (StataCorp LP; College Station, TX, USA).

3. Results

Of the 1874 children participating in CheckPoint, 1126 (mean age 11.4 years, 50.3% girls) had data on all measures (Table 1). The sample mean SEP was 0.26 SD above (i.e. more advantaged) the mean SEP of all families retained at LSAC wave 6 and the mean SEP of all families enrolled at LSAC wave 1 (mean 0, SD 1).

The results of all analyses are presented in Table 2. Data are presented as the association per SD higher of BMI z-score, standardized GlycA, BP, LDL, HDL, triglyceride, cIMT, PWV and arterial elasticity with standardized regression coefficient change in retinal parameters. Higher BMI, SBP and PWV showed weak evidence of an association with lower (i.e. worse) arteriolar D_f (SMD ranging from -0.07 to -0.09 , 95% CIs -0.15 to -0.01), whereas higher HDL was associated with greater arteriolar D_f 0.07 (95% CI 0.01 to 0.13). Only higher SBP was associated with higher (i.e. worse) arteriolar tortuosity (SMD 0.09, 95% CI 0.02 to 0.16). There was no evidence of associations between any exposure measures with venular D_f or tortuosity.

4. Discussion

4.1. Principal findings

In this population-based cross-sectional study of 11–12 year-olds, we found weak evidence of associations between some cardiovascular health measures and retinal arteriolar, but not venular geometry. However, there was no clear generalized pattern across all factors considered. For example, some risk factors (i.e. higher BMI, HDL and SBP) were weakly associated with arteriolar D_f , while only higher SBP was associated with arteriolar tortuosity. Of the clinical arterial phenotypes examined, only PWV showed weak evidence of an association with retinal arteriolar D_f .

4.2. Strengths and limitations

To the best of our knowledge, this is the first study in children to examine the relationship between pre-clinical cardiovascular risk factors and retinal geometric parameters. Our study drew on a large, national sample of Australian children spanning diverse geographical areas. Intermediate phenotypes of small and large vessels were assessed contemporaneously at the right-sided circulation.

Longitudinal studies would perhaps reveal stronger associations and their temporal patterns. Further, our sample is slightly more socio-economically advantaged compared to the original LSAC samples and is predominantly Anglo-European, which may limit the generalizability of findings to other populations.

4.3. Interpretation in light of other studies

D_f and tortuosity are indicators of vasculature complexity. In adults with cardiometabolic conditions, such as hypertension and diabetes, microvascular remodeling results in less structural complexity but more tortuous vessel patterns (Klein et al., 2018; Zhu et al., 2014). There are

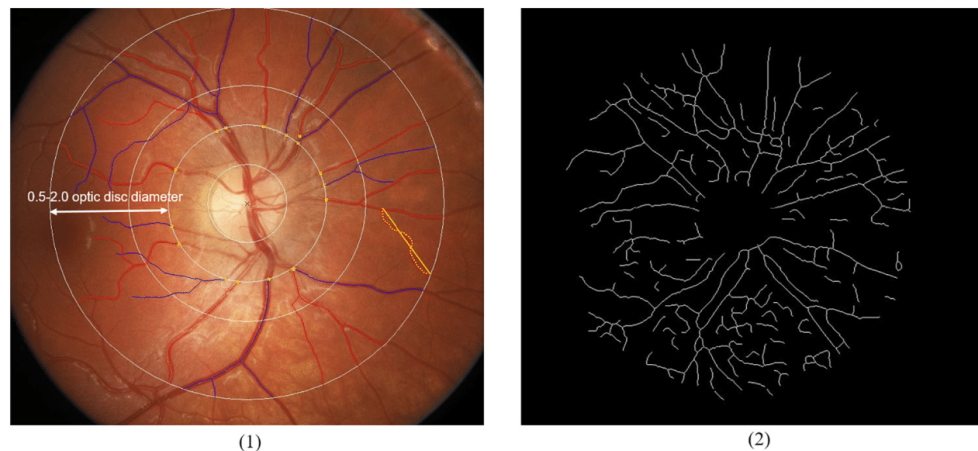


Fig. 1. Retinal geometric parameters measured using Singapore I Vessel Assessment (SIVA). (1) Arterioles are highlighted in red and venules in blue. The measured area of retinal microvascular parameters is demarcated by white circles 0.5 to 2.0 disc diameters away from the disc margin. Simple tortuosity is calculated by the actual length of vessel divided by the Euclidean distance between the first and last points of that vessel; (2) Fractal dimension was calculated from the skeletonized line tracing using the box-counting method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sample characteristics of the analytic sample; all values are mean (SD) except sex (%).

Variable	Value (n = 1126)
Age (years)	11.4 (0.5)
% females	50.3
Family socioeconomic position	0.26 (1.0)
BMI z-score	0.28 (0.97)
Glycoprotein acetyls (mmol/L)	0.99 (0.12)
Low-density lipoprotein cholesterol (mmol/L)	1.36 (0.31)
High-density lipoprotein cholesterol (mmol/L)	1.48 (0.27)
Triglyceride (mmol/L)	1.03 (0.44)
Systolic blood pressure (mmHg)	108.0 (7.8)
Diastolic blood pressure (mmHg)	62.8 (5.4)
cIMT (μ m)	575.4 (41.0)
Arterial elasticity (%/10 mmHg)	4.8 (0.8)
Pulse wave velocity (m/s)	4.5 (0.5)
Fractal dimension	
Arteriole fractal dimension	1.24 (0.05)
Venule fractal dimension	1.21 (0.05)
Tortuosity	
Simple tortuosity arteriole	1.12 (0.03)
Simple tortuosity venule	1.10 (0.02)

few studies in children and results are not consistent. One study of 166 Malaysian children (mean age 9.6 years) found that venular D_f and tortuosity were slightly higher in children with obesity (mean difference: D_f 0.015 (95% CI 0.001, 0.030), tortuosity 0.006 (95% CI 0.001, 0.012)) (Tai et al., 2018). In contrast, our study revealed evidence of an association with only with arteriolar D_f , with per z-score higher BMI associated with 0.08SD (0.10 unit) lower arteriolar D_f .

A smaller study of 88 children with type 1 diabetes (mean age 13.6 years) reported that greater cIMT was associated with greater arteriolar but not venular tortuosity (Pena et al., 2018). This was not evident in our sample. If large arterial dysfunction precedes structural changes (Ballethofer et al., 2003), it is plausible that the association of arterial phenotypes with retinal geometry emerge from mid-childhood and may become more evident later in life and in those with cardio-metabolic disease conditions (Liu et al., 2019d).

4.4. Implications

Several studies have demonstrated that retinal microvascular parameters correlate with changes in the microcirculation in the kidney, brain, and heart, highlighting the clinical value of evaluating

Table 2
Differences in retinal outcomes per SD higher of cardiovascular risk factors and pre-clinical arterial phenotypes; regression estimates adjusted for age, sex and SEP.

Exposures	Fractal dimension				Simple tortuosity			
	Arteriole		Venule		Arteriole		Venule	
	SMD (95%CI)	p	SMD (95%CI)	p	SMD (95%CI)	p	SMD (95%CI)	p
BMI z-score	-0.08 (-0.13, -0.02)	0.01	-0.01 (-0.06, 0.05)	0.83	0.00 (-0.06, 0.07)	0.92	-0.02 (-0.09, 0.05)	0.57
GlycA	-0.06 (-0.13, 0.00)	0.05	0.02 (-0.04, 0.09)	0.52	0.04 (-0.03, 0.12)	0.22	0.05 (-0.02, 0.12)	0.17
LDL cholesterol	0.00 (-0.06, 0.06)	1.00	-0.02 (-0.09, 0.04)	0.43	-0.00 (-0.06, 0.06)	0.99	0.01 (-0.06, 0.07)	0.83
HDL cholesterol	0.07 (0.01, 0.13)	0.02	0.01 (-0.05, 0.07)	0.68	-0.05 (-0.11, 0.02)	0.14	-0.01 (-0.08, 0.05)	0.74
Triglyceride	-0.05 (-0.11, 0.02)	0.15	-0.03 (-0.09, 0.04)	0.43	0.06 (-0.00, 0.13)	0.06	0.06 (-0.00, 0.13)	0.07
Systolic BP	-0.07 (-0.13, -0.01)	0.03	-0.00 (-0.06, 0.06)	0.92	0.09 (0.02, 0.16)	0.01	-0.01 (-0.07, 0.06)	0.86
Diastolic BP	-0.02 (-0.08, 0.04)	0.52	-0.00 (-0.07, 0.06)	0.93	0.07 (-0.00, 0.14)	0.06	0.01 (-0.06, 0.09)	0.68
cIMT	-0.02 (-0.08, 0.04)	0.44	-0.05 (-0.11, 0.01)	0.12	-0.01 (-0.07, 0.06)	0.80	-0.03 (-0.09, 0.04)	0.46
Arterial elasticity	0.05 (-0.01, 0.11)	0.11	0.01 (-0.05, 0.08)	0.64	-0.03 (-0.10, 0.04)	0.35	0.01 (-0.06, 0.08)	0.71
PWV	-0.09 (-0.15, -0.03)	0.00	-0.01 (-0.07, 0.04)	0.63	0.05 (-0.01, 0.12)	0.10	0.02 (-0.05, 0.09)	0.58

Abbreviations: SMD, standardized mean difference; SEP, socioeconomic position; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure; cIMT, carotid intima-media thickness; PWV, pulse wave velocity. Results with p-value less than 0.05 were in bold.

the retinal microcirculation (Grassi and Mancia, 2018). Advanced deep-learning algorithms from retinal images can predict a range of cardiovascular risk factors, including age, sex, SBP and BMI (Poplin et al., 2018). Although some large population-based studies report associations between retinal geometric parameters and CVD in late adulthood (Liew et al., 2011), these parameters have only recently been described and there are fewer and less consistent data compared to retinal microvascular caliber, particularly in children. Likewise, we found a smaller size of associations with these geometric parameters than we have previously reported for retinal microvascular caliber in the same cohort (Liu, 2019; Liu, 2019). This might reflect lower reliability in geometric parameters than calibers (Huang et al., 2018). Longitudinal studies with repeated measurements of both cardiovascular risk factors and retinal parameters across different life stages could clarify the relationships and inform the usefulness of the geometric measures of retinal vasculature.

5. Conclusions

In a largely healthy sample of Australian children, CVD risk factors were only weakly associated with retinal arteriolar geometric parameters. In childhood, emerging relationships between microvascular parameters and cardiometabolic risk may be better described by retinal vessel caliber than by retinal vessel geometry.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mvr.2019.103966>.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Table. Details of measures

Construct, equipment	Brief protocol
<i>Exposures</i>	
Anthropometry: body mass index (BMI) Portable rigid stadiometer (Invicta, IP0955, Leicester, UK); 4-limb segmental Bioimpedance Analysis (InBody230, Biospace, Seoul, Korea);	Height (cm): measured in bare feet. Mean of two measurements used; where the two differed by more than 0.5 cm a third measurement was taken and the average of the three measures was used. Weight (kg): to the nearest 50g with participants in light clothing and without shoes or socks. BMI was calculated as weight (kg)/height (m ²).
Inflammation and cholesterol markers: GlycA, low-density lipoprotein (LDL), high-density lipoprotein (HDL) cholesterol and triglyceride. NMR spectroscopy (Nightingale Ltd, Vantaa, Finland)	Semi-fasting (median 4.2 hours postprandial) peripheral blood was processed within four hours of collection at an on-site processing laboratory. Serum samples were shipped on dry ice to Finland for metabolomic analyses. GlycA, LDL, HDL cholesterol and triglyceride were directly derived from high-throughput proton NMR spectroscopy. Levels of these markers were calculated using Nightingale 2017 quantification algorithms and were reported in mmol/L.
Blood pressure and pre-clinical arterial function: pulse wave velocity (PWV), carotid arterial elasticity. SphygmoCor XCEL (AtCor Medical, Australia)	Following 7 min in supine position at rest, systolic and diastolic blood pressures were measured at the brachial artery up to three times, with mean values reported. Assessors measured with participants supine the wave of pulse from carotid to the suprasternal notch to the right femoral. PWV was calculated as the ratio of the distance travelled by the pulse wave and the time delay between the waveforms (m/s). Carotid arterial elasticity (%/10mmHg) was calculated using intima-intima lumen diameter from carotid artery images divided by the different value of systolic and diastolic blood pressure. ²³⁷
Pre-clinical arterial structure: carotid intima-media thickness (cIMT). B-mode ultrasound (Vivid-I, GE Healthcare, Chicago, Illinois, US); Medical Imaging Applications (Coralville, Iowa, US)	Carotid artery images were obtained via B-mode ultrasound machine. The right carotid artery was measured as participants lay supine with their head turned 45° to the left to expose the right side of neck. cIMT was analysed using Carotid Analyzer. cIMT (μm) is defined as the distance from the lumen-intima interface to the media-adventitia interface.
<i>Outcomes</i>	

Construct, equipment	Brief protocol
Retinal parameters: fractal dimension (D_f) and simple tortuosity Fundus camera (EOS 60D SLR); Singapore I Vessel Assessment (SIVA, National University of Singapore, Singapore)	Two optic disc-centred digital photographs from each eye were taken by a fundus camera in a darkened room. The right eye was selected as the first choice of scoring. Three graders, masked to participant identity, performed the retinal vessel analysis using a validated semi-automated software, SIVA.⁸¹ The software can automatically detect and trace the optic disc, set the grading grid on the fundus photograph within the region between 0.5 and 2.0 disc diameters away from the disc margin, and automatically trace vessel path. Graders needed to check the accurate classification of vessels as either arterioles or venules, with manual vessel tracking required to ensure complete each tracing. The inter- and intra- grader reliability were assessed by three graders analysing a subset of 30 and 46 randomly chosen images respectively. Two-way mixed-effects intraclass correlation coefficients for retinal D_f and tortuosity were 0.54-0.76 and 0.75-0.93. Retinal D_f was calculated using the ‘box-counting method’, which divided each photograph into a series of squares of various side lengths. D_f was defined as the gradient of logarithms of the number of boxes and the size of those boxes. Tortuosity is calculated by the actual length of vessel divided by the Euclidean distance between the first and last points of that vessel (i.e., the length of the straight line connecting the two points).
<i>Covariates</i>	
Age	Age was calculated to nearest week using date of birth, either imported from Medicare Australia’s database at the time of LSAC enrolment (child) and date of assessment.
Sex	Sex was self-reported in the questionnaire by children.
Family socioeconomic position (SEP, LSAC wave 6)	This SEP variable summarized parent-reported combined household income, current or most recent occupation of each parent and highest achieved educational qualification of each parent. Each component of the score was scaled and an unweighted average was calculated over three values in a single-parent household, or over five values in a dual-parent household. The unweighted average variable at LSAC was then standardized within the wave to have a mean 0, and standard deviation (SD) of 1.

Chapter 10. Discussion

10.1 Overview

This chapter critically discusses the results from preceding chapters 6 to 9. It outlines the principal findings, their strengths and limitations, and interprets the findings in light of previous literature. Finally, it outlines the implications for future research.

10.2 Principal findings

10.2.1 Aim 1: Retinal vascular calibre showed small variations in union with large arterial phenotypes in children and adults

In our cross-sectional study, adverse retinal vascular calibre profile was modestly associated with poorer large artery functional phenotypes in both 11–12 year-olds and mid-life adults. From early life, associations were observed for PWV and carotid arterial elasticity after adjusting for CVD risk factors. These associations strengthened with age. In mid-life adults, there was also evidence of a small association between adverse retinal vascular calibre and large artery structural phenotypes (e.g., greater CIMT). Overall, the effect sizes were small for both children and adults.

10.2.2 Aim 2: Decade-long obesity-related measures predicted retinal vascular calibre in children and adults

In our longitudinal study, higher BMI and WHtR from age 4–5 years modestly predicted adverse retinal arteriolar calibre at age 11–12 years. We observed similar effects in time point analyses for the association between BMI and arteriolar calibre in mid-life adults but with higher explanatory power (e.g., R^2). There was less convincing evidence in time point analyses that BMI was associated with venular calibre in either children or adults. However, there was some evidence that WHtR from 8–9 years was associated with venular calibre at age 11–12 years. A gradient of suboptimal decade-long higher BMI trajectories in both age groups and higher WHtR trajectories in children predicted poorer retinal arteriolar calibre (p for trend <0.01), but not venular calibre. We saw the same patterns for BMI trajectories in adults with larger effects. The exception was that the least favourable WHtR trajectories had small associations with adverse venular calibre in children.

10.2.3 Aim 3: Inflammation mediated the relationship between obesity and retinal vascular calibre in children and adults

To our knowledge, this is the first study to investigate whether inflammation mediated the associations between obesity and retinal vascular calibre. In our cross-sectional study, we showed that concurrent BMI and WHtR were associated with both a chronic inflammation marker GlycA and adverse retinal vascular calibre in both children and mid-life adults. We also showed that higher GlycA was associated with both wider venular calibre (in mid-childhood and mid-life adults) and narrower arteriolar calibre (in adults). Inflammation mediated associations between obesity and retinal venular calibre, but not arteriolar calibre. Furthermore, in adults, the mediation effects were higher than in children.

10.2.4 Aim 4: CVD risk factors and large arterial phenotypes showed some weak associations with retinal geometric parameters in children

In our cross-sectional study, CVD risk factors and large arterial phenotypes showed little evidence of associations with venular geometry, but some weak evidence of associations with arteriolar geometry. For example, higher BMI, lower HDL and higher systolic blood pressure showed small associations with lower arteriolar fractal dimension. Yet, only higher systolic blood pressure showed a small association with higher arteriolar tortuosity. Of the preclinical large arterial phenotypes examined, only PWV showed weak evidence of an association with retinal arteriolar geometry.

10.3 Strengths

The study has several strengths that are outlined below.

10.3.1 Sample size and generalisability of the cohort

LSAC is Australia's largest and only ongoing national birth cohort study. The LSAC sample was designed to be generalisable to all Australian children. The cohort was sampled to be representative of all Australian births in 2004, through a 2-stage random sampling design with the postcode as the primary sampling unit. In addition, LSAC's large sample size (5,107 families in wave 1) includes families from a range of socioeconomic backgrounds and geographical areas, which enhance

generalisability. It has rich repeated measures collected biennially, high retention rates (~75% after 12 years) and is fully-funded to continue data collection until adulthood. The Child Health CheckPoint sample (n= 1,874 parent-child dyads) represented over 50% of contactable LSAC families from wave 6. This large cohort with biomarkers and physical measurements provides statistical precision in effect estimation.

10.3.2 Validated and longitudinal repeated measures

All CheckPoint assessments were objectively measured and processed under standard operating procedures, with moderate to high inter- and intra-rater reliability.²⁴⁰ The retinal microvascular parameters were quantified using previously validated computer-based techniques with standardised protocols. Most of the parameters had high reliability. In addition, preclinical large arterial phenotypes, including those related to structure and function, were assessed by trained researchers using standardised protocols. The age of children (11–12 years of age) allowed for good compliance with measurements, such as PWV and carotid ultrasound.²⁴¹ This allowed us to uniquely examine relationships of retinal microvascular parameters with large arterial function and structure simultaneously. The well-phenotyped sample also allowed us to consider the effects of important covariates.

We used a novel inflammation biomarker, GlycA, which has been shown to reflect diverse inflammatory pathways.²²¹ GlycA appears to provide a more stable signal of chronic inflammation than a single acute-phase marker, such as CRP.²²² Thus, it may be more accurate at capturing chronic inflammation in generally healthy populations, especially in children.¹⁹⁶

Height, weight and waist circumference were measured on average five times across the preceding decade (e.g., from child 2–3 years), allowing for the separation of individuals into distinct BMI and WHtR trajectories. The average posterior probability value for each trajectory was 0.82 to 0.97 for each group. This is well above the recommended value of 0.70,²⁴² indicating good assignment accuracy of the identified trajectories.

10.3.3 Contemporaneous child and adult analysis

All biomarkers and physical assessments in CheckPoint were conducted at the same time, using the same equipment and the same protocols, ensuring a high degree of measurement comparability. This approach avoids potential bias and unknown confounding in typical cross-generational studies, such as measurement bias. Many of the associations, such as the shared variation between retinal vascular calibre and preclinical large arterial phenotypes, have been described in adult studies, but are less studied in children.¹¹⁷ Our adult findings validate the existing literature, while the child findings fill the gap in our understanding about when these associations emerge. In addition, the cross-generational design enables us to efficiently infer temporal strengthening of associations with age, which would otherwise take decades to observe.

10.4 Limitations

We also describe some important limitations of the study below.

10.4.1 Observational study design

We draw on observational data where biomarkers and physical assessments were largely collected cross-sectionally. We lack repeated measures of retinal microvascular parameters, preclinical large arterial phenotypes and biomarkers, which limits our ability to draw conclusions regarding directionality or causality. For example, in the Aim 1 analysis, retinal vascular calibre was conceptualised as the exposure for preclinical large arterial phenotypes. However, the reverse could be equally plausible since the order of causality is not clearly established. In other words, it is not possible to definitively conclude whether retinal vascular calibre precedes or follows changes in large arterial phenotypes. However, the directionality of our analyses was hypothesis-driven. Furthermore, before exploring causal relationships, it is warranted to examine cross-sectional associations as a first step and a foundation for future longitudinal studies. The replication of our findings in other studies would strengthen their reliability.

10.4.2 Sample representation of certain groups

10.4.2.1 Adult sample generalisability

The original LSAC sample was designed to represent Australian children born in 2004. Adults were not the original unit of selection, and only parents with a new baby were eligible. Thus, the adult sample is not entirely representative of all Australian mid-life adults. Still, our sample covers a broad social and geographic range across Australia, making it possible to generalise our findings.

Another important limitation is that our sample is under-representative of fathers. It is a well-documented feature of longitudinal cohort studies that mothers are more likely to participate in research studies with their children. Especially in our study, participants attended assessment centres during working hours, and only one parent/caregiver was asked to attend. Our sex imbalance (88% mothers, 12% fathers) may have led to an underestimate in the strength of associations. Men generally have a higher risk of CVD than women.²⁴³ This means we may have seen more variation in cardiovascular phenotypes if our sample comprised more fathers. Nonetheless, when we conducted sensitivity analyses to look at sex effects, we observed similar patterns of associations in mothers and fathers, suggesting that in these instances, the sex imbalance had little influence on associations.

10.4.2.2 Sample attrition

Due to sample attrition, only 75% of LSAC families had wave 6 home visits in 2014. Of these retained families, 53% participated in the CheckPoint assessment in 2015. The main sociodemographic characteristics were analogous between CheckPoint responders and non-responders, except for SEP. This may limit generalisability to families from more disadvantaged socioeconomic backgrounds. We were able to partly offset the influence of social disadvantage by adjusting for SEP in all analyses.

10.4.2.3 Incomplete retinal data

Despite retinal images were collected on only 70% of CheckPoint participants, the sample characteristics of those with or without retinal data were similar (previously described in Table 5-8 in Section 5.6.1.2). Thus, although there is a potential for

selection bias, associations we examined are unlikely to be influenced by the missing retinal data.

10.4.3 Methodological considerations

10.4.3.1 Complete case analysis

I performed all analyses based on participants with available data without handling missing data. Before conducting analyses, we had discussed tackling missing data using methods such as multiple imputations. However, we decided to have complete case analyses throughout all thesis aims. The major reason for this decision is that we considered the CheckPoint sample as sufficiently representative of the wider Australian sample. In addition, methods of dealing with missing data may introduce extra bias. For example, many multiple imputation techniques are based on the assumption that data are missing at random. This assumption could be flawed in our study. For example, children who missed blood collection may have done so due to obesity (e.g., difficulty drawing venous blood). It is possible that in those who failed to have blood collected, GlycA (our inflammatory marker) may be associated with retinal vascular calibre, but in those with blood samples, this association did not exist.

10.4.3.2 Suboptimal inter- and intra-rater reliability

One of the retinal geometric parameters fractal dimension and the large artery structural phenotype CIMT both had moderate reliability. Details are reported in Section 5.4. Briefly, the inter- and intra-grader reliability for retinal arteriolar and venular fractal dimension ranged from 0.58 to 0.67 (95% CIs 0.38 to 0.82). For CIMT, the within-rater intraclass correlation was 0.62 (95% CI 0.54 to 0.71), while the between-rater intraclass correlation was 0.59 (95% CI 0.49 to 0.68). Our fractal dimension measure had lower reliability than other published studies that have used SIVA in adults. This is likely due to the young age of our cohort. However, no other studies in children have provided reliability data on SIVA measures. The fractal dimension is calculated based on the vessel trace. Physically, children's retinal fundus has prominent opalescent sheen in the nerve fibre, which makes the software difficult to delineate each vessel trace precisely. In comparison, adult's fundus has clear retinal nerve fibre layer making it easier to trace. This is likely why children's

reliability for fractal dimension is lower than the published adult reliability. These moderate reliability statistics may inflate measurement errors, which may mask an underlying signal of associations between factors. Thus, the weak association found for CIMT in adults in Aim 1 and fractal dimension for Aim 4 warrant replication in other studies.

10.4.3.3 Potential residual confounding

Physical activity and dietary intake both show complex relationships with cardiometabolic health. In this thesis, we chose not to treat them as potential confounders in our analyses for several reasons: 1) Our previous work in this cohort has shown that an inflammatory diet is not related to retinal vascular calibre in children or adults;²⁴⁴ 2) similarly, the work by our team has shown that frequent takeaway food consumption or sugar-sweetened beverages had little impact on retinal vascular calibre in children or adults.²⁴⁵ Also, we found that children who followed the ‘never healthy’ or ‘always healthy’ diet trajectory over the past decade showed similar retinal vascular calibre profile with those who follow the ‘always healthy’ diet;¹⁴⁵ 3) in cross-lagged wave-on-wave analyses, dietary scores/patterns did not consistently predict weight-to-height ratio and BMI z-score score or vice versa in subsequent waves;²⁴⁶ 4) our physical activity measure had low response rate, with only around half of participants having valid physical activity data (1015 and 1070 of 1874 children and adults respectively). Based on these reasons, we did not consider diet or physical activity as covariates in our models. However, we note that this is a limitation of the study and something that should be addressed in future studies.

In Aim 3, I conducted a mediation analysis to explore whether GlycA mediates obesity and retinal vascular calibre. In the analysis, I only considered GlycA as the mediator. Given the current lack of statistical packages for examining interventional effects with multiple mediators,²⁴⁷ we assumed that there was no post-exposure confounding of mediator-outcome relationships that could arise through other potential mediating pathways and biomarkers in the current study. This assumption could be flawed. For example, LDL levels may be increased in those with obesity

and may contribute to adverse changes of retinal vascular calibre directly,²⁴⁸ or via the contribution to inflammation indirectly (Figure 10-1).²⁴⁹

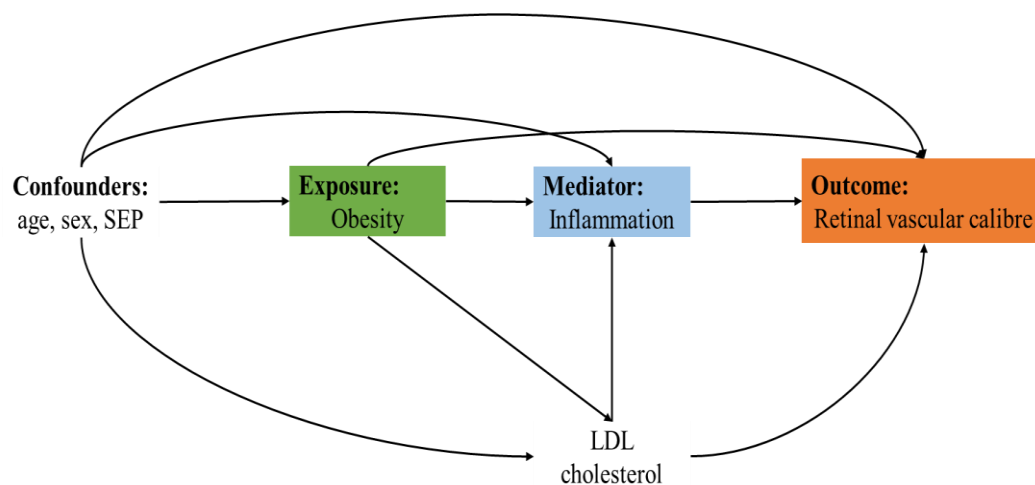


Figure 10-1 *LDL cholesterol as a potential exposure introduced mediator-outcome confounder*

However, our post hoc analysis suggests that LDL may only influence the association of venular calibre in adults, not children. Current research within Clinical Epidemiology & Biostatistics (CEBU) at Murdoch Children’s Research Institute is working on developing new software to account for multiple mediators in one model. This may allow more comprehensive mediation analysis modelling in the future but is beyond the scope of the current thesis.

10.4.4 Lack of other inflammatory biomarkers

Adding biomarkers to large population-based datasets is costly, but choices must be made. Unlike most other studies of inflammation, we examined GlycA rather than single acute-phase inflammatory markers (e.g., CRP). This reflects the CheckPoint Investigators’ decision to use the funding in the first instance to examine GlycA, as it is thought to be a better and more stable marker of chronic inflammation than the single acute-phase protein.¹⁹⁵ The latter is unlikely to be a good marker of chronic inflammation because of its rapid kinetics in response to stimuli such as acute infection.²²¹ Despite this chronic rather than acute inflammation focus, previous studies have shown that elevated GlycA levels correlate moderately with levels of interleukin-6, tumour necrosis factor- α , fibrinogen, and CRP (correlation coefficient

around 0.5 in adults).^{195, 221} Additionally, the magnitude of associations we found between GlycA and retinal vascular calibre is similar to previous findings.

10.5 Interpretation in light of previous studies

This section critically discusses our findings in relation to previous studies.

10.5.1 Aim 1: Retinal vascular calibre showed small variations in union with large arterial phenotypes

To date, only one study has examined the associations of retinal vascular calibre and preclinical large arterial phenotypes in generally healthy children.⁸⁶ The Generation R Study (Rotterdam, The Netherlands) found that in children (median age six years), each SD increase in retinal arteriolar and venular calibre was associated with 0.02 SD (95% CI -0.01 to 0.05) and 0.04 SD (95% CI 0.01 to 0.07) higher carotid-femoral PWV (SD 0.9 cm/s).⁸⁶ In our study of children aged 11–12 years, we found a stronger association of retinal vascular calibre with PWV and replicated this finding with another large artery functional phenotype, carotid arterial elasticity. In turn, we now show that the magnitude of associations between retinal vascular calibre and large artery functional phenotypes roughly doubled between mid-childhood and mid-adulthood. The explanatory power (e.g., model R^2 values) was small at age 11–12 years and much stronger in adults (e.g., 6.1-12.8% and 26.7-29.5% for children and adults, respectively). These results are consistent with our meta-analysis of slightly older adults (mean age 54 years), which showed narrower retinal arteriolar calibre was associated with faster PWV ($r = -0.17$, 95% CI -0.25 to -0.10).¹¹⁷ Thus, the associations of retinal vascular calibre and large artery functional phenotypes may emerge from early childhood and increase with age. The current evidence is summarised below in Table 10-1.

We found little evidence that retinal arteriolar calibre was associated with CIMT in children, but observed a small association in adults. In children of similar age (mean age 13.6 years) with type 1 diabetes ($n = 90$), Pena and colleagues showed that retinal vascular calibre was associated with CIMT.²⁰⁸ For example, each 0.1 mm higher CIMT was associated with 7.9 μm (95% CI 4.50 to 11.30, $p < 0.0001$) wider arteriolar calibre and 9.61 μm (95% CI 4.16 to 15.06, $p < 0.01$) wider venular calibre.²⁰⁸ In our meta-analysis of older adults (mean age 63 years), narrower retinal

arteriolar calibre was weakly associated with higher CIMT ($r = -0.05$, 95% CI -0.09 to -0.02), and effect sizes were greater in patients with cardiometabolic disease.¹¹⁷ Taken together, both age and cardiometabolic disease conditions may contribute to the degree of co-variation between small and large artery structure.

Table 10-1 Evidence summary of the relationship between retinal vascular calibre and preclinical large arterial phenotypes

Studies	Population	Age	sample size	PWV	Arterial elasticity	CIMT
The only child study (Gen R)	General population	Children age 6	4007	+		
From CheckPoint	General population	Children age 11-12	Function: 1232 Structure: 1262	++	+	
		Adults	Function: 1125 Structure: 1226	+++	++	+
From meta-analysis	General population	Adults	Function: 229 Structure: 9194	+++		+
	Cardiometabolic patients	Adults	Function: 637 Structure: 173	++++		++

Higher CIMT represents adverse changes in arterial structure, which has been shown to associate with CVD risk factors such as obesity and hypertension in children and CVD events in adults.^{33, 250} The association between wider (worse) venular calibre and lower (better) CIMT observed in children may be a chance finding, given its small magnitude, unexpected direction and full attenuation on adjustment. Also, clinical implications of CIMT at 11–12 year-olds are still being established, and there may be considerable vascular remodelling in early puberty. Therefore, it is plausible that a marginally greater CIMT may be physiological or adaptive rather than pathophysiological changes.

10.5.2 Aim 2: Decade-long obesity-related measures predicted retinal vascular calibre

We showed that BMI as early as 4–5 years of age adversely affected retinal microvascular variation at 11–12 years of age. This finding is consistent with the literature suggesting that the association between BMI and adverse changes in retinal vascular calibre may commence early in life. The youngest population-based

sample in which this relationship has been previously examined was in children aged 55.5 months (SD 10.3) taking part in the cross-sectional Sydney Pediatric Eye Disease Study.²⁵¹ In this modest community sample (n= 379), each unit (kg/m²) higher BMI was cross-sectionally associated with 1.06 μ m narrower arteriolar calibre (p= 0.01) and 1.12 μ m wider venular calibre (p= 0.02).²⁵¹ Together with our findings, early BMI from 4–5 years maybe not only cross-sectionally associated with but also predictive of future retinal vascular calibre. Our findings are also consistent with the Singapore Cohort Study of Risk Factors for Myopia, which included children of the same age. In Singaporean children (n= 421), the magnitude of the association between BMI and arteriolar calibre was similar to our findings. However, contrary to our findings, they did see small associations between BMI and venular calibre.¹⁶³

In addition, by using two generations of participants with identical outcome measures, we can speculate that the increasing adiposity may have cumulative effects on retinal microvascular parameters from childhood to mid-adulthood. Although the effects were similar in children and adults, the explanatory power (e.g., R²) was higher in adults than in children. Furthermore, we found that suboptimal decade-long BMI trajectories in both generations and WHtR trajectories in children were associated with adverse retinal arteriolar calibre. This supports the hypothesis that over time there are cumulative effects of high BMI/WHtR on retinal arteriolar calibre. The only other study that has examined the effect of BMI trajectories on retinal vascular calibre was from our research team.¹⁶⁶ In a small cohort (n= 187), we did not observe any association of children's BMI trajectories (10 time points from 2 weeks to 14 years of age) and retinal vascular calibre.¹⁶⁶ However, the small sample size and the fact that 90% of children were of normal-weight BMI when retinal images were taken in adolescents may limit the power to detect small associations.¹⁶⁶

Retinal arteriolar and venular calibre had different patterns of association with BMI and WHtR. The association of BMI with narrower arteriolar calibre in children and adults is in line with previous studies and a recent meta-analysis of 15,710 participants.^{150, 165} We found little evidence of associations with retinal venular calibre for adults but did see some weak evidence in children. For instance, we

found that WHtR, an index of central fat distribution, was related to venular calibre in children from 8–9 years, and the least favourable WHtR trajectories had small associations with adverse venular calibre in children. These observations indicate venular associations may appear later and be more closely related to central adiposity. Unfortunately, WHtR was not available for adults to speculate effects from childhood to mid-adulthood.

10.5.3 Aim 3: Inflammation mediated the relationship between obesity and retinal vascular calibre

Our study supports previous findings that inflammation is more consistently associated with retinal venular than arteriolar calibre in children and adults. The Generation R Study of six-year-olds found that each SD higher CRP was associated with 0.10 SD (95% CI 0.06 to 0.14) wider venular calibre.¹⁵⁵ Similar findings were reported in ageing adults in the Netherlands (mean age 68 years), where each SD increase of CRP was associated with 1.61 μm (95% CI 1.00 to 2.22) wider venular calibre (approximately 0.07SD). At the same time, there was little evidence of association with arteriolar calibre.¹⁸⁴ From our unpublished meta-analysis (details in Chapter 2, Section 2.4.5.1), the correlation coefficient between CRP and retinal venular calibre was 0.09 (95% CI 0.05 to 0.12) in the general population and 0.10 (95% CI -0.05 to 0.25) in patients with disease conditions. Analogous findings were also reported in mid-life and older adults (mean age 49–68 years) and with other inflammatory markers, such as fibrinogen and white blood cells count.^{93, 157, 184, 188, 190} Of these inflammatory biomarkers, only higher levels of white blood cell count were associated with narrower arteriolar calibre.^{186, 189, 192} This could be due to the unnecessary dual adjustment of retinal venular calibre in the models.¹⁵⁷ Alternatively, it could reflect that white blood cells are also influenced by additional, non-inflammatory pathways.

In population studies, GlycA is a robust predictor of all-cause of mortality and CVD, even after adjusting for CRP.²²³⁻²²⁵ In adolescents, GlycA has been shown to be associated with obesity and prediabetes.²⁵² To the best of our knowledge, this is the first study to demonstrate that GlycA is associated with retinal venular calibre in

both children and adults, and the magnitude of association is similar to previous findings using other inflammatory markers.

We extend prior studies by exploring whether inflammation (as measured by GlycA) mediates the associations of obesity and retinal vascular calibre. We found that the relationship between obesity and venular calibre was mediated by inflammation in both age groups. Data from both population-based studies and animal experiments support this finding. For example, in a sample of 157 American adolescents, Yau and colleagues reported that low-grade inflammation partially mediated the relationship between cardiovascular fitness and more favourable retinal venular, but not arteriolar parameters.¹⁹³ In animal models, increased adiposity results in adipocyte dysfunction because of increased pro-inflammatory cytokines and leukocyte activation. This may lead to the destruction of venular endothelium and consequently lead to wider retinal venular calibre.²⁵³

In our study, the relationship between obesity and arteriolar calibre is unlikely to be mediated via the inflammatory pathway but could be mediated by other non-inflammatory pathways. For example, obesity is a well-recognised risk factor for elevated blood pressure, a key determinant of retinal arteriolar calibre.²⁵⁴ Structural adaptations of the endothelium and vessel wall in chronic hypertension may contribute to narrowing of the retinal arteriolar lumen.¹⁹² Future studies could address the possible mediating role of blood pressure in this relationship.

10.5.4 Aim 4: CVD risk factors and large arterial phenotypes showed some weak associations with retinal geometric parameters

Fractal dimension and tortuosity are indicators of microvasculature architecture. In adults with cardiometabolic conditions, such as hypertension and diabetes, microvascular remodelling results in less structural complexity but more tortuous vessel patterns.^{22,23} These microvasculature architecture changes may further lead to CVD events, such as stroke.¹⁰³

As introduced in Chapter 2, Section 2.5.1, higher blood pressure and lower HDL cholesterol levels are consistently associated with worse fractal dimension and tortuosity in adults. However, few studies have examined CVD risk factors and retinal geometric parameters in children, and results are inconsistent. One study of

166 Malaysian children (mean age 9.6 years) found that venular fractal dimension and tortuosity were slightly higher in children with obesity compared to children with normal weight (mean difference: fractal dimension 0.015, 95% CI 0.001 to 0.030 and tortuosity 0.006, 95% CI 0.001 to 0.012).¹¹ Our study only revealed evidence of a small association between BMI and arteriolar fractal dimension. On the contrary, the Sydney Childhood Eye Study (n= 2,353, median age 12.7 years) did not observe any associations between higher BMI and fractal dimension (arteriole and venule).²⁰³

In terms of other CVD risk factors, The Child Heart And Health Study in England (CHAHS, n= 986, aged 10–11 years) found that retinal arteriolar tortuosity was positively associated with levels of triglycerides, total and LDL cholesterol, and diastolic blood pressure.²⁰⁴ Yet, in our study, we only observed an association of higher systolic blood pressure with higher arteriolar tortuosity.

Few studies have examined large arterial phenotypes in relation to retinal geometric parameters. One small study (n= 88) of children with type 1 diabetes (mean age 13.6 years) reported that greater CIMT was associated with greater arteriolar but not venular tortuosity.¹² This was not evident in our sample. If large arterial dysfunction precedes structural changes,²⁴ it is plausible that the association of arterial phenotypes with retinal geometry emerge from mid-childhood and may become more evident later in life and when cardiometabolic disease emerges.²⁵

10.6 Implications

10.6.1 Progressive associations across the life course

In this thesis, analyses for Aims 1 to 3 were conducted contemporaneously in children and adults. Findings consistently allow us to speculate that associations strengthen with age.

Retinal vascular calibre variation increases with age and other pathophysiological processes, as outlined in the literature review in Chapter 2, Sections 2.3.3 and 2.4. Similarly, preclinical large arterial phenotypes also increasingly vary with age and share some of the same risk factors as the retinal vascular calibre (e.g., BMI, smoking exposure and blood pressure).²⁵⁵ Thus, cumulative exposure to shared risk

factors may underlie the age-dependent co-variation between small and large vascular phenotypes that we can speculate from our cross-generational cohort.

Compared to children, in adults, we observed larger explanatory power in the effects of obesity-related measures on retinal arteriolar calibre, and larger inflammation-mediated effects between obesity and venular calibre. Together, these findings suggest that cumulative effects of high adiposity on retinal microvasculature increase with age. Furthermore, with cumulative exposure to obesity across the life course, obesity and retinal venular calibre maybe increasingly mediated by inflammation.

10.6.2 Clinical implications

10.6.2.1 Some shared but unique micro- and macro-vascular pathways to CVD

Typically, the potential value of micro- or macrocirculation parameters as predictors of CVD risk have largely been investigated separately. While it is plausible that their genetic determinants could be quite separate, biologically the two vascular systems are functionally related to the pulsatile transmission of pressure and flow.⁴³ Thus, our finding has several potential clinical implications. Firstly, the stronger association between retinal vascular calibre and large arterial phenotypes observed in cardiometabolic patients, compared to the general population, could suggest a synergistic effect on the variation of small and large vascular phenotypes. That is, adverse changes could be accelerated once cardiometabolic diseases manifest. This postulation is consistent with other studies showing that individuals with both type 2 diabetes and hypertension have an increased risk of CVD compared with those with either condition alone.²⁵⁶ Thus, it highlights the potential importance of the microcirculation in CVD management, which has attracted less attention to date.

Secondly, some of the observed associations between the micro- and macro-circulation may arise from shared CVD risk factors.²⁵⁷ For example, we found that adding BMI, LDL cholesterol and smoking exposures to the Aim1 analysis model only had small attenuating effects on the association of retinal vascular calibre with large artery functional and structural phenotypes. This suggests that although the micro- and macro-vascular phenotypes share some common risk factors, each phenotype may contribute to CVD primarily through independent causal pathways.

Therefore, combining the assessment of both micro- and macro-circulation may offer a better prediction of CVD. For example, McGeechan and colleagues found that adding both arteriolar and venular calibre to the Framingham risk score improved prediction of coronary artery disease in individuals without diabetes by 1.7%.²⁵⁸ In individuals with diabetes, Ho and colleagues showed that the prediction value for CVD was higher (3.0%) when retinal arteriolar, venular calibre and diabetic retinopathy were added to other CVD risk factors.²⁵⁹ However, it is unclear whether the inclusion of *both* large arterial phenotypes and retinal vascular calibre adds to clinical risk prediction of CVD and which populations or age groups could benefit most.

There is strong evidence that microvascular alteration plays an important role in the pathogenesis of CVD.⁴⁰ Given that the association between small and large vascular phenotypes was relatively small, each phenotype may contribute to CVD through largely separate causal pathways. Future studies with clinical outcomes could explore this question by combining both measures from large arteries and microcirculation to inform the optimal use of these measures.

10.6.2.2 Tracking obesity from early childhood

Mounting evidence suggests obesity has adverse effects on both preclinical and clinical cardiovascular health.^{260, 261} Our study indicates that greater BMI and WHtR impact adversely on retinal vascular calibre, a recognised early marker of later CVD.¹⁵⁰ Adverse microvascular parameters were predicted by BMI from age 4–5 years onwards and strengthened across the life course. Even though the effects were relatively modest, at the population level, they may have clinical implications. As mentioned in the literature review (Chapter 2, Section 2.3.3.1), where data from 16 community-based studies estimated that the natural history of change in arteriolar calibre, without considering BMI, was about -0.02 μm per decade.⁹⁰ Taken together with our current findings, high or rising BMI appears to accelerate the adverse changes in retinal vascular calibre. For example, if a child's BMI z-score increased by two SD units (e.g., moved from normal into the obese range) at age 6–7 years, then we estimate that arteriolar calibre would be 1.7 μm narrower than the average. Another example is from the Cardiovascular Risk in Young Finns Study, who

reported that the improved ideal cardiovascular health (a score comprising BMI, diet status, physical activity status, smoking status, blood pressure, and fasting plasma glucose) from childhood to adulthood was positively associated with adult 0.61 μm (95% CI 0.01 to 0.24) wider (better) arteriolar calibre.²⁶² Thus, the effect of BMI we estimated may represent substantial effects on the future cardiovascular health.

Our findings emphasise the importance of tackling obesity from early childhood. This is of particular importance, as obesity tracks strongly from childhood to adolescence and into adulthood, with adverse effects occurring across the life course, but are more likely to be reversible at a younger age.^{176, 263}

Evidence has found that using traditional CVD risk factors to predict obesity-related metabolic syndrome across adolescence is unstable because of the profound physiological changes during this specific period.²⁶⁴ Some studies have then investigated the association of obesity with preclinical large arterial phenotypes.^{265, 266} However, there are cost and methodological concerns regarding preclinical large arterial phenotypes, as previously discussed (Section 2.2.4.3). Retinal microvascular parameters could be a possible marker to observe obesity-related damage in the cardiovascular system. With the development of technology, it may be possible to have a real-time analysis of the quantified retinal microvascular parameters (see later Section 10.7.2).

10.6.3 Mechanistic implications

In the mediation analysis, we found a large proportion of the association between obesity and retinal venular calibre was mediated via GlycA, 19-41% of the total effect in children and 70-100% in adults. This suggests that children have less cumulative exposure to inflammation and that, with age, associations between obesity and adverse microvascular parameters may be increasingly mediated by inflammation. A large randomised control trial targeting interleukin-1 β (a prototypical inflammatory cytokine) has provided direct support for the ‘inflammatory hypothesis’ of CVD in humans.¹⁸⁰ Obesity is a major driver of inflammation²⁶⁷, and anti-inflammatory interventions may have a role in mitigating the adverse impact of obesity on the microvasculature. Currently, most obesity interventions focus on nutritional intake and physical activity but do not result in

sustained improvements in preventing or managing weight gain.²⁶⁸ Addressing downstream adverse effects, such as inflammation, may be useful adjunctive interventions.²⁶⁹ Further studies are required to understand the key therapeutic targets and the optimal age to intervene safely.

Obesity is a major cause of hypertension. The Framingham Offspring Study has estimated that approximately 78% of the hypertension cases in men and 65% in women can be directly attributed to obesity.²⁷⁰ As mentioned earlier in the literature review (Section 2.4.3.4), high blood pressure has been shown to adversely impact on retinal microvasculature, particularly on narrowing retinal arteriolar calibre. Thus, obesity-related high blood pressure could be another important pathway leading to adverse retinal microvascular variation, as well as poor cardiovascular health. This was not explored in the current thesis because the statistical software used did not allow us to include multiple mediators. However, this is something that will be explored in the future as new statistical techniques now allow for multiple mediators to be examined.

10.7 Future directions

The microcirculation is increasingly being recognised for its role in the pathogenesis of CVD (Chapter 2, Section 2.2.5), but has attracted relatively less attention in CVD management. Technological developments now allow retinal microvascular parameters to be quantified from noninvasive, low cost and highly reproducible retinal images. However, the scoring remains timely and costly, which somewhat limits their use. In addition, few studies have tracked retinal microvascular variation from early in life in combination with adverse CVD outcomes later in life. Such data would enhance the practical meaning of retinal microvascular parameters across the life course. Below potential future research directions are discussed.

10.7.1 Replicate findings across the life course with clinical CVD outcome data

To date, the bulk of the evidence examining the retinal microvasculature is from late-adulthood, with far fewer data in childhood to mid-adulthood. To examine how retinal microvasculature varies with different factors across the life course, we need longitudinal studies with repeated measures of the retinal microvasculature and associated measures over time.

Several large cohort studies investigate the development of atherosclerosis and CVD have measured retinal microvascular parameters.^{98, 185} However, these studies have failed to measure retinal microvascular parameters at each follow-up together with clinical outcomes. Tracking changes in retinal microvascular parameters would provide information on whether these parameters progress with CVD development, and whether they provide additional information beyond other risk assessment measures.

10.7.2 Advanced technology assisting with retinal image scoring

Currently, the assessment of retinal microvascular parameters is commonly performed via semi-automated computer-based software. There are several concerns relating to the measurement. Firstly, the quantification process is not entirely objective. Subjective decisions in the assessing process may influence the quality of data. Although the reliability of retinal vascular calibre ranged from 0.79 to 0.99 in our study, measurement bias cannot be fully avoided. Secondly, various software has been developed for retinal image scoring, which uses different algorithms. Different software limits direct comparisons across studies.⁷⁸ Thirdly, the quantification process is very time-consuming (~45 minutes per retinal image using SIVA software). Thus, if retinal microvascular parameters are to be used more broadly in research at scale, or even one day at a clinical level, a standardised process using an objective tool is required. Recent innovations in deep learning technology could be a solution.

A deep learning system has been developed to detect individuals with diabetic retinopathy from retinal images with 91% sensitivity (95% CI 87% to 93%) and 92% specificity (95% CI 91% to 92%).²⁷¹ Recently, Peng and colleagues also developed a deep learning system to predict traditional CVD risk factors from retinal images.⁷³ As introduced in Chapter 2, Section 2.3.2.2, their findings showed that the deep learning system could accurately predict a series of traditional CVD risk factors, such as age, smoking and systolic blood pressure, with AUC up to 0.97.⁷³

My co-supervisor, Professor Wong is leading a project of developing the deep learning-based SIVA software. This would allow retinal microvascular parameters to be scored in real-time. The adult version has been validated in over ten studies.

The intraclass correlation coefficient for retinal arteriolar calibre is 0.88 and for venular calibre is 0.94 (unpublished data, available upon request). Using our manually scored CheckPoint SIVA child data, I am now participating in the validation of the child version of this software. We know that there are prominent sheens of retinal nerve fibre layers on children's images, which influences the scoring of child images. Thus, validation using children's data is necessary.

The deep learning-based software has the potential to further support the retinal microvascular parameters being used at scale, both in research and clinical settings. Future plans could be extending the deep learning technology to quantify retinal geometric parameters in addition to the vascular calibre.

10.7.3 More research on retinal geometric parameters

Our study showed that in children (Aim 4, Chapter 9) only some of the CVD risk factors and preclinical large arterial phenotypes were associated with the retinal geometric parameters. There was no clear overall pattern across the factors considered. This may be the result of the moderate scoring reliability of these geometric parameters which could mask weak associations. The development of deep learning-based scoring, as mentioned above, could help address this issue. Other studies may soon be able to validate our findings using the deep learning scored results, which would enhance our understanding of whether these additional geometric parameters add any new knowledge beyond retinal vascular calibre.

Another reason for our mixed findings could be that the geometric parameters reflect the overall architecture variation of the retinal microvasculature, which may take time to develop. If this is the case, then it could explain why the association of CVD risk factors with geometric parameters is more consistent in adults than in children. Currently, research evidence is still limited. More research could work to firstly understand the epidemiology of the geometric parameters and secondly to reproduce our analysis in people at different life stages and see if the associations present cumulative life course trends.

Novel geometric parameters in older adults have been shown to predict adverse CVD events,^{101, 102} just like retinal vascular calibre. However, whether the geometric parameters perform over and beyond the retinal vascular calibre in risk prediction

has not been studied, as most studies only quantified one or the other. Future studies could investigate if CVD risk is better described by retinal vascular calibre or geometric parameters.

10.7.4 Body composition and retinal microvascular parameters

BMI has predominantly been investigated when discussing the associations of body composition and retinal microvascular parameters.^{155, 165, 175} However, BMI does not distinguish between fat and muscle.¹⁷³ A few studies have specifically looked at the possible impact of fat distributions on retinal microvasculature.^{153, 163, 173} For example, the Generation R study found that higher total body and abdominal fat mass in 4,145 children aged six years were associated with worse arteriolar but not venular calibre.¹⁵⁵ However, investigating one component of body composition alone (e.g., truncal fat mass, non-truncal fat mass and fat-free mass) with retinal microvascular parameters is less than ideal. It is problematic to have all components in one statistical model due to the multicollinearity between these measures. Thus, it is unclear whether retinal microvascular parameters are most strongly influenced by body mass or different distributions of body composition.

The novel analytic technique of compositional data analysis presents a way to answer this question.²⁷² Body composition comprises truncal fat, non-truncal fat and fat-free mass, of which their proportions sum to 100%. Body composition data can be expressed as log-ratios in the compositional analysis model to allow all the compositional parts to be explored simultaneously, along with body mass, in relation to health outcomes. Our CheckPoint study has measured body composition using the BIA device, which provides an opportunity to explore how body composition profiles and body mass predict microvascular variations. Therefore, by considering body composition as compositional data, I plan to examine the association of body composition with retinal microvasculature in children 11–12 year-olds and mid-life adults in future research. This knowledge could further enhance our understanding of the pathogenesis of the microvasculature.

Chapter 11. Conclusions

Retinal microvascular parameters offer a ‘window’ into the microcirculation, which are known to predict future CVD events in late-adulthood. This thesis extends our understanding of the retinal microvascular parameters early in the life course. Findings suggest that by age 11–12 years, microvascular pathophysiology starts to contribute to CVD pathogenesis.

Preclinical phenotypes of the large arteries and microcirculation have some shared, but mainly unique pathways to CVD, with shared pathways becoming more evident with age across the life course. We also showed that the adverse impact of obesity on retinal microvasculature begins early in life, more markedly on arteriolar than venular parameters, with modest venular effects mediated largely by inflammation. In children, the retinal geometric parameters did not add novel information about CVD risk beyond retinal vascular calibre. All in all, microvascular parameters, retinal vascular calibre, may offer additional value to CVD prevention efforts from childhood onwards, especially when combined with other risk assessment measures.

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Appendices

Appendix A: See Here SOP

Appendix A is composed of two parts: standard operating procedures (SOP) for *See Here* data collection and for retinal microvascular parameter data management.

The thesis author, in consultation with Prof Mingguang He, Prof Tien Yin Wong and the wider CheckPoint team, developed these procedures and contributed to the initial draft and revisions.

CHILD HEALTH CHECKPOINT

STANDARD OPERATING PROCEDURE (SOP)

Data Collection: See Here



1. PURPOSE

To detail how the retinal photographs are to be undertaken safely, accurately and consistently.

Data management procedures are described in the *Data Management SOP – Vision* and *Data Management SOP – Cardiovascular (retinal photography)*.

2. SCOPE

This procedure is relevant for retinal photography at the Assessment Centres only.

This procedure is not relevant for Home Visits.

3. ABBREVIATIONS

Abbreviation	Definition
CERA	Centre for Eye Research Australia
ID	Identification
REDCap	Research Electronic Data Capture tool
SOP	Standard operating procedure

4. DEFINITIONS

Terminology	Definition
Assessment Centre	Assessment Centres were set up in seven major and eight regional Australian cities to conduct a series of physical health measurements in a single visit

5. RESPONSIBILITIES

Investigator/content expert

- Lead protocol development for the *See Here* assessment.
- Train staff to undertake assessments in a safe, accurate and consistent way.
- Provide advice to assessment staff and the Assessment Centre manager if required.

Assessment staff

- Attend training for visual acuity measurement and retinal photography.
- Carry out visual acuity measurements and retinal photography.

Assessment Centre manager

- Carry out and/or oversee the training for visual acuity measurement and retinal photography.
- Oversee assessment staffs' practices to maintain the agreed SOP.

6. TRAINING

Retinal photography training will be conducted by Ms Lauren Hodgson from the Centre for Eye Research Australia (CERA). Training will be conducted at the Murdoch Childrens Research Institute, Melbourne and involve three 90-minute education sessions, followed by testing to meet certification requirements (see Appendix 13.1).

Staff taking photographs for the CheckPoint study must be certified for the relevant procedure(s), before taking patient photographs.

7. Equipment

Equipment	Retinal photography (in separate dark room): CR-DGi (with EOS 60D SLR camera back) Laptop with software & USB Power board
Consumables	Retinal photography: Low lint tissues Alco wipes Hand sanitiser
Furniture	Adjustable stools Trolley (for monitor) Adjustable height trolley 2 x adjustable height stools Table for laptop

8. Procedure

Start of day processes

Preparation for retinal photography

- Ensure that the camera back is attached to the camera body; and the camera and laptop is connected to power
- The equipment must be turned on in the following order: big camera, small camera, then laptop.

- The ‘DH Client’ software and the REDCap *See Here* forms should be opened ready to record assessment details.

Participant assessment

- Greet participant by their first name and introduce yourself:

“Hi(participant), my name is(staff name)”

- Check participants Name Badge.
- Check participants ID on lanyard.
- Open participants REDCap form.

Retinal photography

- Before the participant sits, ensure the camera table has been pulled back so they don’t touch the lens with their nose.
- Greet the participant by their first name and introduce yourself:

“Hi (participant), my name is (staff name)”

- Check the participant’s name badge.
- Check ID on lanyard.
- Open participant’s REDCap form.
- Explain Retinal Photography:

“Welcome to *See Here*. Grab a seat on this chair for me. In this station, we will take some pictures of your eyes. You might have had this done at the optometrist before. It won’t hurt, you’ll just see a bright flash, and it’s done! We’ll take two pictures of each eye.

- Ask the participant if they have any known eye conditions and enter into REDCap.
- Note: Make sure the participant has removed glasses, if applicable.
- Adjust the height of the chair to ensure the participant can sit comfortably when their chin is resting on the chin rest. Adjust the table height using the up/down button at the base of the table (at edge of table).
- Check the eye is in line with the white line on the metal pole attached to the side of the chin rest. If adjustments are needed, use the white roller to lift/drop the chin rest, so that the participant is comfortable and can sit normally.
- Explain to the participant:

“Could you please rest your chin on the chin rest for me. Rest your forehead against the head rest. I need you to stay nice and still in this position for me for a few minutes, and this includes not talking, as moving your jaw will affect the photos. Are you comfortable?”

- If the participant is not comfortable, continue to adjust the chin rest and/or chair.

- With the participant in position with their right eye in front of the camera, ask them to look at the orange dot:

“First we’ll start with your right eye. Make sure you keep your head still, with your chin on the chin rest and forehead touching the head rest. Look straight ahead at the flashing orange dot for me”.

- Move the camera platform to focus on the right eye.
- Take the right eye macula-focused image:
 1. Line up the two sides of the split circle to be a continuous circle around the right pupil by moving the platform in or out.
 2. Line up the inner circle on the screen around the outside of the pupil with the three white dots surrounding the pupil.
 3. Note: If the participant has small pupils, use the small pupil SP white lever on the right side of the camera. This reduces the field of the camera and focuses the photo on the centre of the pupil.
 4. Press the black square button to zoom into the eye.
 5. Line up the two lines on the screen using the knob on left side of camera. Ensure the lines are in focus.
 6. Locate the two white dots using the joystick to move the camera.
 7. To focus use the joystick for in/out movement; to move dots up/down use roller on base of joystick.
 8. Once the two dots are located, they both need to be inside the square indents on the left and right of the camera monitor. Ensure they are in focus.
 9. When you’re ready to take the image, ask the participant to blink. Use the button on top of the joystick, capture the image once they open from their blink. If the participant blinks during the image, re-take the image.
- DO NOT delete any photos during this sequence, they can be removed later.
- Take the right eye disc image once the participant has recovered from the first flash:
 10. Ask the participant to look at the orange dot again.
Complete steps 1-4 for macula centred image above.
 11. When the participant is ready, press the white left button next to the black square zoom button. This will move the orange dot outside of the camera to line the optic disk in the centre of the picture.

12. If observing right eye then move the dot 2-3 across to the left by pressing the white left button next to the black square zoom in button 2-3 times. If participant is struggling to follow the dot move it across only 2 times.
 13. Once the participant is focused on the dot, repeat steps 5-9 for macular centred image above to capture the right disc image.
- Allow participant to relax for a minute or two then readjust position/height of the table for the left eye and repeat steps 3-13 for the left eye to capture the left eye Macular and Disk centred images
 - To end the photo session, click 'END'. This allows you to review all photos taken. You then have the option of:
Taking more photos (if time permits). Click on "Continue photographing this patient".
If no more photos are required, click on "End program" or to review images, click "Go to patient's visit list".
 - Copy the image code onto REDCap and tick which images you've captured. You should have a macula centred and disc centred image for the right and left eye (see below for examples):



Figure 1: Right Eye, Field 1



Figure 2: Left Eye, Field 1



Figure 3: Right Eye, Field 2

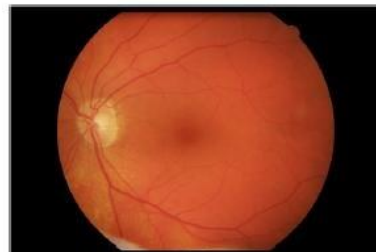


Figure 4: Left Eye, Field 2

Note: FIELD 1 = disc centred image (see Figures 1 and 2); FIELD 2 = macular centred image (see Figures 3 and 4)

- To save the images:
Click "select all" to save images.
Images will be highlighted with a pink frame.
Click "export selected".

Save files in the following file pathway: *CheckPoint > Participants Today > Child ID > Retinal_Photos > Child or Parent > Save files here.*

Select image quality as “high” (approx. 1.5MB).

Click “send” to confirm save.

Saved images should be named as the image code followed by a 3-digit number. E.g. CC01) JTZ001.

- Thank the participant for their time and direct them to their next station:

“Thank you for your help here. I will save these pictures for you on a USB, so you can take them home with you to show your friends and family what the inside of your eye looks like! Grab your stuff and I’ll take you to your next station”

- Ensure REDCap is updated. Make a note in the “Comments” box of any abnormal images that require following up.
- Mark ‘Complete’ on REDCap Form.
- Wipe down the chin rest and head rest with an alcohol wipe.
- Lock the moveable base of the camera platform.
- Replace dust covers.
- Switch off the laptop.

End of day procedures

- Check REDCap data entry completed for all participants.
- At the end of each session, retinal photographs need to be saved onto the server. The server will be backed up at the end of every session. Save the images in “CheckPoint” then in the participant’s ID folder. Name the file *[ID number][p (if parent)]_retinalpic*.
- Mark the station as ‘complete’ on REDCap Form.
- Say to participant:

“Thank you, I have finished now. I’ll take you to your next station.”

9. CHANGES TO PROTOCOL

Changes to protocol over time

- Retinal Photography was performed at the Main Assessment Centres only.

10. STATION TIPS AND TRICKS

Retinal photography tips and tricks

- The digital camera program (EOS client) must be on for the camera to transfer files to the computer.
- If images aren’t transferring from camera to computer attempt the following:

Look for a dialogue box in the EOS digital camera program that says 'could not write to selected folder. Select another folder.

Note this dialogue box may be behind other programs, so find it on the taskbar (next to start) or press 'alt + tab' to switch between the windows on the screen.

Once found, Click Ok

Another window will appear under the EOS program attempting to choose a new folder. Click Cancel.

Another window will appear saying 'shot may not be saved. Continue?' Click yes.

- If files are still not transferring, attempt restarting the computer, and cameras.
- If REDCap is down, save files in folders on the desktop naming the folders as the participants ID (with a p for parents). Then when possible transfer them manually into the CheckPoint folders for each participant.
- If the flash is too bright for participant you can dim this by xxx.

General orientation of the Retinal Photography camera

- The joystick is best used for small movements
- If large movements are needed, especially at the start when first lining up the camera, slide the whole slide table
- The wheel on the right will focus the image
- The square button in front of the joystick moves from the camera view from the external eye to internal eye view
- The round button in front of the joystick moves the camera left and right
- The dial around the joystick moves the camera up and down
- Digital images should have enough resolutions for grading. Ideal image resolution for adequate grading is approximately 3000 pixels x 2000 pixels more than 150 dpi.
- When saving images as JPEG, make sure they are saved in high quality (low compression rate). For example, a digital photograph with resolution of 3072 pixels x 2048 pixels, 180 dpi, in 24 bit colour depth will have approximate image size of 2Mb per photo.

11. DOCUMENT VERSION CONTROL

Version	Effective Date	Change
1	17 January 2014	New Document.
2	15 August 2014	Major revisions.
3	19 August 2014	Revisions post-first run through.
4	25 August 2014	Decimal VA vs logmar; clean text.
5	3 November 2014	Post DR version.
6	16 December 2014	Minor modifications.
7	20 May 2015	Consistency with Summary doc.
8	27 March 2018	Update formatting to match template, review accuracy and completeness of information.
9	22 June 2018	Merge with Retinal photography <i>See Here</i> SOP.
9	28 June 2018	Formatting and consistency updated.

12. REFERENCES

<http://www.michaelbach.de/fract/checklist.html>

13. APPENDICES

Certification

Staff are encouraged to contact the CERA grading consultant, Ms Lauren Hodgson (labh@unimelb.edu.au) with any photography related questions.

Certification consists of (1) review of standard photography procedures and (2) demonstrated ability to perform the photographic procedure by submission of photographs of acceptable quality.

Photographers should submit colour photographs of five patients (10 eyes) taken using this procedure. The colour photographs may be taken of patients in whom photography is being carried out for clinical purposes or in normal volunteers. The digital images should be prepared for grading. A signed Photographer Certification Request Form is also required.

Photographers who meet certification criteria will receive written confirmation of certification. Photographers who do not meet these criteria will receive feedback from the photographic consultants, and will be required to submit additional sets of photographs. After three unsuccessful attempts for certification, no additional photographic submissions will be accepted until a plan for improving photographic quality has been developed in collaboration with the Principal Investigator.

CHILD HEALTH CHECKPOINT

STANDARD OPERATING PROCEDURE (SOP)

Data Management: Retinal Photos



1. PURPOSE

This Standard Operating Procedure (SOP) describes how the Retinal photos data is processed after data collection. This includes transfer of the source data to the Murdoch Children's Research Institute (MCRI), measure extraction and data derivation.

Data Collection processes are described in the *Data Collection SOP* – [see here](#).

2. SCOPE

This SOP relates to Retinal photos data only; this includes data from REDCap (Research Electronic Data Capture tool) and image analysing software.

The retinal photographs were undertaken by children and an attending parent at Main Assessment Centres only.

3. SUGGESTED CITATION

Growing Up in Australia's Child Health CheckPoint. Standard Operating Procedure: Data Management, Retinal photos. Melbourne: Murdoch Children's Research Institute, 2019. doi: 10.25374/MCRI.8957084

4. RESPONSIBILITIES

CheckPoint Data Management Team (Data Team)

- Safe transfer of files onto MCRI server.
- Safe transfer of files into Source Data (restructuring).
- Movement of files through from Source Data to Measurement Extraction to Derived Data folders.
- Undertake and supervise data validation tasks.

CheckPoint Data Management and Statistics Committee

- Provide guidelines and policies on data management for the project.
- Monitor the performance of data management activities.
- Support and assist in the resolution of issues that arise during data management.

Student/Research Assistant (RA)

- Undertake review of the scoring literature.
- Propose scoring to be used.
- Undertake data cleaning and validation tasks as instructed (eg. data comment review).

- Review and propose scoring system and derived variables to be used
- Liaise with investigators to resolve issues arising.

Investigators

- Provide guidance and assistance in the measure extraction, data derivation and analysis processes.

Key Contacts

- Melissa Wake (Investigator)
- Mingguang He (Software provider)
- Tien Yin Wong (Software provider)
- Mengjiao Liu (PhD Student)
- Lauren Hodgson (CERA RA)

5. ABBREVIATIONS & DEFINITIONS

Assessment Centre	Assessment Centres were set up in Australian cities to conduct a series of physical health measurements in a single visit. 'Main' Assessment Centres were set up in seven major Australian cities. Participants were offered all CheckPoint assessments during a 3½ hr visit.
Derived Data	These data represent the outputs of processing measure extraction data. This processing varies between measures and can include activities such as variable naming and labelling, incorporating changes from comment cleaning and flagging potentially unreliable data, recoding variables, deriving new variables such as z-scores, totals and averages, and checking distributions and ranges. These data can be included in the final data set.
Measurement Extraction	These data represent the outputs of processing source data. This processing varies between measures and can include activities such as file conversion (consolidating accumulative files, transcription, changing file types), validating IDs and cross checking duplicate data, scoring data such as images or loops, reviewing data quality and excluding poor quality data and checking for missing data. These data can be included in the final data set.
REDCap	Research Electronic Data Capture A research data entry Program.
SOP	Standard Operating Procedure.

MCRI	Murdoch Children's Research Institute.
Source Data	These data represent the first point at which research data was collected. These data take many forms such as images, data directly entered into the Research Data Capture application, written documentation and data generated from physical assessment machines. These data are stored in their original format with no changes, such as cleaning, made.
Stata	A statistical software package.
IVAN	Integrative Vessel Analysis, the software analysing retinal photos.

6. TRAINING

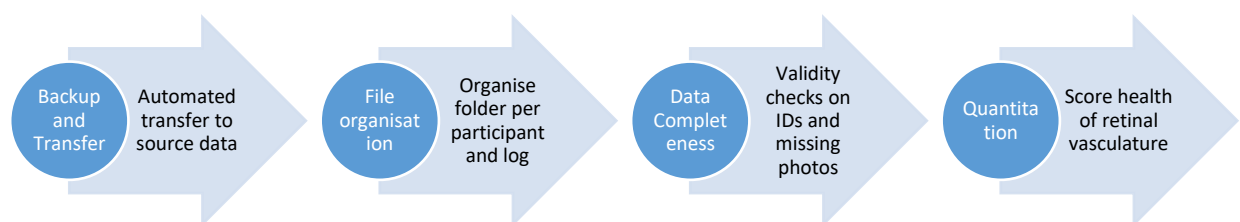
- Students/RAs - familiarity with data collection process and retinal photography scoring training (CERA)
- Data team - familiarity with file compression (7-Zip), Python modules and Excel functions (sorting, conditional formatting) and Stata14

7. PROGRAMS

- REDCap ("Main Wave Data Collection" Project)
- Space on MCRI Servers (JPEG files, copies for file name cleaning, file logs, extracted REDCap data)
- Retinal photography software installed computer
- Microsoft Office
- Stata installed computer

8. PROCEDURE

8.1 Summary Steps

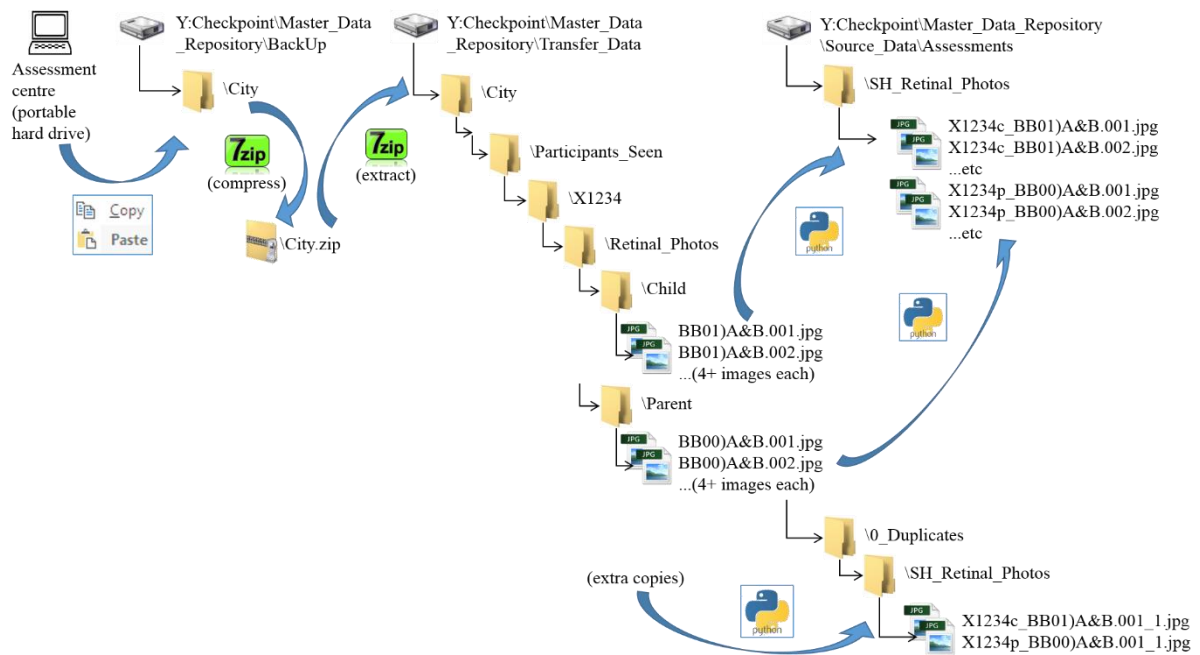


8.2 Preparation of REDCap data

File Conversion

Data source:	REDCap
Procedure:	Data Team download data from REDCap
Program:	REDCap, Stata
File type produced:	DTA Stata dataset (file name, if you have it)

- Retinal photos are saved as high-quality JPEG files, 1-8 photos per participant
- File naming procedure:
 - At AxC, each participant has arbitrary letter/number/symbol combination, with photos numbered sequentially, eg. BB01)A&B.001.jpg
 - Automated file transfer to source data retains original name and adds Participant ID with suffix to start, eg. A1234c_BB01)A&B.001.jpg
- Process regarding transfer of files from collection to Source Data
- MCRI data team copy/clear hard drive and return to Assessment Centre
 - compressed copy of original city backup is made prior to transfer
- Automated restructuring script adds participant ID and suffix, and transfers unique jpeg files into \SH_Retinal_Photos, and duplicates are numbered and placed in \0_Duplicates\ SH_Retinal_Photos
- The Data Team exported these variables from REDCap, used Stata to append 'Main Wave' project exports into a single document, and saved as DTA Stata dataset: red_allergyYYYYMMDD.dta
- Type of Data
 - One entry per family, with separate pages for child (See Here Rpic) and parent (See Here Parent Rpic)
 - Includes known eye conditions, images captured, image code, number of photos, reason missing and other retinal photo comments



Procedure:

1. From the REDCap Main Wave Data Collection project, export the following variables:
 - a. checkpoint_id ID
 - b. ccpt_refuse_p__8 Parent refused measures for child (choice=Retinal photo)
 - c. p1ci_refuse_p__8 Parent refused measures for parent (choice=Retinal photo)
 - d. ccsh_eyecond_d Does the child have any eye conditions they know of?
 - e. ccsh_neyecon_d What is the name of the condition?
 - f. ccsh_disc_d__1 Disc images captured (choice=Left Eye)
 - g. ccsh_disc_d__2 Disc images captured (choice=Right Eye)
 - h. ccsh_macula_d__1 Macula images captured (choice=Left Eye)
 - i. ccsh_macula_d__2 Macula images captured (choice=Right Eye)
 - j. ccsh_icode_d Image code
 - k. ccsh_norpic_d Number of retinal photos taken
 - l. ccsh_rpmiss_d Reason Retinal Photo Missing
 - m. ccsh_rpcomm_d Retinal photo comments
 - n. p1sh_eyecond_d Does the parent have any eye conditions they know of?
 - o. p1sh_neyecon_d What is the name of the condition?
 - p. p1sh_disc_d__1 Disc images captured (choice=Left Eye)
 - q. p1sh_disc_d__2 Disc images captured (choice=Right Eye)
 - r. p1sh_macula_d__1 Macula images captured (choice=Left Eye)

- s. p1sh_macula_d__2 Macula images captured (choice=Right Eye)
 - t. p1sh_icode_d Image code
 - u. p1sh_norpic_d Number of retinal photos taken
 - v. p1sh_rpmiss_d Reason Retinal Photo Missing
 - w. p1sh_rpcomm_d Retinal photo comments
2. Reshape data to long format, with parent and child on separate rows (including ID and c/p)
 3. Save as Y:\checkpoint \Master_Data_Repository \Measure_Extraction \sh_retinal \1.FileOrganisation \quality control docs \CheckPoint_MainWavfe_DataCollection_retinal photos_all_yyyymmdd.csv

Comment Cleaning

Process regarding steps of comment cleaning:

Data source:	REDCap export (red_allergyYYYYMMDD.dta)
Procedure:	Review and actioning of free-text comments entered into data entry forms by Data Collection staff at the time of assessment. These comments were made to share relevant information with other CheckPoint team members during the visit, or record details of the assessment that could have impacted on data quality. The Data Team, students and RAs reviewed comments and allocated an action: the data be dropped, changed, replaced, or retained unchanged.
Program:	Microsoft Excel and Stata
File type produced:	XLSX list of changes, DO file

- Data RA produces output of all string fields and comments from related station/s (*See Here*), with template columns for student to complete
- STATA script to produce output should replace known child and parent names with placeholder to protect participant privacy
- Student reads through string fields/comments for parent and child, and:
 - a. score action to be taken, with appropriate syntax
 - i. replace a value
 - ii. remove a value
 - iii. no action (NA)
 - iv. wrong ID
 - v. investigate/ask CIs
 - vi. remove identifying info in comment
 - vii. report special case to analyst
 - b. score explanation to justify above action

- i. 1 if already recorded in data or irrelevant
 - ii. 2 if removing identifying info (DOB, name, address, phone number)
 - iii. 3 if wrong participant
 - iv. 4 if replace incorrect values
 - v. 5 if check machine data vs. REDCap
 - vi. free text if absolutely need to type explanation
- c. consolidate areas that need further clarification, and consult with relevant investigators
- Full list of instructions and examples can be found in README_comclean.xlsx
- Data RA incorporate results of comments cleaning in FinalClean STATA syntax

8.3 Preparation of IVAN Data

File Conversion

Data source:	IVAN
Procedure:	Students (summer students and interns)
Program:	Windows Explorer
File type produced:	JPEG (1 folder per participant with 4-8 photos each)

- Data management team make a copy of all transferred retinal images from Source Data to the Student working folder in Measure Extraction
- Create new folder for each participant with retinal photos, in the format "A1234c" (child) or "A1234p" (parent)
- In windows explorer, drag and drop photos for each participant to their relevant folder
- Open each file to verify all 4 image types have been taken (left and right disc and macula), remove any blank or unreadable images (blinks); also compare available images with information exported from REDCap, and list any changes necessary in final photo log

NOTE - this process was completed manually due to student availability and original requirement to remove blinks and blank photos; for automated processing of images where number of images is not a factor, image organisation can be automated in Python without checking each image

Quantitation Analysis

Procedure:	Student/RA
Program:	IVAN
File type produced:	CSV

8.4 Data Completeness Indicator

Process regarding preparation steps for validity checks:

Program:	Python, Excel
File type produced:	Zip folder of all BIN and CSV files Excel spreadsheet of filenames and sizes

Key Parameters:

1. All valid attended IDs
2. ID/suffix on photo matches folder
3. Number of images in folder matches number taken in REDCap
4. Image code on filename matches image code in REDCap

Procedure:

1. Student organised photos moved to appropriate location in Measure Extraction
2. Export retinal photos information from REDCap (see Measure Extraction - REDCap below for variables) to
//Measure_Extraction/sh_retinal/1.FileOrganisation/quality control docs
3. Export list of attended IDs from participant tracking Access database to above location
4. Run Python script "Python script_retinal photos QC"

```
import os
import glob
import logging
import pandas

## 1. Create log of photos present
source = 'Y:/Master_Data_Repository/Measure_Extraction/sh_retinal/1.FileOrganisation/Retinal photos for scoring/*'
logging.basicConfig(format='%(message)s',
                    filename='Y:/Master_Data_Repository/Measure_Extraction/sh_retinal/1.FileOrganisation/quality control
docs/Log_Retinal photos available_yyyymmdd.csv',
                    level=logging.INFO)
logging.info('CP_ID,SUFFIX,FOLDER,NUMBER_IMAGES,IMAGE_CODE,IMAGE_NUMBER,FILENAME')
print('CP_ID,SUFFIX,FOLDER,NUMBER_IMAGES,IMAGE_CODE,IMAGE_NUMBER,FILENAME')
for folder in glob.iglob(source):
    foldername = os.path.basename(folder)
    ID = foldername[0:5]
    cp = foldername[5]
```

```

source_dir = os.path.dirname(folder)
folder_path = os.path.join(source_dir+'/'+foldername+'/*.*jpg')
folder_abspath = os.path.abspath(folder)
number=0
for file in glob.iglob(folder_path):
    number=number+1
for file in glob.iglob(folder_path):
    filename = os.path.splitext(os.path.basename(file))[0]
    image_code = filename[7:15]
    image_number = filename[17:19]
    print (ID+', '+cp+', '+foldername+', '+str(number)+'+', '+image_code+', '+image_number+', '+filename)
    logging.info(ID+', '+cp+', '+foldername+', '+str(number)+'+', '+image_code+', '+image_number+', '+filename)
print('Part 1 (photos log) complete')

## 2. Merge REDCap info with attended IDs
attended_IDs = 'C:/Users/katherine.lange/Desktop/test data/requests/Retinal photos log/Attended IDs_AC
only_20160308.csv'
redcap_info = 'C:/Users/katherine.lange/Desktop/test data/requests/Retinal photos
log/CheckPoint_MainWave_DataCollection_retinal photos_all_20160321.csv'
output_info = 'C:/Users/katherine.lange/Desktop/test data/requests/Retinal photos log/Retinal photos_info_20160321.csv'
a = pandas.read_csv(attended_IDs)
b = pandas.read_csv(redcap_info)
merge = a.merge(b, on='CP_ID')
merge.to_csv(output_info, index=False)
print ('Part 2 (info merge) complete')

## 3. Merge RC attended info with photos present
# NOTE - this will exclude participants with photos taken but not found, will need to be logged seperately
photos_log = 'C:/Users/katherine.lange/Desktop/test data/requests/Retinal photos log/Log_Retinal photos
available_YYYYMMDD.csv'
output_log = 'C:/Users/katherine.lange/Desktop/test data/requests/Retinal photos log/Retinal photos_log_YYYYMMDD.csv'
c = pandas.read_csv(output_info)
d = pandas.read_csv(photos_log)
merge = c.merge(d, on='CP_ID')
merge.to_csv(output_log, index=False)
print('Part 3 (consolidation) complete')
print('Program complete')

```

5. Automated script above creates 3 documents:
 - a. Log_Retinal photos available_YYYYMMDD.csv is a list of all photos available, including ID, suffix, folder (should be participant ID+suffix), number of images in folder, image number and filename (without extension)
 - b. Retinal photos_info_YYYYMMDD.csv merges known IDs from attended IDs list to retinal photos information input in REDCap
 - c. Retinal photos_log_YYYYMMDD.csv merges list of retinal photos available with attended IDs and information input in REDCap
6. Open Retinal photos_log_YYYYMMDD.csv and re-save as Excel workbook with "QC" in new filename, eg. Retinal photos_log_QC_YYYYMMDD.xlsx
7. In new tabs, also copy/paste the information from Log_Retinal photos available_YYYYMMDD.csv (photos available) and Retinal photos_info_YYYYMMDD.csv (REDCap info for attended IDs)

8. Under available photos list, check all photos have a valid attended ID, and investigate/edit/remove any errors as appropriate
9. Under tab of merged available photos and REDCap info for attended IDs:
 - a. create new columns for foldername match, suffix match, code match and number of photos match
 - b. foldername match = exact(S2,LEFT(W2,6)), where S2 is the foldername and W2 is the photo filename
 - c. suffix match = exact(E2,R2), where E2 is the suffix from attended IDs/REDCap info and R2 is the suffix from available photos log
 - d. code match = exact(G2,U2), where G2 is the image code copies to REDCap and U2 is the image code section from photo filename
 - e. number of photos = exact(H2,T2) where H2 is the number of photos input on REDCap and T2 is the number of photos in each folder
 - i. if number of photos taken is missing from REDCap, use sum of which photos were taken (eg. is disc and macula taken for both left and right, number=4)
10. For foldername mismatch with ID on filename, move files to correct folder or rename folder as appropriate (same for suffix mismatch)
11. For mismatched image codes, investigate for typographical errors, multiple photo sets for same participant, or other errors.
 - a. If errors resolvable, make list of changes to make to final photo log
 - b. If unresolvable, remove photos to a separate folder (removed from analysis)
12. For mismatched number of photos, if number of photos available is less than number taken, check retinal photos laptop for additional photos that may have been mis-named or copied to the wrong AxC folder
 - a. Student also given list of attended participants missing retinal photos, to see if any additional photos locatable on retinal photos laptop
13. Repeat Python photo log and QC procedure until all errors resolved as much as possible

Log creation and preparation for scoring:

1. Once errors resolved, create photo log for scoring by running Python script "Python script_retinal photos for scoring" (very similar to above script with different file locations and without)
2. Open and re-save merged log as an excel workbook "merged log cleaning"
3. Create a new tab named "edit" and copy/past the log to this tab (so original data is retained)
4. Delete columns B (date of check-in), G (image code, from REDCap), H (Number of images, from REDCap), M (reason retinal photo missing), R (copy of CP_ID), S (copy of suffix), W (Image number) and Z (filename)
5. Use conditional formatting to identify duplicate Participants

- a. make changes to log according to document indicating changes to be made, and delete the additional record
6. Make other changes to log according to document indicating changes to be made (from student file organisation AND data completeness checks)
7. Check number of photos is equal to or greater than the list of photos taken; check photos and edit log as appropriate
8. Rearrange columns and re-format headings according to CERA log template

<i>Participant</i>	<i>ID</i>	<i>C/P</i>	<i>Session Date</i>	<i>City</i>	<i>Image Code</i>	<i>Left disc</i>	<i>Right disc</i>	<i>Left macula</i>	<i>Right macula</i>	<i>Number of photos</i>	<i>Notes</i>	<i>Eye condition?</i>	<i>Name of eye condition</i>	<i>Glasses or contact lenses?</i>
--------------------	-----------	------------	---------------------	-------------	-------------------	------------------	-------------------	--------------------	---------------------	-------------------------	--------------	-----------------------	------------------------------	-----------------------------------

9. Copy retinal photos for scoring, along with cleaned log file, to an external hard drive and deliver to CERA representative

9. DECISION MAKING

10. DOCUMENT CONTROL

Version	Effective date	Change
1	01/04/2016	New document.
2	29/09/2016	Data team SOP reviews (updated all fields).
3	31/05/2019	Formatting and consistency updated.

11. REFERENCE

1. Weiss MD, Salpekar J. Sleep problems in the child with attention-deficit hyperactivity disorder: defining aetiology and appropriate treatments. *CNS Drugs*. 2010 Oct;24(10):811-28.
2. Noble GS, O'Laughlin L, Brubaker B. Attention Deficit Hyperactivity Disorder and Sleep Disturbances: Consideration of Parental Influence. *Behav Sleep Med*. 2012;10(1):41-53.
3. Owens JA. The ADHD and sleep conundrum: a review. *J Dev Behav Pediatr*. 2005 Aug;26(4):312-22.
4. Miano S. The Complex Relationship Between ADHD and Pediatric Sleep Disorders. *The ADHD Report*. 2012;20(6):1-6.

12. APPENDICES

Appendix B: Retinal data scoring manuals

In this PhD project, we assessed the retinal microvascular parameters used the two computer-based scoring systems, Integrative Vessel Analysis (IVAN, University of Wisconsin, Madison, US) and Singapore “I” Vessel Assessment (SIVA, Singapore Eye Research Institute in collaboration with National University of Singapore, Singapore). Appendix B contains manuals relating to the two systems.

Vessel Measurement System, the IVAN grading protocol, University of Wisconsin

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Vessel Measurement System

DESCRIPTION

The VMS is a semi-automated system used to measure retinal vessel widths from a digital retinal image.

The automated components include placement of the overlying grid centered on the optic disc, vessel type identification, and width measurements for vessels. The color blue is used to denote venules and the color red is used to denote arterioles in the screen displays. The data table on the control window displays the mean width and standard deviation for each measured vessel and locates each vessel by its clockwise angle in degrees. The vessels are automatically typed as arteriole or venule; in the case of uncertainty, the vessel is typed as an arteriole.

The manual components include the option to override any of the initial automated decisions or measurements. This would include adjusting the placement of the grid, changing the vessel type, deleting vessels, re-measuring vessels, and adding significant vessels missed in the initial automation.

The VMS is operated off of a 2-monitor workstation with the image files organized by reading lists. The location of the application is G:\RVU\Software\ivan\IVAN\VM_Lite. This file contains input parameters including the allowable range of reading lists and pixel conversion factors. The pixel conversion factor should be 5.11. The data is saved on the server at G:\RVU\Grading Databases\IVAN Data*.CVS (comma separated values) and is formatted in an Excel spreadsheet.

The Modified ARIC grid is composed of 3 concentric circles which demarcate an average optic disc, Zone A defined as the region from the disc margin to 1/2 disc diameter from the disc, and Zone B defined as the region from 1/2 disc diameter to 1 disc diameter from the disc. All retinal vessels are measured in Zone B.

Only the width data from the 6 largest venules and 6 largest arterioles are required to calculate the AV ratio using the Knudtson formula. No branch (daughter) width data are required.

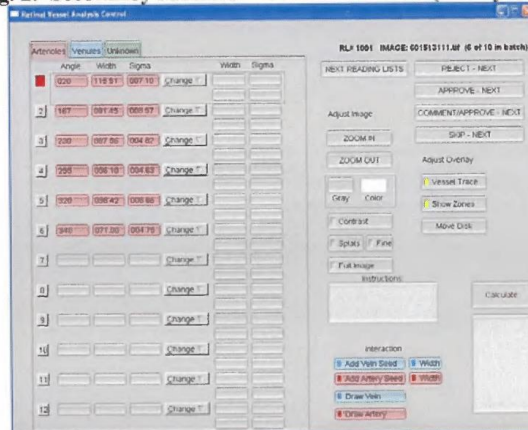
PROCEDURE

1. Log onto workstation with study name first and grader's name the second.
The application opens on the primary monitor with 3 windows: the image display window, the control window, and the vessel data parameters file.
2. Drag control window from behind image window to secondary monitor. The image display should be kept on the larger primary monitor. The vessel data parameters file is left hidden below the image display.

Fig. 1. Primary Monitor – Image Display



Fig. 2. Secondary Monitor – Control Window (Artery View)



Adjust Image Tools (See examples on following pages)

Zoom In/Zoom Out — zoom in to incrementally magnify a selected area of the image or zoom out to display the entire cropped image (figs. 3h, 3i)

Gray/Color — choose red-free or color image display (figs. 3a, 3b)

Contrast — equalizes color contrast; especially useful for eliminating choroidal pattern (toggle) (fig. 3g)

Splats — displays computer model of image with “splat” units; useful in determining likelihood of an accurate measurement in a specific area (toggle) (fig. 3d)

Fine — displays a more detailed visual of splat units (toggle) (fig. 3e)

Full Image — pops-up original (uncropped) F1 image; useful for vessel identification (toggle) (fig. 3c)

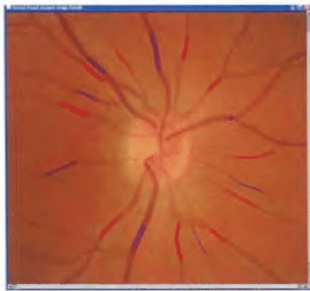
Vessel Trace — hides vessel width overlay for unobstructed view of vessels; toggles back to display same overlay (fig. 3b)

Show Zones — hides grid overlay for unobstructed view of image; toggles back to display same grid (fig. 3f)

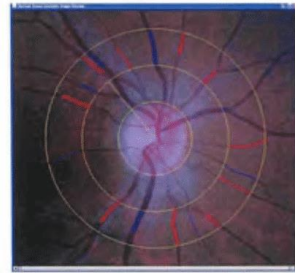
Move Disc — brings up control window that allows incremental movement of grid overlay by clicking directional arrows; center OK button saves grid placement and refreshes vessel measurements. Red Cancel button restores original grid placement and vessel measurements



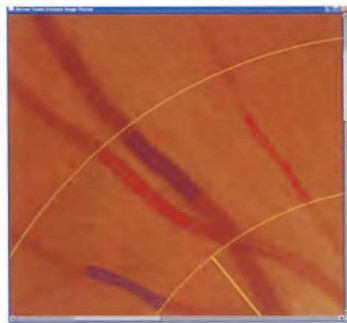
Fig. 3. Adjust-Image Tools Examples (cont'd.)



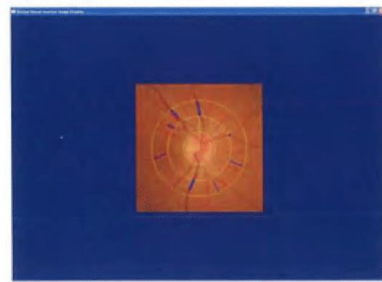
3f. Show Zones



3g. Contrast



3h. Zoom In



3i. Zoom Out

Vessel Measurement Tools – Change Menu

To make the following changes to vessels, the vessel must first be accessed. Vessels are accessed by clicking on the seed point (the proximal end of the vessel trace) on the image display or by clicking on the vessel number button on the data table. Both access methods highlight the vessel trace, direct the pointer to the vessel trace seed point (and angle location), and highlight the data for the accessed vessel on the data table. In this way, the visual vessel trace and the data (angle, width, Sigma) for each vessel are easily correlated.



Change Menu

Change Type – automatically switches vessel type and updates vessel trace color and type on data table.

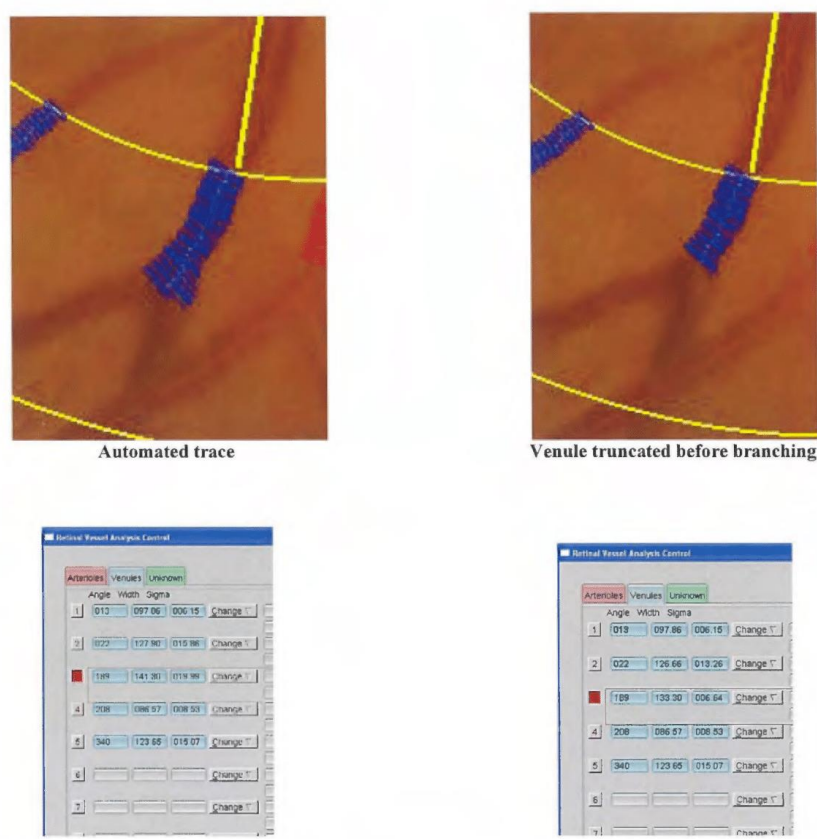
Delete – deletes vessel trace from display and vessel measurement and type from data table. The vessel data is then listed as type Unknown in the data table.

Extend – to add on a second segment distal to the original (bypasses unmeasurable area); the Extend segment is added by seeding a start point with 2 clicks along the vessel. The data table regenerates the mean and standard deviation based on the combined segments. (Fig. 4)

Truncate – make a single click at a point on the vessel to terminate the distal segment before a branching or crossing or measurement discrepancy. (Figs. 5 and 6)

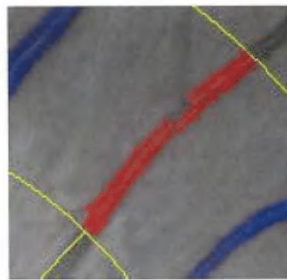
Proximal Chop – make a single click at a point on the vessel to terminate the proximal segment. (Fig. 6)

Fig. 4. Truncate Example

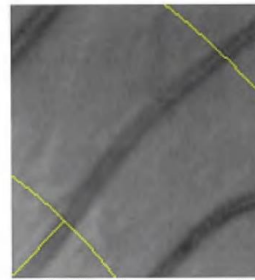


Note the effect of truncating the automatic venule measurement before the branch on the width and standard deviation of Venule #3.

Fig. 5. Proximal Chop / Truncate Example



5a. Automated display



5b. Vessel trace removed



5c. Final measurement using proximal chop and truncate

Arterioles Venules Unknown					
	Angle	Width	Sigma		Width
1	008	083.74	005.56	Change T	
4	044	076.68	017.92	Change T	
3	113	070.58	009.65	Change Type	
				Draw Daughters	
				Add Daughter Seeds	
4	147	069.61	008.23	Change T	
5	227	068.18	013.43	Change T	
6	236	042.79	002.63	Change T	
7	287	047.95	003.58	Change T	

5d. Automated data for Artery #2

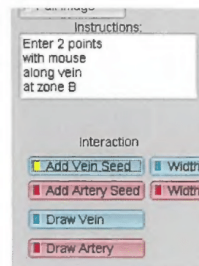
Arterioles Venules Unknown					
	Angle	Width	Sigma		Width
1	008	083.74	005.56	Change T	
4	044	077.63	004.39	Change T	
3	113	070.58	009.65	Change T	

5e. Modified data for Artery #2

The automated display of Artery #2 has outliers at the proximal and distal ends (Fig. 5a), the reasons for which are more clear in Fig. 5b. The outlier segments are removed by using Proximal Chop and Truncate and the vessel trace appears accurate (Fig. 5c). As shown in Figs. 5d and 5e, the mean width was not affected significantly by these corrections, but the Sigma value was improved and with the visual accuracy of the vessel trace is an assurance of credible data.

Vessel Measurement Tools-Interactions

The **Instructions** window displays the mouse click requirements for each of the following vessel measurement tools:



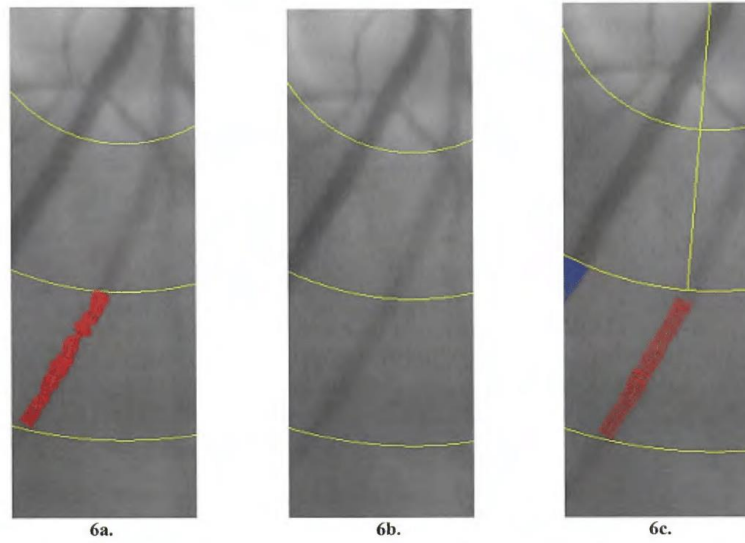
Instructions for Add Vein Seed

Add Vein/Artery Seed – enter mouse click at desired starting point of segment and second mouse click along the direction of the vessel; automated measured segment will display and may need further modification with Change Tools.

Draw Vein/Artery – enter first mouse click at desired starting point and enter 4 more mouse clicks along vessel to be measured with intent to include as much as Zone B as possible; measured segment will display and may need further modification with Change Tools.

Widths – enter 1 mouse click along edge of vessel and second mouse click at opposite width edge as perpendicular as possible; automated measured segment will display and may need further modification with Change Tools. This tool is especially useful for vessels with central light reflex. (Figs. 6a-6c)

Fig. 6 Width Tool Example



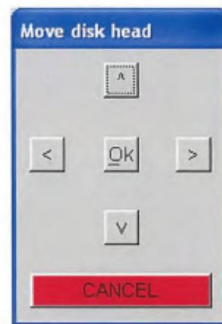
The automated measure (Fig. 6a) has obvious outliers due to the softly focused vessel edges (Fig 6b). Seeding or drawing the arteriole produces the same pattern. Using the Width tool produces a visually accurate trace that smoothes out the vessel edges (Fig. 6c).

GRADER INTERACTIONS

The grader is responsible for visual evaluation of the automated display and modifying as necessary. This includes approving the placement of the grid, correcting vessel types, and modifying vessel measurements according to the following guidelines.

Grid Placement

The grader will visually determine if the inner circle is best centered on the optic disc. If the placement should be changed, OK should be clicked. The vessel measurements will automatically recalculate according to the new Zone B.



Identification of Arterioles and Venules

The grader identifies each vessel within Zone B as either an arteriole or a venule and makes changes as necessary. Most vessels with a caliber greater than or equal to 50u can be identified as either arterioles or venules from the digital image. For smaller vessels, the option to view the entire uncropped image may be helpful or referring back to the images in the EyeQ database may be necessary. Only the data from the largest 6 venules and largest 6 arterioles are necessary so if the largest 6 are easily determined the smaller vessels can be deleted without further considerations. If more than 6 are automatically and correctly displayed, no deletions are necessary because the calculating process will ignore the unnecessary values.

The grader uses the guidelines below to identify each vessel:

- (1) **Color on digital image.** Arterioles are a lighter orange-red color with a strong central light reflex. However, the central light reflex may not always be apparent, especially in arterioles less than 50u in caliber. Veins are a darker purple-red color with little or no central light reflex.
- (2) **Course.** Arterioles tend to be straighter and smoother in outline; they are more regular in both path and outline. Venules are generally more tortuous, and more irregular in outline and diameter. Venules are broader in diameter at the disc margin than the corresponding arterioles.
- (3) **Alternating vessels.** In principle, arterioles alternate with venules. Therefore, if the grader has measured a distinct venule, the next vessel is more likely to be an arteriole.
- (4) **Crossing.** As a general rule, arterioles do not cross arterioles and venules do not cross venules. This is a reliable guideline more than 1 DD from the disc margin (but may not always be true on or near the disc). Therefore, if a vessel of unknown identity crosses a venous branch within or distal to Zone B, then the unknown vessel is an arteriole. If it crosses an arteriolar branch within or distal to Zone B, then it is a venule. This rule is crucial in identifying small vessels.
- (5) **Parent vessel.** Smaller branches can be identified by tracing them proximally to their branching from a parent vessel, the identity of which may be evident from the first two guidelines. Angles between vessels may be useful in differentiating crossings and branchings. Crossings are frequently almost perpendicular (90°) or, if the two vessels are coursing in parallel, the angle of the crossing may be very shallow (less than 30°). Branchings are usually somewhat less than perpendicular (with the angle between the two branches from 30° to 45°).

Vessel Measurement Guidelines

The grader determines the validity of a measurement by the visual display and the width and standard deviation values. More significance should be given to the visual display.

An acceptable measurement is required to have the following:

1. The visual vessel trace should have no obvious outliers from the visible edges of the vessel.
2. The length of the measured segment should be as long as possible through Zone B for each particular vessel. A reasonable length will depend on the branching of the vessel and the photographic quality of the digital image.

All measurements must be taken proximal to all visible branching regardless of the length of the trunk or the length of the measured segment. If the trunk length in Zone B is short, it may be difficult to get a reliable measurement. In this case, a Comment should be added to the saved data indicating the location of the possibly suspect vessel width. *If the trunk is ungradable, then both branches should be measured instead of the trunk.*

The grader should not always attempt to optimize the automated measurement since it may be difficult to agree on what segment represents a “normal” width. Perceived variations in vessel edges should be allowed, especially if the measured segment is relatively long through Zone B, but only if the variation is not what should be considered an outlier. Variations may be due to normal anatomical variation of the vessel, undulations of the vessel, or minor image quality issues.

If it is clear that a vessel width is affected by a crossing (e.g. arterio-venous nicking) or obscured by nerve fiber, then those affected segments should be deleted and the grader should determine the most representative segments to be measured.

More than 6 vessels of each type may be measured and saved; the formula will automatically be based on the 6 largest measurements of each vessel type.

To maintain consistency and reproducibility, it is necessary to keep grader interactions to a minimum. The following priority order should be used when correcting the initial automated display:

Grader Interaction Order of Priority

1. No interaction; accept the automated measure through Zone B
2. Automated measure **truncated** and/or **proximal chopped** before a branching, or a crossing if necessary, or an area of outlying measurements.

Automated measure deleted (if displayed) and:

3. Vessel **seeded**. The starting point of the seed should be as close to the inner gridline of Zone B as possible to produce as long a measured length as possible. If the seed is unsuccessful, the splat view may be used to indicate the appropriate measuring area.
4. Vessel **drawn** in a similar manner as the seed. Vessel Draw is often a more expedient and accurate way to measure problematic vessels.
5. **Width** tool used to seed problematic vessels, e.g., larger vessels with central reflex or vessel edges affected by image quality.

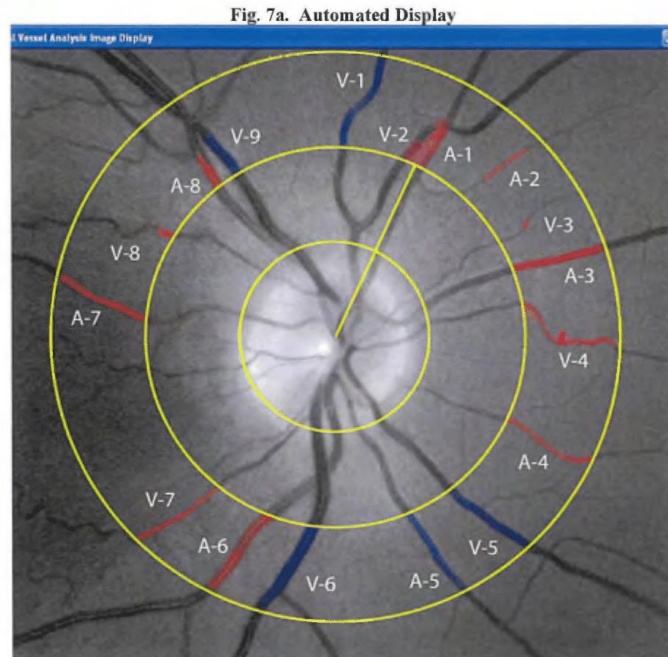
Note:

Errors introduced by the grader may significantly affect the calculated results or reproducibility of the data. For this reason, it is important to adhere to the grading guidelines and to give special attention to the following:

- Grid placement
- Vessel typing
- Measuring trunk vs. branches
- Deleting all automated “garbage” values
- Not overlooking significant vessels
- Potential for more than one automated measure on one vessel
- Typing and measuring small vessels in order to get 6 largest vessels of each type

It is recommended that the grader completely review all the vessel traces and corresponding vessel data on the control window before saving.

COMPLETE GRADING EXAMPLE

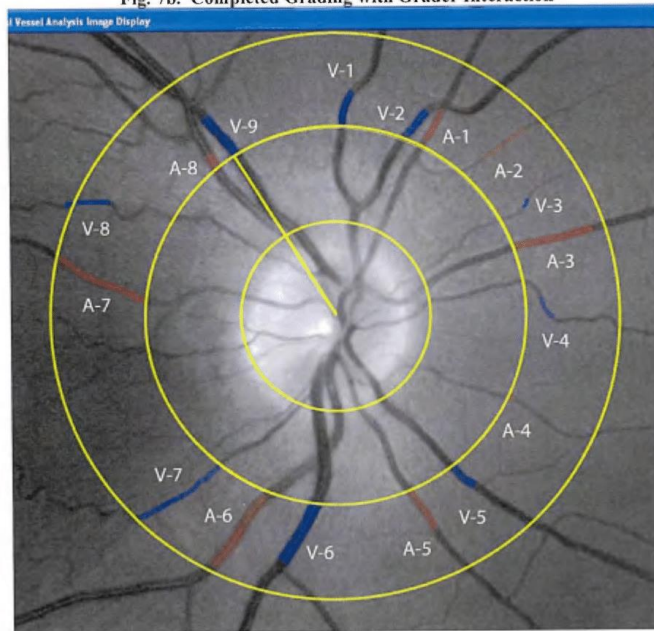


Grader Operations

A1 – delete/artery seed/truncate (before branch)	V1 – truncate (before branch)
A2 – no change	V2 – delete/vein seed/truncate (before crossing)
A3 – no change	V3 – change type
A4 – truncate (before branch)	V4 – change type/proximal chop/truncate (outliers)
A5 – change type/truncate (before branch)	V5 – truncate (before branch)
A6 – no change	V6 – truncate (before branch)
A7 – no change	V7 – change type
A8 – truncate (before outlier)	V8 – delete /vein seed
	V9 – no change

COMPLETE GRADING EXAMPLE

Fig. 7b. Completed Grading with Grader Interaction



COMPLETE GRADING EXAMPLE

Fig. 7c. Final Artery Data

Retinal Vessel Analysis Control

RL# 1001 IMAGE: 601729010.tif (5 of 5 in batch)

Arterioles	Venules	Unknown				
Angle	Width	Sigma				
1	027	060.47	005.22	Change T		
2	043	036.61	003.60	Change T		
3	068	072.90	004.54	Change T		
4	115	045.89	002.95	Change T		
5	157	069.69	004.39	Change T		
6	199	103.15	004.78	Change T		
7	275	064.92	008.27	Change T		
8	322	103.50	003.64	Change T		
9				Change T		
10				Change T		
11				Change T		
12				Change T		

Adjust Image: ZOOM IN, ZOOM OUT, Contrast, Splats, Full Image

Adjust Overlay: Vessel Trace, Show Zones, Move Disk

Instructions: Calculate

Interaction: Add Vein Seed, Add Artery Seed, Draw Vein, Draw Artery

Fig. 7d. Final Vein Data

Retinal Vessel Analysis Control

RL# 1001 IMAGE: 601729010.tif (5 of 5 in batch)

Arterioles	Venules	Unknown				
Angle	Width	Sigma				
1	002	073.65	003.41	Change T		
2	021	060.29	003.95	Change T		
3	060	037.18	003.71	Change T		
4	079	042.46	003.12	Change T		
5	143	080.59	002.90	Change T		
6	186	120.32	003.90	Change T		
7	218	048.25	005.15	Change T		
8	297	045.87	003.95	Change T		
9	328	102.07	005.13	Change T		
10				Change T		
11				Change T		
12				Change T		

Adjust Image: ZOOM IN, ZOOM OUT, Contrast, Splats, Full Image

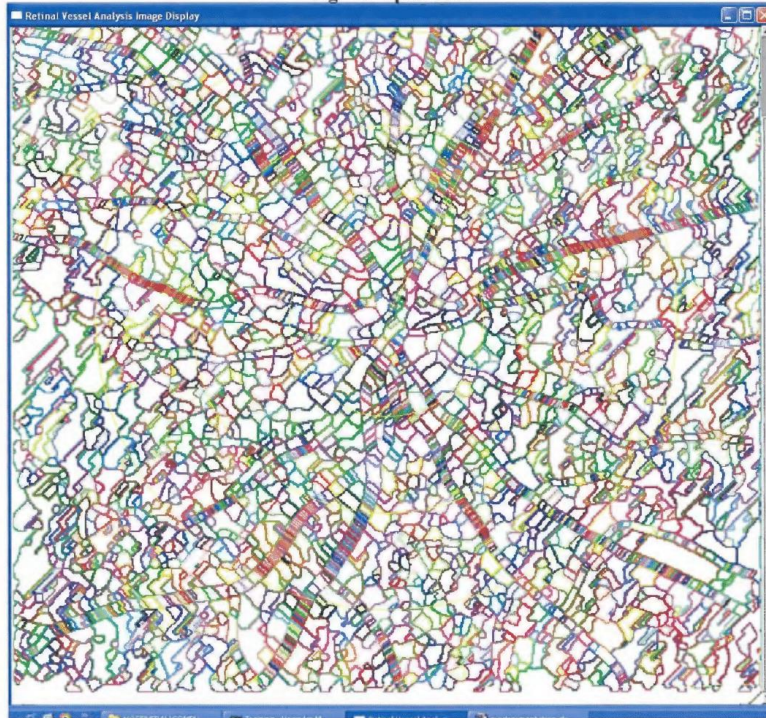
Adjust Overlay: Vessel Trace, Show Zones, Move Disk

Instructions: Calculate

Interaction: Add Vein Seed, Add Artery Seed, Draw Vein, Draw Artery

COMPLETE GRADING EXAMPLE

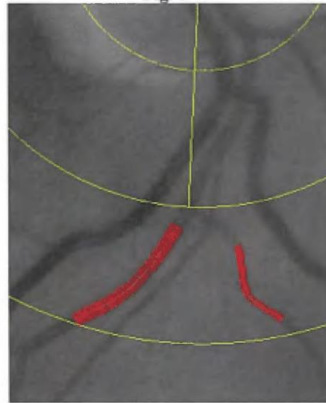
Fig. 7e. Splat View



With practice, the grader can discern the vessel pattern and the feasibility of accurate measurements from viewing the splat model.

BRANCHING EXAMPLES

Fig. 8

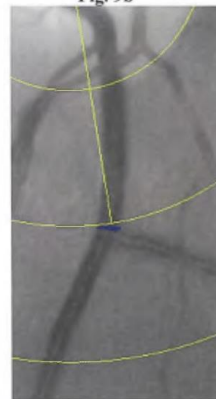


When the Zone B gridline falls at the point of branching (see Fig. 8), then the vessel branches should be measured as trunks.

Fig. 9a



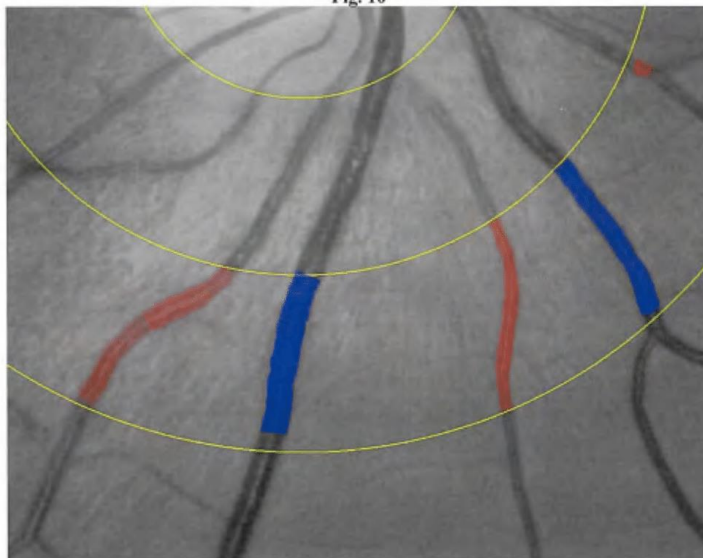
Fig. 9b



All trunks should be measured before branching even if the measured segment is very short. The automated vessel trace in Fig. 9a is truncated before the branch to produce the very short but acceptable measured segment in Fig. 9b.

BRANCHING EXAMPLES

Fig. 10



If suspected branches are impossible to identify with certainty and are of small size, then the grader should not truncate the vessel and instead measure as long a segment as possible. (see Fig. 10)

DATA SAVING OPTIONS

Saving of Data

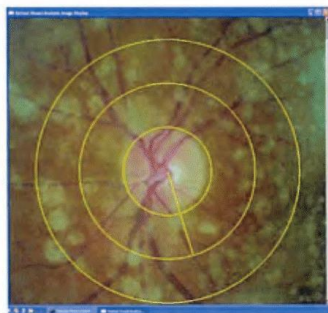
At the completion of grading, there are 3 options for saving the data:

1. REJECT – NEXT
2. APPROVE – NEXT
3. COMMENT/APPROVE - NEXT

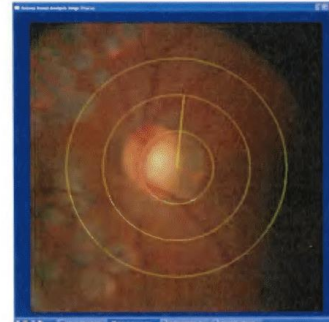
The REJECT-NEXT option is used for images that are ungradable and no comment is necessary. Images that are ungradable very often will have no acceptable automated vessels on the initial display and the grader will have difficulty discerning any vessels. **Eyes that have <4 acceptable measurements of either vessel type will be considered ungradable.**

The APPROVE-NEXT option is used for images that are gradable and no comment is necessary. The COMMENT/APPROVE - NEXT option is used for images that are gradable but had grading discrepancies that may affect the consistency of the data. These comments will be evaluated by the statisticians.

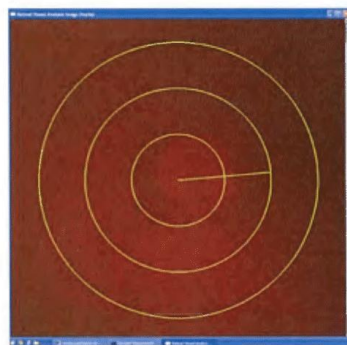
REJECT-NEXT EXAMPLES (Ungradable)



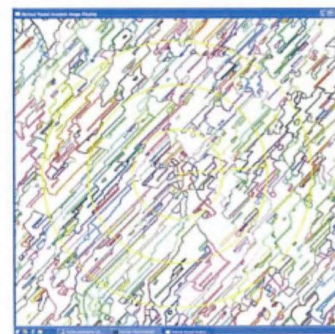
11a. Confounding pathology



11b. Out of focus/too dark/other



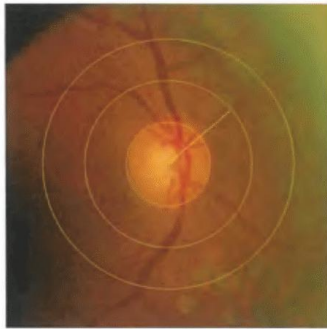
11c. Too dark



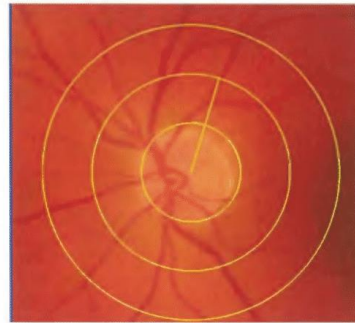
11d. No vessels visible in splat display

Pathologies like asteroid hyalosis (Fig. 11a) may obscure vessels and compromise measurements. Eyes with narrowed and sheathed arterioles clearly due to a pathology other than a cardiovascular condition, e.g. retinitis pigmentosa, should be rejected. Poor illumination may cause the image to be too dark for automated measures or too dark for the grader to visually validate the vessel trace (Figs. 11b and 11c). Gradability is easily determined by the splat display (Fig. 11d).

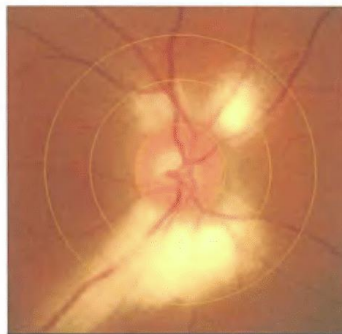
Approve with Comment Examples



12a. Poor illumination/treatment



12b. Poor focus



12c. Pathology/Ungradable arteries-veins

Affects of laser treatment on vessels should be documented with a comment (Fig. 12a). Poor focus often blurs the edges of vessels and causes falsely large measurements (Fig. 12b). Anatomical conditions such as myelinated nerve fiber may obscure significant vessels. The example shown in Fig 12c has more than one ungradable largest arteriole and venule and would not qualify for data analyses.

The SIVA grading protocol, Singapore Eye Research Institute collaborated with National University of Singapore, Singapore

We used SIVA version 3.0 to score the retinal geometric parameters; the protocol provided here is only relevant to this version.

5.4 SIVA 4.0 Grading

5.4.1 Welcome to SIVA

The Singapore I Vessel Assessment (SIVA) is a semi-automated program allowing graders to objectively assess digital retinal fundus photographs for a range of quantitative retinal vascular parameters.

Technical requirements of retinal images for SIVA grading are available at [Section 1.4.2 Image requirements].

During image grading, SIVA divides the retina into three zones with reference to the optic disc location and size.

Zone A: Region from the disc margin to $\frac{1}{2}$ disc diameter from the disc margin.

Zone B: Region from $\frac{1}{2}$ disc diameter to 1 disc diameter from the disc margin.

Zone C: Region from 1 disc diameter to 2 disc diameters from the disc margin.

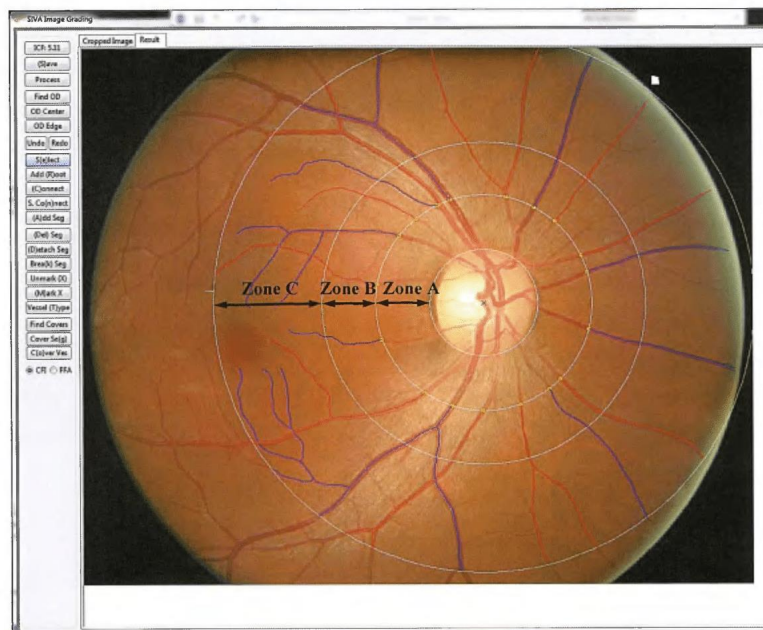


Figure 5.4.1.1 Measurement zones in SIVA

SIVA parameters are calculated based on retinal vessels in Zone B and Zone C as measurements in these zones are more stable from curvature of the eye.

附件

5.4.1.1 The SIVA interface

The SIVA grading interface has two major panels. The left panel displays the current image that is being graded, with the function buttons on the far left.

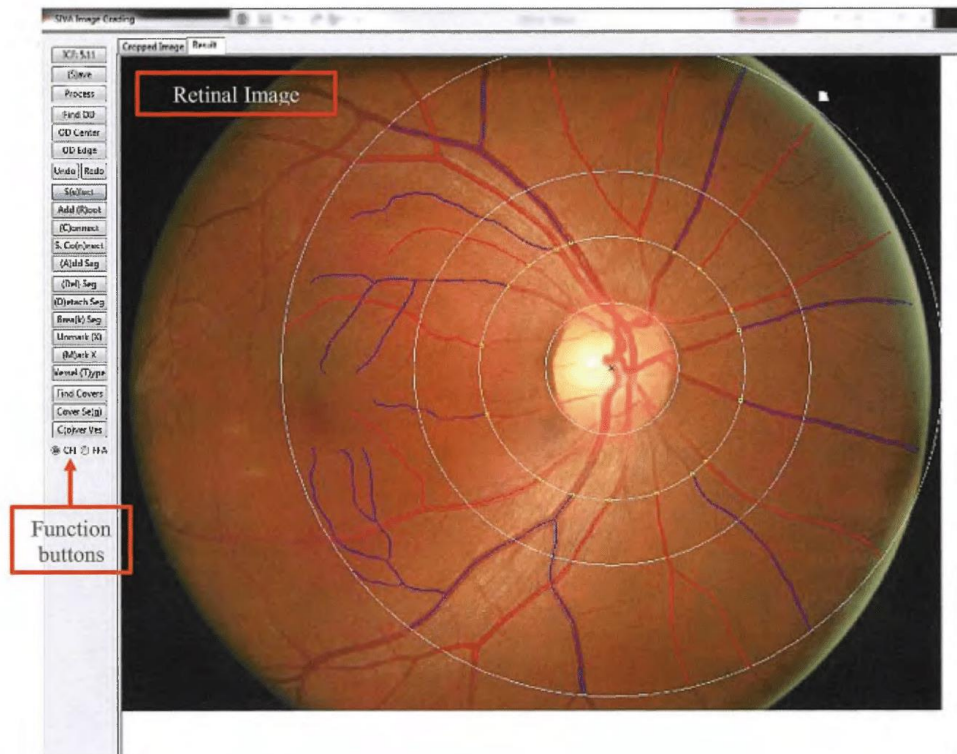


Figure 5.4.1.1.1 SIVA interface left panel

The right panel displays the vessel segment being selected or edited, the comment code panel, the vessel data panel, and a comments box.

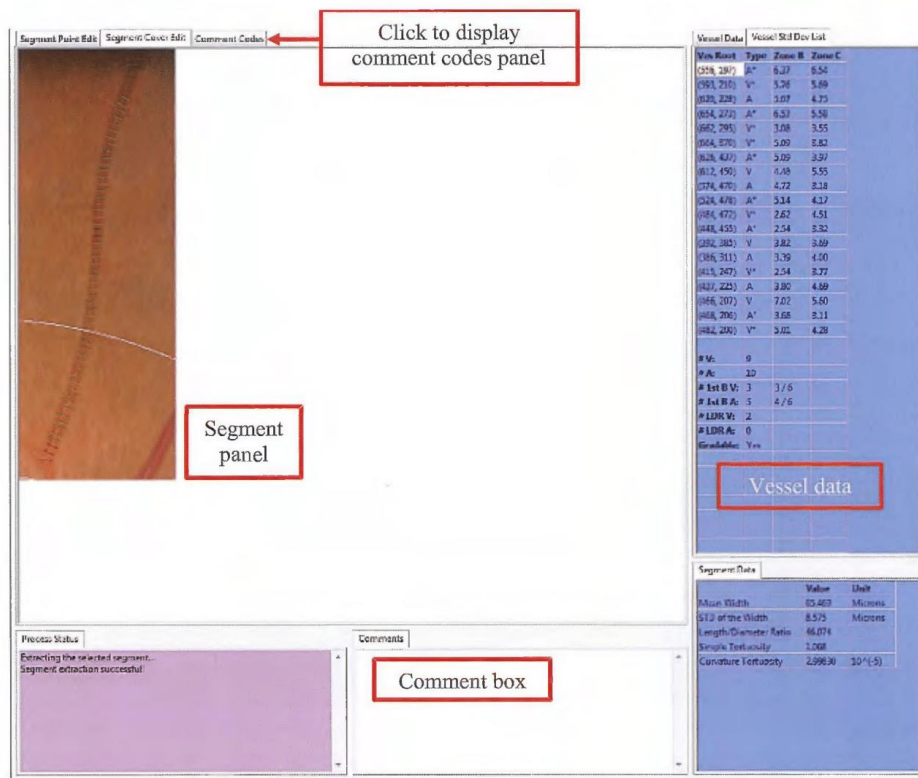


Figure 5.4.1.1.2 SIVA interface right panel

5.4.1.2 SIVA function buttons

Functions of the buttons on the left panel of the SIVA interface:

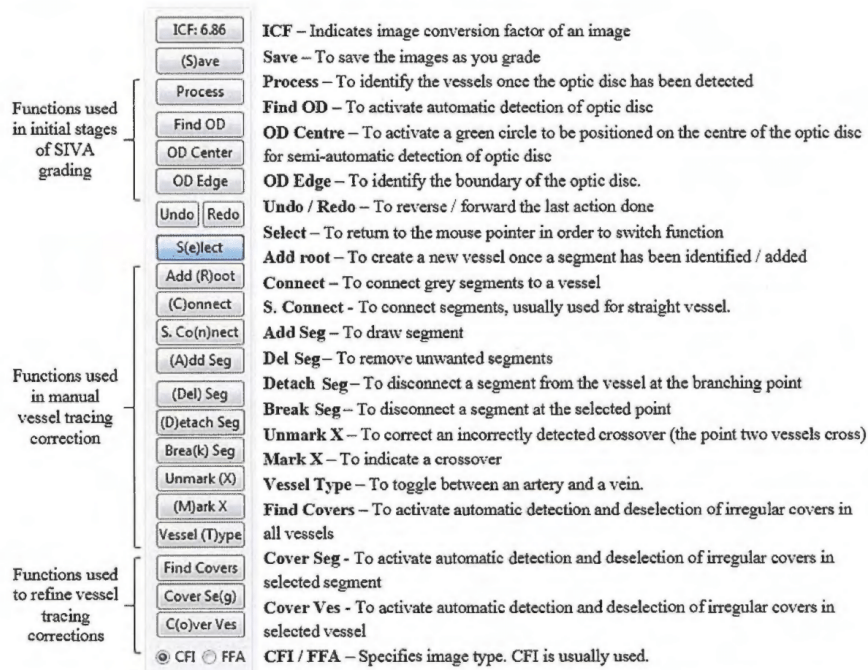


Figure 5.4.1.2.1 SIVA function buttons

Additionally, these hot keys can be used to toggle between various display modes and functions:

Hot Key	Function
F1	Toggles between colour, red-free and grayscale display mode
F2	Toggles zoning grid on and off
F3	Toggles vessel trace on and off
F4	Toggles vessel covers on and off
F5	Toggles crossover points on and off
F11	Extra segment info
F12	Toggle line image
Ctrl and +	Zoom in 放大
Ctrl and -	Zoom out 缩小
Ctrl, Alt, and =	Zoom in segment
Ctrl, Alt, and -	Zoom out segment
Ctrl and alphabet in brackets	Activates function button on left panel

5.4.1.3 Visual inspection for image gradability

Image field requirements

For best results, retinal images chosen for SIVA grading should be Field 1 (Optic disc-centred) images with the optic disc as centralised as possible. This ensures that both Zone B and C are well within the limits of the field of view.

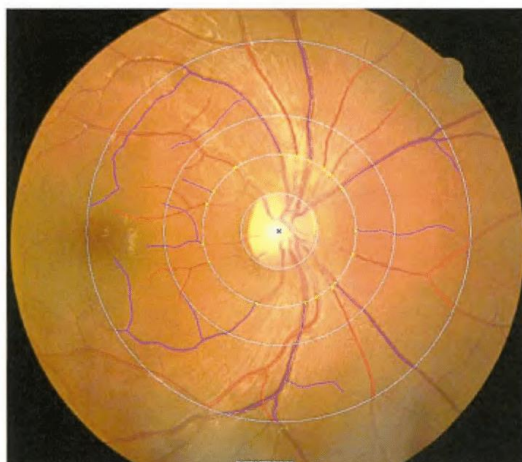


Figure 5.4.1.3.1 Gradable retinal image with centralised optic disc

Images are acceptable for SIVA grading if the cut-off region takes up less than half of a quadrant in Zone C.

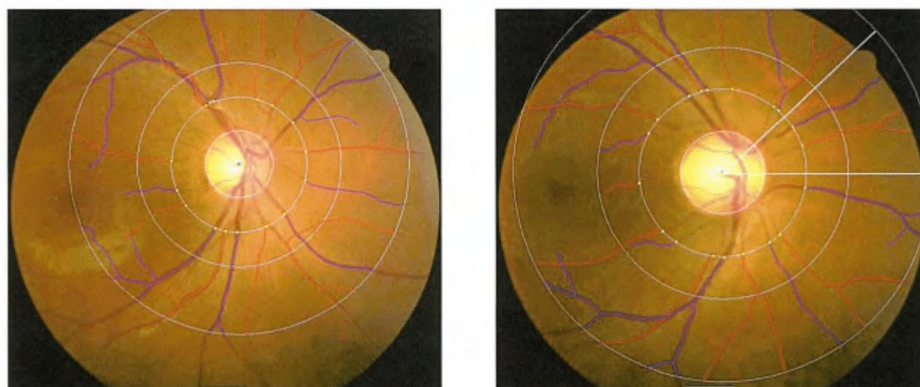


Figure 5.4.1.3.2 Gradable retinal image with partial Zone C cut off

Images are not suitable for SIVA grading if the cut-off region takes up more than half of a quadrant in Zone C.

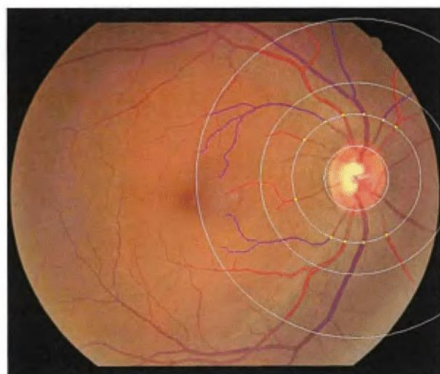
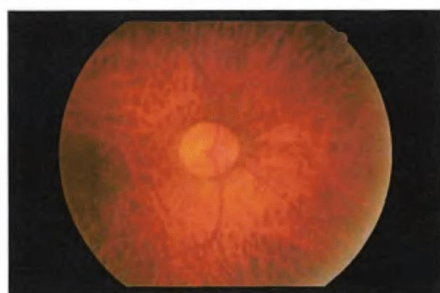


Figure 5.4.1.3.3 Ungradable retinal image with partial Zone C cut off

Vessel number requirements

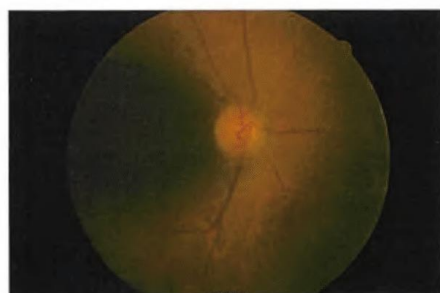
For best results, a minimum of at least **4 arterioles AND 4 venules** must be clearly visible for grading. Examples of ungradable retinal photos are provided below.



Cataract and retro-illumination obscuring vessels



Photocoagulation scars obscuring vessels



Not fully dilated pupils, field too dark for grading



Confounding pathology

Figure 5.4.1.3.4 Ungradable retinal images and reasons (*italics*)

For selected images with compromised visibility of the retina, the image remains acceptable for grading if at least 4 arterioles AND 4 venules can be clearly seen. It is advisable to document these images as “Force grade” in the Comment codes panel.



Figure 5.4.1.3.5 Force grade images with partial obstruction

5.4.2 Optic disc detection

5.4.2.1 Open image

To open images for grading in SIVA 4.0, please refer to [Section 5.3.2 Navigate the Grader’s Client].

5.4.2.2 Automated OD detection

While opening the image in SIVA 4.0, the optic disc and vessel paths are automatically detected and traced.

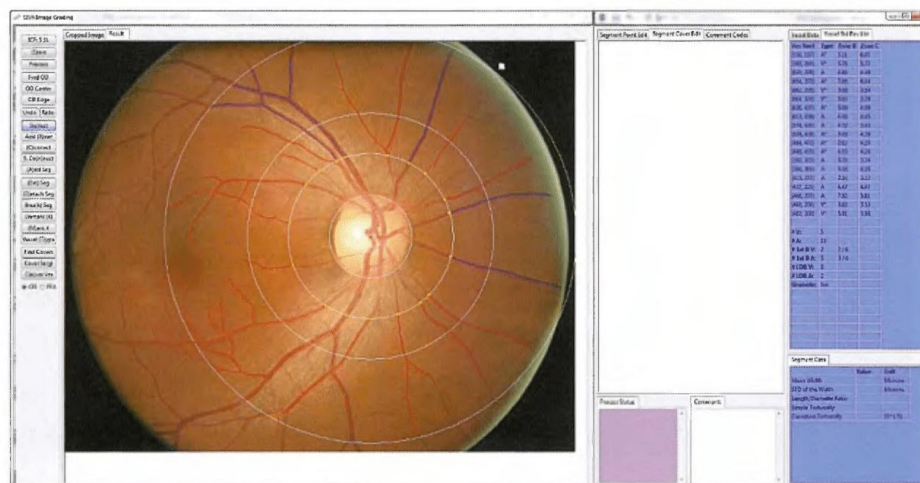


Figure 5.4.2.2.1 Automatic optic disc detection and vessel tracing by SIVA

ODs are considered correctly located and measurement grid correctly placed when:

- The innermost circle lies directly on or outside of the optic disc rim 边缘、圆圈
- The entire optic disc lies within the innermost white circle

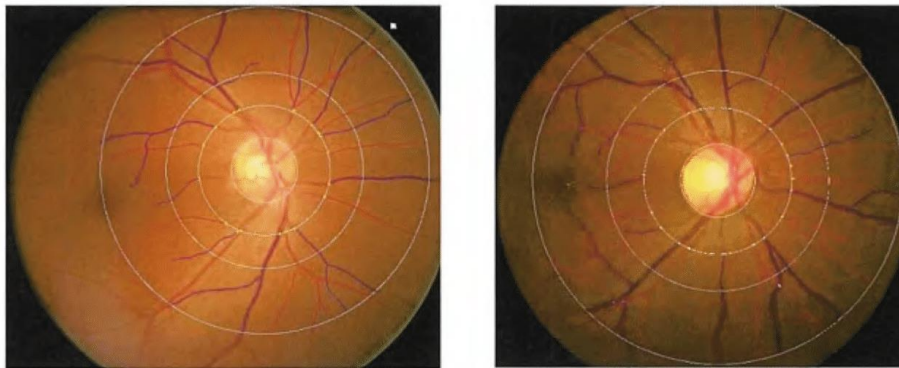
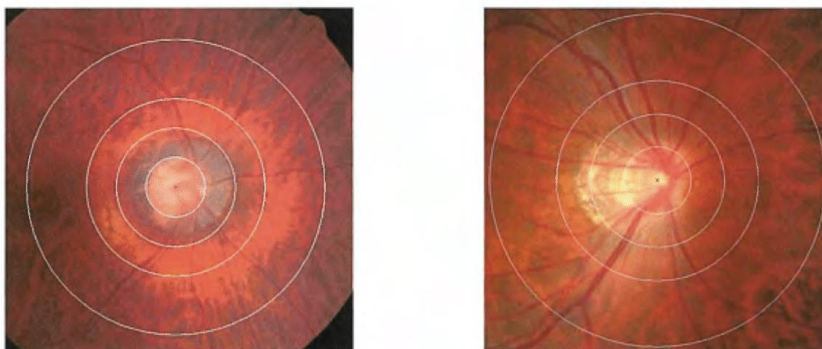


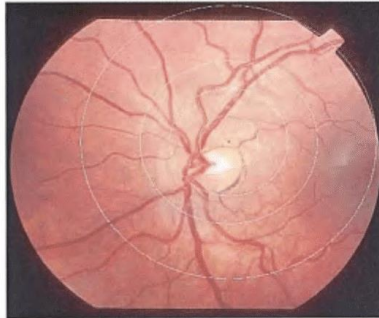
Figure 5.4.2.2.2 Correctly detected and placed grids on optic disc

For ODs with myopic, or peripapillary atrophic changes, identification of accurate OD location and measurement grid placement may be challenging (see examples of correct location and placement below).

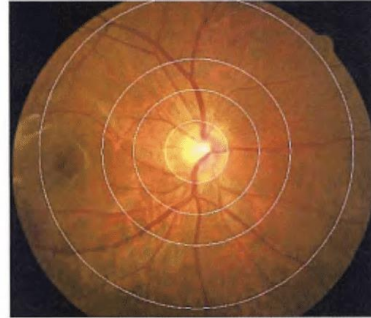


Disc with peripapillary atrophy 视乳头萎缩 Myopic disc 近视
Figure 5.4.2.2.3 Correctly detected and placed grids on abnormal optic discs

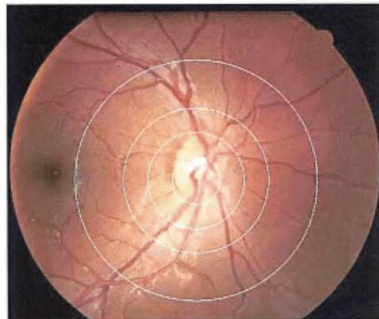
If the optic disc is not correctly located and/or measurement grid not correctly placed (as in the examples below), manual correction will be required.



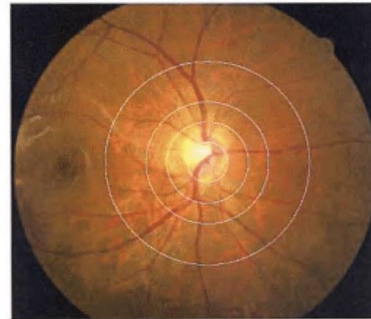
Grid not on optic disc



Grid not centred on optic disc centre



Grid not on disc margin



Grid does not cover entire optic disc

Figure 5.4.2.2.4 Incorrectly detected and placed grids

5.4.2.3 Manual OD correction

Specify OD centre

- Click “OD Center” on the left function panel. A green circle will appear to replace the mouse cursor. 光标
- Using your mouse, move the green circle on the centre of the OD, and left-click to fix the green circle.

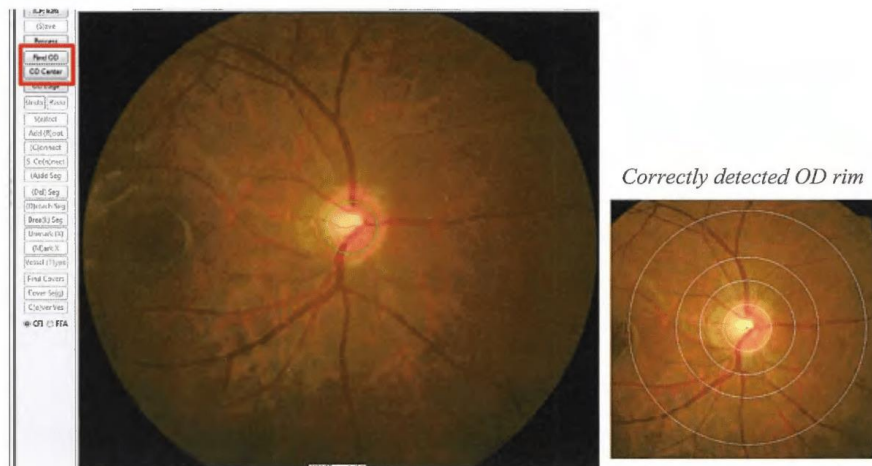


Figure 5.4.2.3.1 Specify OD centre

- Click “Find OD” to prompt the software to detect the OD rim based on the new OD centre position.
- Check if the white circle on the measurement grid outlines the OD rim completely. If yes, click “Process” to prompt the software to automatically trace vessels.
- If the grid is still not correctly placed, retry by repeating the previous three steps.
- If OD detection is still inaccurate after 5 attempts, please proceed to specify OD edge [Specify OD centre and edge].

Specify OD centre and edge

- Click “OD Center” on the left function panel. A green circle will appear to replace the mouse cursor.
- Using your mouse, move the green circle on the centre of the OD, and left-click to fix the green circle.
- Click “OD Edge”, using the mouse cursor, click again to mark the OD rim.

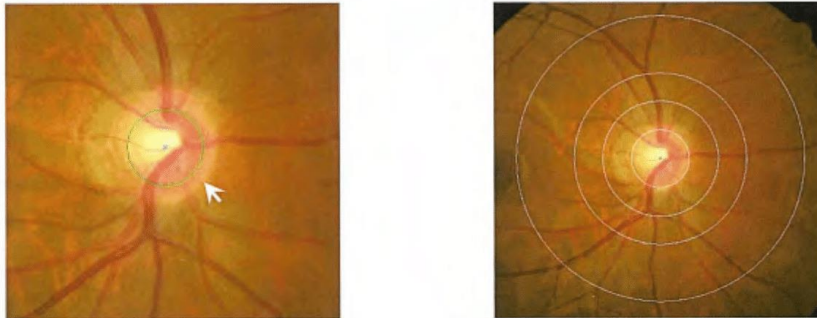


Figure 5.4.2.3.2 Specify OD edge

- Check if the innermost white circle on the measurement grid marks the OD rim correctly.
- Repeat previous steps until OD is correctly marked.
- Click “Process” to prompt the software to automatically trace vessels.

5.4.3 Vessel tracing

5.4.3.1 Automated vessel tracing

SIVA automatically detects and traces vessels within the measurement grid (0.5 to 2.0 OD diameters from OD centre). This helps to improve grading efficiency by reducing amount of work required from each grader and improves consistency by detecting similar vessels for the same subject.

Automated vessel tracing at this stage is not completely accurate and needs to be manually corrected.

Automated vessel tracing before manual correction: *Vessel tracing after manual correction:*

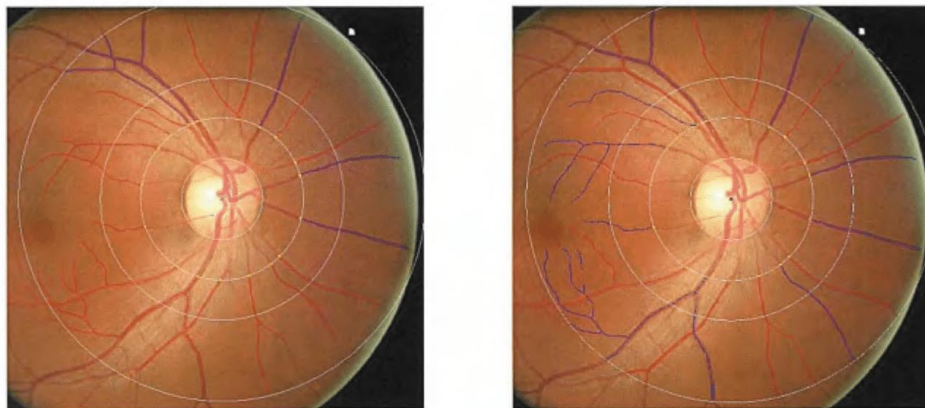


Figure 5.4.3.1.1 Vessel tracing using SIVA

In order to ensure that all vessels on the image are checked and graded, it is good practice to begin grading by inspecting the vessel starting from the 12 o'clock position, and correcting each vessel in a clockwise manner. This sequence of vessels is also reflected in the "Vessel Std Dev List" panel.

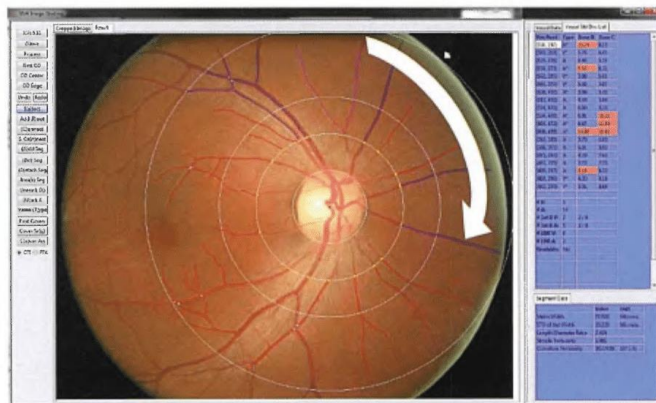


Figure 5.4.3.1.2 Grading sequence for vessels

Identifying erroneous vessel tracing (bumps, crossovers, branching, incomplete tracing)

For each vessel traced automatically by SIVA, there may be some inaccuracies. Some of the common errors are:

A) Incorrect vessel tracing

1) Root of vessel not marked

Beginning of all vessel paths should start at the 2nd innermost white circle, and is marked by a yellow square.

2) Vessel path incompletely traced

All vessels should be traced till the edge of the outermost white circle or till the end of the vessel path.

3) Bumps on vessel path

Vessels tracing should be smooth and follow shortest path along the vessel.

4) Vessel paths inaccurately traced at branching or crossover points

Vessel tracing near branching or crossover points have higher tendency to be inaccurate.



Figure 5.4.3.1.3 Common vessel tracing errors

B) Wrongly labelled branching or crossover points

C) Wrongly labelled vessel type

Please refer to the section below for guidelines on how to differentiate between arterioles and venules.

Differentiating between arterioles and venules

Some key guidelines in differentiating between arterioles (labelled red) and venules (labelled blue):

- Venules are in general darker in colour compared to arterioles.
- Venules are generally wider than arterioles.
- Arterioles do not cross each other, same with venules.

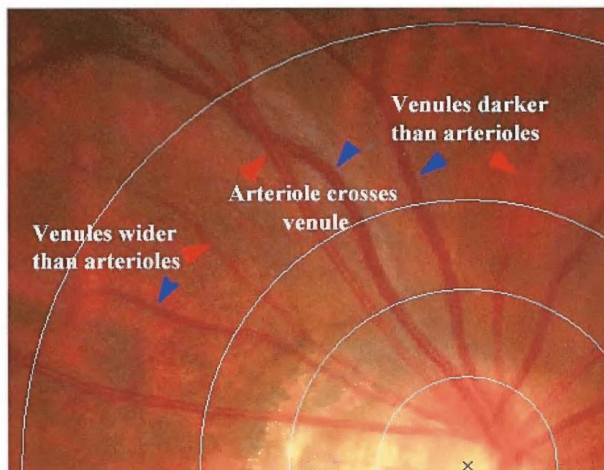


Figure 5.4.3.1.4 Differentiating arterioles (red arrowheads) and venules (blue arrowheads)

5.4.3.2 Manual vessel tracing correction

Add root

Vessels without a root identified are marked in grey, and inactive.

- Ensure that the vessel path reaches the second innermost white circle (Zone B inner border).
If not, use [\[Add segment\]](#) to extend vessel tracing. *ctrl+A*
- Click “Add (R)oot”. *ctrl+R*

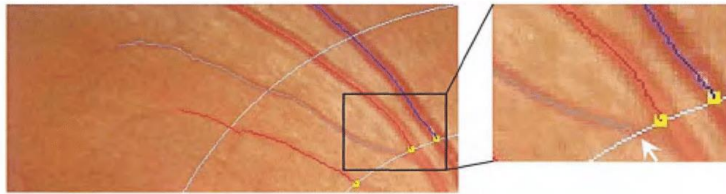


Figure 5.4.2.3.1 Add vessel root

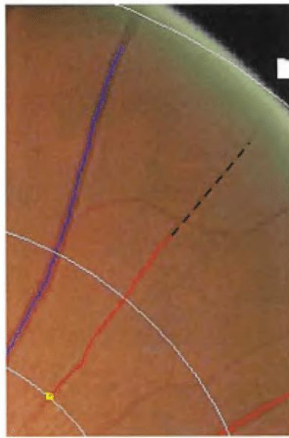
- Using the cursor, left click at the end of the vessel path to mark it as a root. This point should be as close to the Zone B inner border as much as possible.
- Once the vessel root has been marked, the vessel line will change to red (for arterioles) or blue (for venules).

Add segment

Use this to trace vessel paths not automatically traced by SIVA

- Click “(A)dd Seg”.
- Using the cursor, hover over the centre of the vessel to be traced.
- Left click to start tracing, and continue to left click at points along the vessel to trace the vessel path.
- With each left click, a green square marks the last point placed.

Before add segment



During add segment



Figure 5.4.2.3.2 Add segment

- Continue till you reach the end of the vessel or the outermost white circle (Zone C outer border).
- To end, click “Select” or press “Esc”.

Connecting segments

Use this to connect vessel segments. Can be used to connect adjacent segments or connect branch segments to the main vessel.

- Click "(C)onnect". This changes the cursor to a green crosshair (+).
- Using the cursor, click on the end of the grey (inactive) vessel segment you want to connect. The selected vessel segment is now marked yellow.
- Left click the position on which you want the yellow vessel segment to connect to.

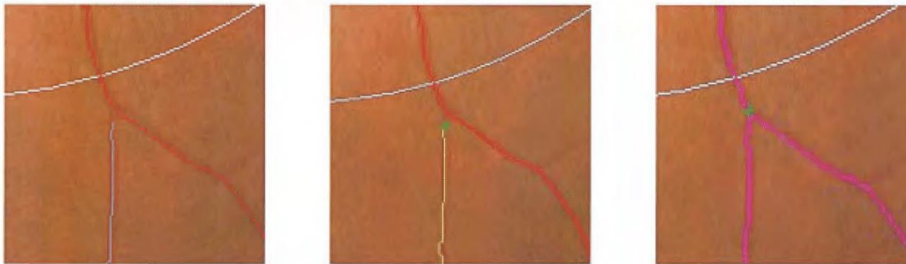


Figure 5.4.2.3.3 Connect segment

Detach segment

Use this to separate vessel segments not correctly attached or traced at a connected point.

- Click “(D)etach Seg”. This changes the cursor to a green crosshair (+).
- To detach all branching vessels from the parent vessel, left click at a point on the parent vessel as close to the branches as possible.

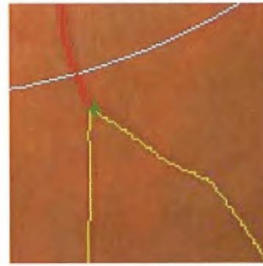
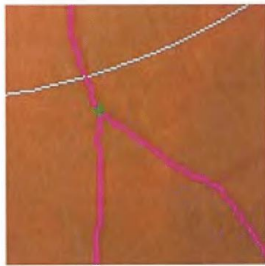


Figure 5.4.2.3.4 Detach branching vessels

- To detach a single branching vessel from the parent vessel, left click at any point on the branching vessel you want to detach.

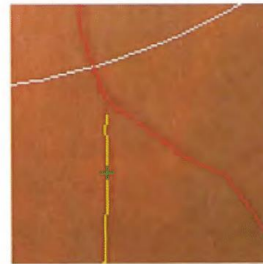
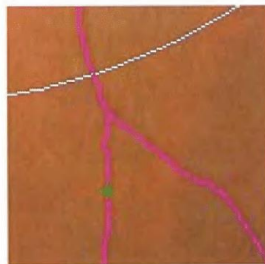


Figure 5.4.2.3.5 Detach branching segment

Break segment

Use this to break segments that need to be removed or re-traced.

- Click “Select”. Using the cursor (+), left click at the point on the vessel trace you want to break.
- Click “Brea(k) Seg”. The vessel trace will be disconnected at the point selected.



Figure 5.4.2.3.6 Break segment

Delete segment

Use this to remove unwanted vessel segments.

- Click “Select”. Using the cursor (+), left click on the vessel segment to be deleted.
- Click “(Del) Seg” to delete.

Mark/unmark crossover

Crossovers are defined as the point at which two vessels cross each other.

In the figure below, vessels in the red box are correctly traced, but wrongly identified as all venules due to the crossover point (white circle) being mis-identified as a branching point.

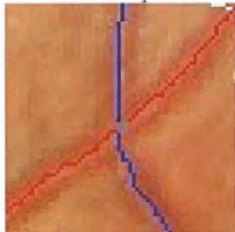


Figure 5.4.2.3.7 Mis-identified vessel crossovers

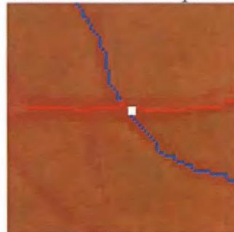
All points at which vessel traces cross or join which each other are marked by square dots. Toggle F5 to display/hide the square dots.

- Automatically detected crossover points are marked by grey square dots. 自动一灰.
- Branching points are marked by orange square dots. 分支一橘.
- Manually marked crossover points are marked by white square dots. 交叉一白.

*Automatically detected
crossover point*



Marked crossover point



*Branching point
(unmarked crossover)*

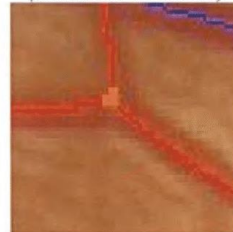


Figure 5.4.2.3.8 Crossover and branching points

To label a point as a crossover point:

- Click "Select". Using the cursor (+), left click on the square dot to select the crossover point.
- Click "(M)ark X" to change the square dot to white. *Handwritten: M, 2X*

To label a point as a branching point:

- Click "Select". Using the cursor (+), left click on the square dot to select the crossover point.
- Click "Unmark (X)" to change the square dot to orange. *Handwritten: X, 172*

Changing vessel type

To change vessel type from venule to arteriole, or arteriole to venule:

- Click "Select". Using the cursor (+), left click any point on the vessel to select the vessel.
- Click "Vessel (T)ype" to change vessel type. *Handwritten: T.*

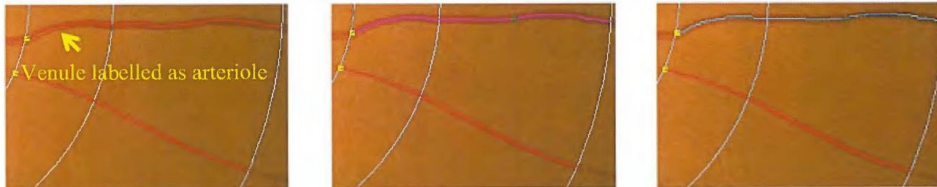


Figure 5.4.2.3.9 Change vessel type

5.4.3.3 Vessel covers

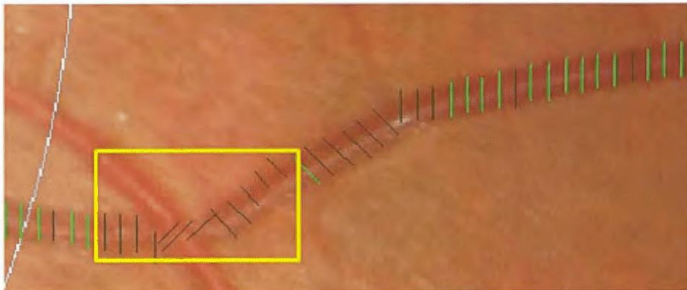
After vessel tracing is complete, vessel covers need to be inspected to ensure that vessel calibre (width) measurements are accurate.

Vessel covers should be placed perpendicularly to the vessel walls, and cover the entire width of the vessel.

Find covers

After vessels have been correctly identified and traced:

- Click “Find Covers” to prompt SIVA to lay covers on all vessel segments automatically.
- SIVA will also automatically deactivate vessel covers that it deems irregular.
- Active covers are green (–) and are used for measurements.
- Deactivated covers are grey (–) and are not used for measurements.



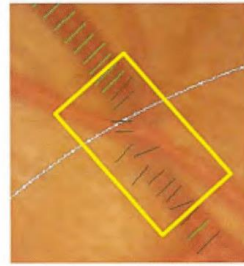
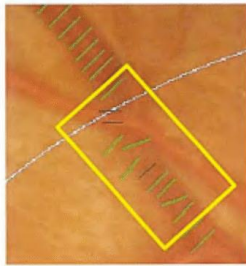
Irregular vessel covers are deactivated

Figure 5.4.3.3.1 Vessel covers

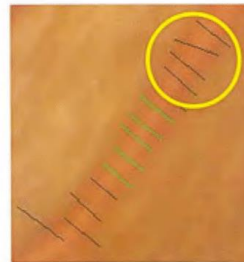
Using a similar strategy with checking vessel tracing, check all vessel segments for vessel covers.

Vessel covers should be deactivated if:

- Vessel covers are not laid perpendicular to the vessel walls
- Vessel covers do not cover the entire vessel width
- Vessel covers exceed vessel walls
- Vessel covers cover branching points



Full width of venule obscured under crossing arteriole, and covers not perpendicular to vessel walls



Covers overestimate arteriolar width

Figure 5.4.3.3.2 Deactivate irregular vessel covers

Deactivating and activating vessel covers

To deactivate vessel covers:

- Toggle F3 to display/hide vessel covers.
- Left-click and drag cursor over vessel covers to deactivate.

To activate vessel covers:

- Toggle F3 to display/hide vessel covers.
- Double-click and drag cursor over vessel covers to activate.

Minimum requirements for vessel covers

During the grading process, it is advised to make as little changes as possible to ensure that SIVA grading is reliable. Graders should leave all accurate vessel covers active.

To ensure that vessel calibre is accurately estimated, the following guidelines should be adhered to:

- Parent vessels (excluding branching vessels) must have at least 3 active covers within Zone B.
- Branching vessels must have at least 3 active covers within each segment.
- Vessels with no branching vessels must have at least 5 active covers, with at least 3 within Zone B.

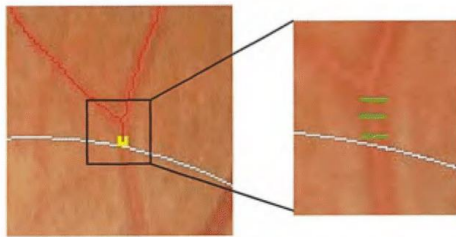


Figure 5.4.3.3.3 Minimum covers for parent vessel

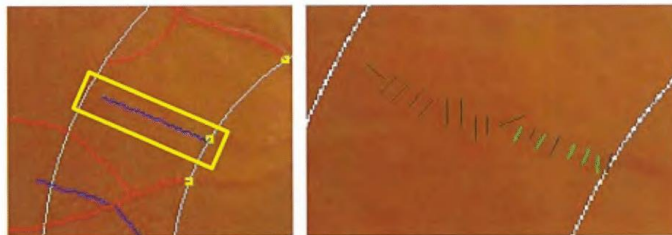


Figure 5.4.3.3.4 Minimum covers for non-branching vessel

- For parent vessels with branching points very close to the Zone B inner border with less than 3 covers automatically detected, treat each branching vessel as separate vessels with new vessel roots.

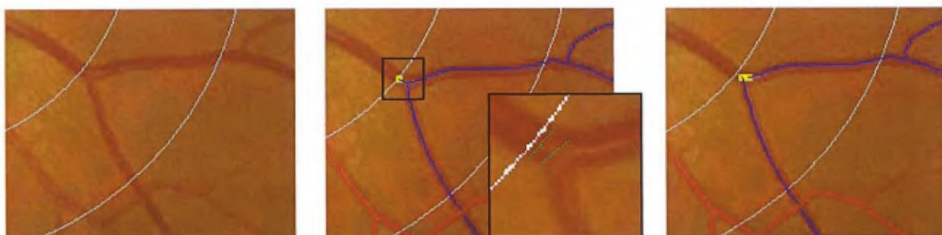


Figure 5.4.3.3.5 Parent vessel with branching points close to Zone B border

Adjusting vessel covers

Use with caution. Only manually adjust covers when all automated covers are completely misplaced, or completely under- or over-estimate vessel widths, and insufficient alternative covers are available.

- In the “Segment Point Edit” tab, black square dots mark the ends of each cover.
- Left-click on a cover ending (black dot changes to blue dot) and drag to adjust length of cover to desired length and angle.
- Left-click again to fix position of cover (blue dot changes back to black).
- To move more than one cover ending, activate them by clicking on them, click-and-drag to move cover endings in tandem.
- Right-click to confirm.

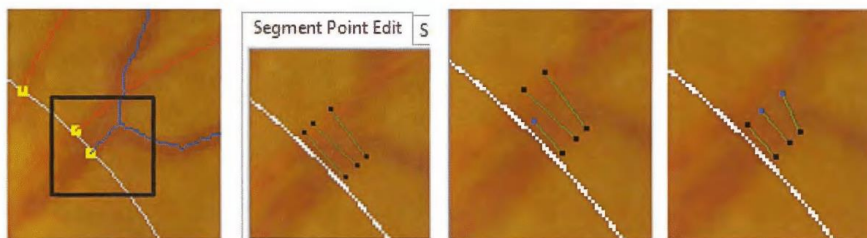


Figure 5.4.3.3.6 Adjust vessel covers

- When all covers in the vessel segment are wrong, choose only 3 covers to adjust manually.

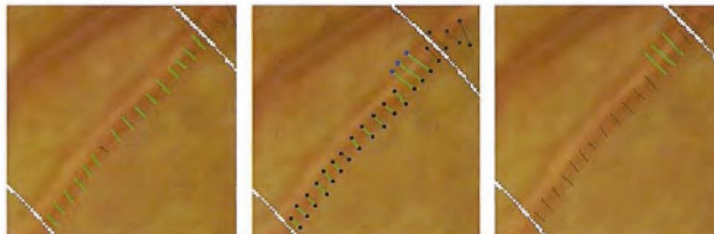


Figure 5.4.3.3.7 Adjust vessel covers

5.4.4 Comment codes

The “Comment Codes” tab is where graders can document any issues encountered during the grading process. A summary of the comment codes is provided below:

Category	Code	Check box if:
Quality	1	Poor quality image
Forced Grading	10	Length of covers were manually adjusted
Not Big 6	2	Not big 6 detected for artery
	3	Not big 6 detected for vein
	4	Less than 6 arteries detected
	5	Less than 6 veins detected
	A	Not big 6 detected in right eye, replace grading with left eye
	B	Not big 5 detected in left eye, replace grading with right eye
Optic Disc	6	OD/CE Used: OD detected automatically was not accurate and grader adjusted OD manually
Pathology	7	Confounding pathology present that affected accuracy of grading
Ungradable	9	Ungradable - less than 4 arteries or 4 veins traced
	9C	Ungradable - more than half of 1 quadrant in Zone C cut off
	9D	Right eye ungradable, replace grading with left eye
	9E	Left eye ungradable, replace grading with right eye
Replace	F	No right eye, replace with left eye
	G	No left eye, replace with right eye
Other	8	Other comments
	8C	Gradable – but less than half of 1 quadrant in Zone C cut off
	8A	Possible alternate choice in vessel trace

5.4.5 Send, save and discard grading data

- When image grading is complete, click “Save” to save image grading.
- Close window using “×” button.



Figure 5.4.5.1 Save grading results

- In the pop-up window, select “Send”, “Save” or “Discard”.

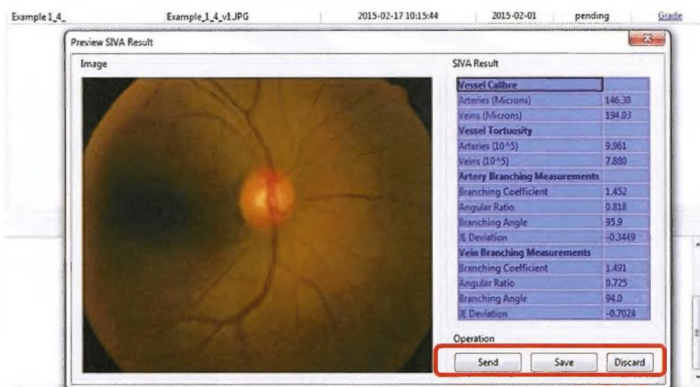


Figure 5.4.5.2 Send, save or discard grading results

- If grading is finalised and ready for sending, select “Send”
- If grading is not finalised, to save results for grading to be continued at a later time, select “Save”.
- If grading is erroneous and needs to be discarded, select “Discard”.

Appendix C: Additional papers arising from this PhD

Three publications arise from this PhD that I contributed equal to or less than 50% of the work. Details of these papers are:

- 1) Lovern C*, **Liu M***, Lycett K, Wake M, Wong TY, He M, Burgner D. Are markers of inflammation related to retinal vascular calibre? A systematic review and Meta-analysis. This paper will be submitted to the *JAMA Ophthalmology* for peer review.

* Lovern C and Liu MJ contributed equally to this work.

- 2) Dascalu J, **Liu M**, Lycett K, He M, Wong TY, Wake M. Epidemiology and concordance of retinal vessel calibre in 11–12 year old children and their parents. *BMJ Open* 2019; **9**: 44-52.
- 3) Wang J, **Liu M**, Sung V, Lycett K, Burgner D, Wong TY, Wake M. Associations of retinal vessel calibre and hearing status in childhood and in midlife: A cross-generational population-based study. *JAMA Otolaryngol Head Neck Surg.* 2020.

The association between markers of inflammation and retinal microvascular calibre: A systematic review and meta-analysis

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Key points

Question: Are inflammatory markers associated with retinal microvascular calibre?

Findings: In a meta-analysis, we found a moderate association between C-reactive protein and white blood cell counts (WBC) and wider (i.e. worse) retinal venular calibre, and weak associations with narrower (i.e. worse) arteriolar calibre. Findings were similar in studies using other inflammatory markers.

Meaning: The association between inflammatory markers and greater venular calibre suggests a pathological mechanism contributing to the reported associations between retinal microvasculature calibre and cardiovascular disease. Retinal vascular calibre may provide insights into the inflammatory pathophysiology of preclinical vascular phenotypes.

Abstract

Importance: Adverse changes to the microcirculation play an important role in the pathogenesis of cardiovascular disease. Observational and trial data support inflammation as a key causal mechanism in cardiovascular disease pathogenesis. However, the population-level relationship between inflammation and microvascular parameters is largely unknown.

Objective: To investigate the relationship between systemic inflammatory markers and retinal microvascular phenotypes.

Data Sources: We identified studies in Medline, Embase and PubMed for the following topics 1) retinal microvascular calibre, 2) inflammatory markers, and 3) observational studies.

Study Selection: Two researchers independently identified studies drawn from general population samples and individuals with cardiometabolic and related diseases ('patients'). If data on retinal calibre and an inflammatory marker was available, the study was included.

Data Extraction and Synthesis: Of 1,920 studies identified, 28 met inclusion criteria (general population 19, patients 9). Two researchers independently extracted data following the MOOSE guidelines.

Main outcome(s): Correlation coefficients of inflammatory markers and retinal microvascular diameters.

Results: A variety of inflammatory markers were reported, most commonly C-reactive protein and white blood cell count. Of these studies, twenty on CRP (general population 5, patients 5) and eight on WBC (general population only)

reported either correlation coefficients or linear regression coefficients that were amenable for meta-analysis. We also undertook a narrative review of all 28 studies. There was consistent evidence of a modest association between CRP and venular calibre, with similar effect sizes in the general population ($r=0.08$, 95% CI 0.04 to 0.11) and in patients ($r=0.03$, 95% CI -0.06 to 0.12). There was weaker evidence of an association between CRP with retinal arteriolar calibre ($r=0.01$, 95% CI 0.00 to 0.03) in the general population and in patients ($r=-0.06$, 95% CI -0.18 to 0.06). Similarly, WBC had stronger associations with venular ($r=0.18$, 95% CI 0.04 to 0.32) than arteriolar calibre ($r=0.05$, 95% CI 0.01 to 0.09) and the association with arteriolar calibre was in the opposite direction to that hypothesized. Narrative review of other biomarkers showed consistent findings.

Conclusions and Relevance: Inflammation has a greater effect on retinal venular than arterial calibre. The findings suggest a potential mechanism contributing to microvascular damage which has been overlooked in CVD pathogenesis.

Introduction

Despite the historical focus on obstructive atherosclerosis of the large muscular arteries, adverse changes to the microcirculation are increasingly recognized to contribute to the pathogenesis of CVD¹. For example, in large studies of patients presenting with non-fatal myocardial infarction (MI), over one fifth had non-obstructive lesions of the coronary arteries², suggesting that additional pathogenic mechanisms are involved^{3,4}. In ischemic heart disease, structural and functional changes affect the entire coronary circulation, including the microcirculation, which is increasingly emerging as a therapeutic target¹. Understanding the mechanisms underlying adverse microvascular changes is therefore important to guide prevention and interventions.

Assessment of the microcirculation *in vivo* has traditionally been difficult and invasive, which precluded investigation in population-based studies^{5,6}. Recent technological advances now allow noninvasive quantification of microcirculatory parameters (particularly arteriolar and venular calibre) from retinal imaging. The retinal microcirculation shares anatomical and physiological properties with cerebral and coronary microvasculature^{7,8}, may reflect changes in the systemic microcirculation⁹⁻¹⁶ and is predictive of CVD events^{15,17}.

Inflammation has a pathogenic role in all stages of the development of CVD^{18,19,7,9}. Two recent randomised control trials have provided direct evidence supporting the 'inflammatory hypothesis' of CVD in humans by demonstrating a reduction in CVD beyond that associated with lipid-lowering by treatment of high risk patients with anti-inflammatory agents. IN the CANTOS trial, patients with previous MI treated with Canakinumab, which targets the pro-inflammatory cytokine interleukin-1 β , had

fewer secondary CVD events^{20,10}. In the COLCOT trial, colchicine, a non-specific anti-inflammatory agent, reduced the risk of both repeat MI and stroke in those who presented with a non-fatal MI²¹.

Inflammation has also been implicated in microvascular dysfunction. Arterioles, capillaries, and venules undergo functional and structural changes during inflammation, such as a diminished capacity of arterioles to dilate and the adhesion of leukocytes and platelets in venules^{22, 23}. These changes contribute to functional outcomes, particularly the development of heart failure¹.

Several studies have examined associations between various markers of inflammation with retinal microvascular calibre but findings have been inconsistent^{14, 24-26}. As far as we are aware, there has been no systematic review or meta-analysis of inflammation and retinal microvascular parameters in either the general population or in those with CVD and related conditions. These data may clarify the extent to which inflammation contributes to microvascular parameters in both general and high-risk populations and indicate whether interventions targeting inflammation may mitigate adverse microcirculatory changes. We therefore performed a systematic review and meta-analysis to quantify the associations of various inflammatory markers with retinal microvascular calibre.

Methods

Protocol registration

We performed the systematic review and meta-analysis according to the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines²⁷. The protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO; CRD42018102932) in July 2018.

Literature search

We searched Medline, Embase and Pubmed databases for studies reporting the association of inflammation and retinal vascular calibre. The literature search strategy was composed by content experts and critically evaluated by our institutional librarian. MeSH terms, key words and search limits for the following three topic areas were combined: 1) retinal microvascular calibre, 2) inflammatory markers, and 3) observational studies (Supplementary Table 1S). The search was limited to studies published in English or Chinese, with all studies up to February 2020 included.

Study selection

The screening process was conducted by two reviewers (ML, CL) independently via the web-based systematic review software package ‘Covidence’ (Veritas Health Innovation, Australia)²⁹. Where there was a conflict, an adjudicator (DB) made the final decision. After abstract and title screening for relevant studies, each reviewer then independently screened full texts using the pre-defined inclusion criteria. Studies were included if they were observational designs, had retinal vascular calibre quantified from digital retinal fundus photographs via computer-assisted methods, and inflammatory markers assessed (e.g. C-reactive protein (CRP), white

blood cell count (WBC), tumour necrosis factor-alpha (TNF α), erythrocyte sedimentation rate, fibrinogen and Interleukin 6 (IL-6)). Articles were only included if a full text with sufficient data was available; conference abstracts and study protocols were excluded. Additional studies were sought by hand-searching the reference lists of relevant studies.

Data extraction

Data were extracted independently by each reviewer from included full texts into Epidata software (version 3.1)³⁰. These data included the first author, year of publication, study population, study name, sample size, age of participants, measurement methods, statistical methods, correlation coefficients, main estimates with confidence intervals (CI), and estimates of the association with covariates if applicable. To avoid over-adjustment by potential intermediate variables and to improve comparability between studies, we preferentially extracted minimally adjusted results (e.g. age and/or sex-adjusted).

Data synthesis and meta-analysis

A variety of inflammatory markers were reported, but we focussed on the two most commonly assessed inflammatory parameters (CRP and WBC), for which there were sufficient data to undertake a meta-analysis. Additional inflammatory markers were included in the narrative review. The large variation in reporting of results made combining data across studies challenging. To conduct a meta-analysis, we aimed to combine correlation coefficients or standardized β from unadjusted or minimally adjusted regression models, as these are directly comparable. When raw regression coefficients were presented, we were able to calculate the standardized β , such that we could directly compare associations across studies given that the

correlation coefficient and a standardized β from an unadjusted model are equal²⁷.

This also allowed us to include studies where retinal microvascular calibre was assessed as the exposure, rather than the outcome, given that standardized β avoids the issue of directionality. When studies reported data in other formats, authors were contacted directly in an effort to obtain more suitable data.

We transformed correlation coefficients to Fisher's z scores, and pooled estimates using the random-effects model of z-transformed correlations^{31,32}. The 95% confidence intervals (CI) of the pooled weighted Fisher's z-scores were calculated, after which all the values were back-transformed to the metric of the correlation coefficients to facilitate interpretation^{31,32}. Statistical heterogeneity was investigated using the I square index (I^2), which measures the percentage of the variation across studies that is likely due to heterogeneity and cannot be explained by chance^{32,33}. Levels of heterogeneity were regarded as 'low' (0%-25%), 'moderate' (26-74%) or 'high' ($\geq 75\%$). We used the R software (version 3.4.2), "meta" package to analyse data³⁴.

For other biomarkers with insufficient studies to do meta-analyses, study characteristics and main findings (e.g. magnitude of association and level of statistical evidence) were compiled into tables.

Results

Study selection and characteristics

Figure 1 shows an overview of review process according to the PRISMA guideline.²⁴ We screened the abstracts and titles of 2383 non-duplicate studies, of which 41 studies met our inclusion criteria. After full-text screening, we extracted data from 28 studies.

Table 1 presents the characteristics of the included studies. Most were cross-sectional and most were conducted in Europe (10 studies) or the United States (9 studies). Of the 28 studies, 19 were performed in the generally healthy populations, mostly with large sample sizes (median sample size 3447), and nine were in patients with disease conditions (e.g. hypertension, obesity and rheumatoid arthritis) with smaller sample sizes (median sample size 112). Three studies were in children, one performed in early childhood (median age six years), and two in mid-childhood (mean age 11 years). Of 25 studies in adults, most were with middle-aged adults (aged 43 to 68 years). Various inflammatory markers were included, most commonly CRP (20 studies) and WBC (8 studies); other inflammatory markers were interleukin (IL)-6, fibrinogen, tumor necrosis factor (TNF), albumin, and erythrocyte sedimentation rate (ESR).

Differences in the presentation of results and in the variety of inflammatory markers made direct comparison of studies challenging. We therefore conducted the meta-analysis with CRP and WBC respectively as sufficient data for meta-analysis were available, and performed a narrative review for other inflammatory markers.

Associations of CRP and retinal vascular calibre

There were 20 articles examining associations between CRP and retinal microvascular calibre (Table 2), of which 11 were conducted in the general population. All studies, including two in children, reported that higher CRP was associated with wider (ie, worse) venular calibre, but not associated with arteriolar calibre. Other studies investigated the association of CRP and retinal vascular calibre in patients with disease conditions. Apart from one study in rheumatoid arthritis patients reported an association of higher CRP levels with smaller arteriole-to-venule ratio (standardised β -0.23, 95% CI -6.20 to -0.07), the remaining studies found little evidence of associations with either arteriolar or venular calibre.

Ten of the 20 studies (six in the general population (n= 18666) and four in patients (n= 455) reported either correlation coefficients or linear regression coefficients that were suitable for meta-analysis. The association with venular calibre was only seen in the general population group (r= 0.08, 95% CI 0.04 to 0.11), but not in the patient group (r= 0.03, 95% CI -0.06 to 0.12) (Figure 2 (B)). The pooled results showed little evidence of association between CRP with retinal arteriolar calibre (r= 0.01, 95% CI -0.00 to 0.03 in the general population and r= -0.06, 95% CI -0.18 to 0.06 in patients, Figure 2(A)).

Associations of WBC and retinal calibre

There were eight studies examining WBC and retinal microvascular calibre, and all were in the general population. For venular calibre, all seven studies reported an association between higher WBC and wider venular calibre^{14, 35-41}. Seven examined arteriolar calibre in relation to WBC, of which three reported an association between higher WBC and greater (i.e. better) arteriolar calibre.

Meta-analysis of WBC data was performed using data from four studies of venular calibre (n= 14967) and four studies of arteriolar calibre (n= 10367 participants). WBC was associated with both venular and arteriolar calibre, with stronger associations seen for venular calibre (r= 0.18, 95% CI 0.04 to 0.32, Figure 3). The association with arteriolar calibre was in the unexpected direction (r= 0.05, 95% CI 0.01 to 0.09).

Other inflammation biomarkers

Other inflammation biomarkers were also reported, including IL-6, fibrinogen, plasminogen activator inhibitor-1 and serum albumin, which were studied in at least three publications (Supplementary table 2S). Most of these studies showed little evidence of associations between inflammatory markers and retinal arteriolar calibre or arteriole-to-venule ratio. Although there were mixed findings across different markers with venular calibre, the majority of studies showed some evidence to support this association (Supplementary table 2S). One study in children aged 10-11 years did not observe an association between IL-6 and either venular or arteriolar calibre or arteriole-to-venule ratio.

Discussion

Principal findings

This systematic review and meta-analysis of 23 studies investigated whether inflammation was associated with retinal microvascular calibre in both the general population and patients with disease conditions. In both the narrative review and meta-analysis, we found consistent evidence across different populations that higher inflammatory markers were associated with wider (i.e. worse) venular calibre. In the meta-analysis, we found a modest correlation between CRP and WBC and venular

calibre. The effect was similar in the general population and patients for CRP and venular calibre. There was weaker and less consistent evidence for an association between inflammatory markers and arteriolar calibre, with the meta-analysis showing weak associations in the unexpected direction – increased inflammatory markers associated with wider (i.e. better) arteriolar calibre.

Strengths and limitations

This is the first systematic review and meta-analysis to summarise the evidence from population- and patient-based studies of the association between inflammation and retinal microvascular calibre. We were able to perform meta-analyses for two widely used inflammatory markers, CRP and WBC. The sample size in the meta-analysis was large (CRP n=23,720, and WBC n= 14,967) and studies were from a variety of high-income countries. To reduce reporting bias, we contacted authors for their data to pool as many studies as possible.

There are also some limitations. The studies were all cross-sectional design, making it difficult to draw causal inferences⁴². There was insufficient data for a subset analysis in different ethnic groups and included participants are mostly Caucasian, so the results may not be generalisable to other populations. As CVD risk factors vary with ethnicity¹¹, the association between inflammatory markers and retinal calibre may also vary¹¹. CRP is an acute-phase reactant and it is unclear whether how well it reflects chronic inflammation in largely healthy populations. GlycA, a novel composite glycosylation marker of five acute-phase reactants has been suggested to better reflect chronic, cumulative inflammation⁴³ and may provide predictive value beyond CRP⁴⁴. Studies examining the association between GlycA and other chronic inflammatory biomarkers, and retinal calibre are warranted.

Finally, we hypothesized that the association between inflammation and retinal microvascular calibre would be stronger in patients with disease conditions, but the strength of association was similar in generally healthy population and in patients in the meta-analysis. This may reflect treatment for the underlying disease, but we could not adjust for anti-inflammatory medications in the patient group as these data were not available.

Possible mechanistic pathways

Inflammation is central to the pathogenesis of cardiovascular disease^{11-13, 25}. The retinal vessels are exposed to the same risk factors and mediators as systemic vessels and adverse changes in retinal vascular calibre (narrower arterioles, wider venules) predicts future CVD, independent of traditional risk factors^{11, 14-16, 45}. If inflammation increases CVD risk in part by adverse effects on the microcirculation, we would expect heightened inflammation to be associated with narrower arteriole diameters if arterioles drive the association between retinal calibre and CVD^{11-15, 25, 26, 36, 45-48}. Conversely, venular diameter may be more relevant in the inflammatory pathophysiological process in CVD^{26, 46, 49, 50} because inflammation causes endothelial dysfunction in venules^{12, 13, 15, 47, 51} and there are common risk factors for atherosclerosis and wider venular diameter, for instance hyperlipidaemia, platelet aggravation, hypercoagulability, and hyperglycaemia^{12, 13, 15, 39} which is in keeping with the findings of our meta-analysis.

The underlying mechanism explaining the link between wider venular calibre, inflammation and CVD risk is likely to be endothelial dysfunction. There are multiple pathways involved in endothelial dysfunction. One theory stems from the response to hypoxia, whereby hypoxia-inducible factor 1- α causes the release of several

proteins, including endothelin-1 and vascular endothelial growth factor (VEGF)^{13, 16, 48}. Endothelin-1 causes vasoconstriction, which increases peripheral resistance and causes further damage to the vascular walls. VEGF causes neovascularisation. Increased levels of VEGF can result in over-vascularisation with reduced flow, causing more hypoxia⁴⁸. Endothelial dysfunction in response to inflammation is more marked in retinal venules than in arterioles. Animal models have highlighted the potential pathophysiological mechanisms underlying inflammation-related microvascular perturbations and suggest that these are more marked in venules. Functional changes (largely endothelial dysfunction) lead to structural pathogenic changes (vascular remodelling and rarefaction)^{4, 14, 26, 45}. Increased inflammatory mediators, such as TNF-alpha and IL-1 cause impaired nitric oxide activity, which results in wider retinal venules^{13, 38, 40, 47}. In cross-sectional studies, a dose response has been observed between higher levels of inflammatory markers, markers of endothelial dysfunction and venular diameter, but not arteriolar diameter^{38, 40}. Another potential mechanism for wider venular diameters is that LDL and activated leukocytes cause damage to the vascular endothelial surface^{25, 38-40, 45, 52} and loss of the glycocalyx layer³⁸.

Implications

In this population-based systematic review and meta-analysis, we found that inflammation contributes to adverse changes in retinal venular calibre with less consistent effects on arteriolar calibre. Inflammation has been shown to be involved in every stage of CVD pathogenesis^{7, 9, 53}. There is increasing evidence that retinal microvascular calibre predicts CVD events¹⁵. Our results suggest that inflammation

may be a contributory shared mechanism underlying adverse changes to both the microcirculation and to large arteries.

Microcirculation is a major site of end-organ damage in CVD, including the heart, brain, and kidney. Targeting inflammatory pathways may mitigate damage to the microcirculation, which may lower the risk of later CVD events. For example, statins have been shown to reduce cardiovascular event risk more than expected by only lowering cholesterol. Statins may exhibit this effect by reducing systemic inflammation, as seen by a reduced CRP⁵⁴. Longitudinal observational studies and intervention trials are warranted.

Overall, the magnitude of association is small, with correlation coefficients from 0.1 to 0.2. The clinical significance of our finding is uncertain. Intervention studies targeting inflammatory biomarkers could add microvasculature as an outcome in addition to large vessel measures to inform the clinical value of the findings.

Conclusion

Our systematic review and meta-analysis showed that inflammation has a greater effect on retinal venular than arterial calibre, with similar size of associations seen in generally healthy populations and patients with disease conditions. This suggests that inflammation is a pathway underlying adverse changes to the microcirculation, which could be a potential target in CVD risk management.

Figure 1. Flow diagram detailing numbers of studies searched, screened and included in the review.

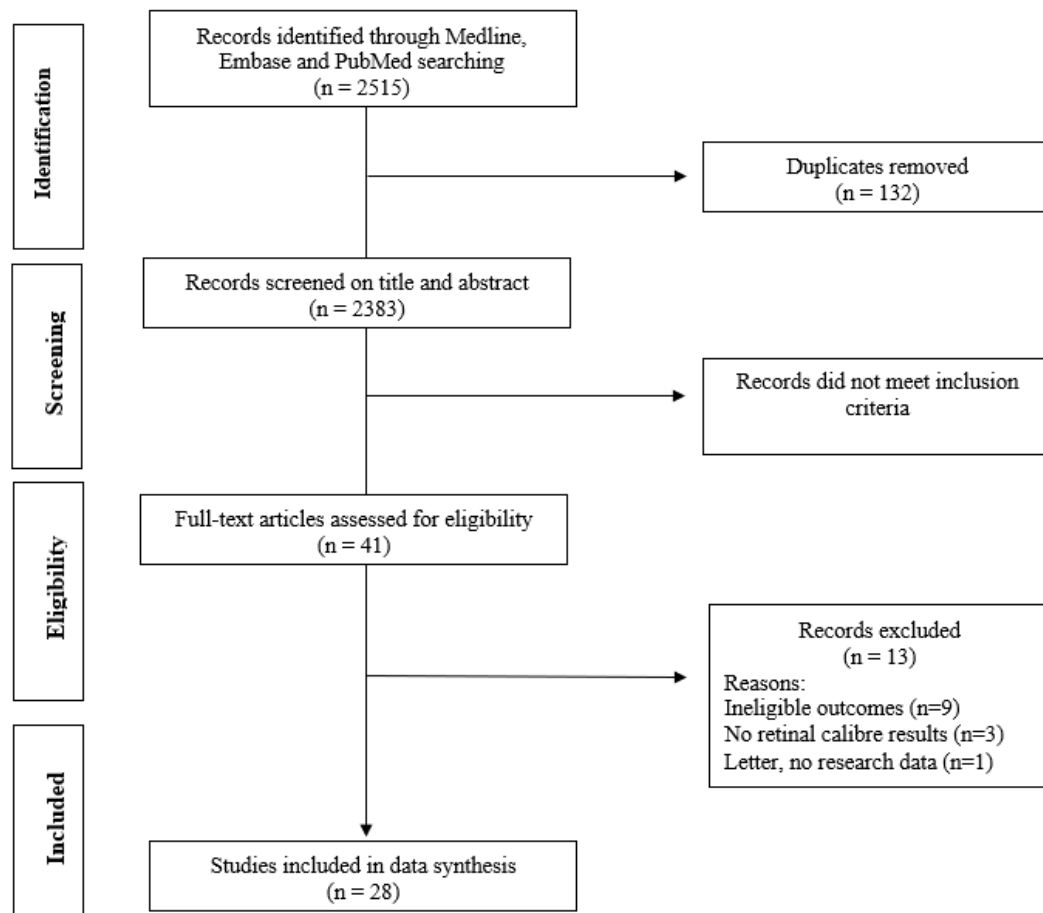


Table 1. Study characteristics

Study ID (Author-year)	Country (disease if applicable)	Sample size (Female %)	Age range, mean/median age (y)	Inflammation biomarkers	Study quality
<i>General population</i>					
Buttner, M-2019	Germany	1682 (19.2)	ng, 44.8	C-reactive protein; white blood cell count	Moderate
Xinxin, L-2018	China	3447	>40, 56.6	C-reactive protein	Moderate
Yau, PL-2017	US	157(n.s.)	ng, 19.29	Inflammation balance score ^a	Low
Lammertyn, L-2015	South Africa	170(51.8)	25-60, 49.2	Fibrinogen; PAI-1	Moderate
Gishti, O-2015	Netherlands	4145(ng)	5.8-8, 6	C-reactive protein	Moderate
Siegrist, M-2014	Germany	376(ng)	10-11, 11.1	IL-6	Moderate
Liew, G-2014	Australia	3009(ng)	>49, 59.4	White blood cell count	High
Daïen, V-2013	France	1224(58.7)	>60, ng	C-reactive protein; Orosomucoid	Moderate
Myers, E-2012	US	4600(ng)	43-86, ng	C-reactive protein; white blood cell count	Moderate
Hanssen, H-2012	Germany	578(43.1)	ng, 11.1	C-reactive protein	Moderate
Kawasaki, R-2012	US	4849(52.8)	45-84, ng	C-reactive protein	Moderate
Yim-Lui-2010	Singapore	3583(53.5)	24.6-94.9, 49.4	C-reactive protein	Moderate
Klein, B-2011	US	4730(55.5)	ng, 61.74	White blood cell count	High
Liew, G-2008	US	8794(54.6)	ng,60	White blood cell count; fibrinogen; Albumin	High
Liew, G-2007	Australia	3000(57.1)	>49, 65.5	White blood cell count	Moderate
De Jong, FJ-2007	Netherlands	5279(58.7)	>55, 67.9	C-reactive protein; fibrinogen; Lp-PLA2	High
Tien, YW-2006	US	5979(52.3)	45-84, 61.9	C-reactive protein; IL-6; Fibrinogen; PAI-1	High
Klein, R-2006	US	383(54.8)	57-71, 62	C-reactive protein, IL-6, white blood cell count, albumin, amyloid A, tumour necrosis factor	High

Study ID (Author-year)	Country (disease if applicable)	Sample size (Female %)	Age range, mean/median age (y)	Inflammation biomarkers	Study quality
Ikram, MK-2004	Netherlands	5674 (59%)	≥55, 68	White blood cell count, erythrocyte sedimentation rate	High
<i>Disease patients</i>					
Babaoglu, H-2019	Turkey (Rheumatoid arthritis)	47 (83)	ng, 52.0	C-reactive protein	Low
Jabs, D-2019	US (AIDS)	454(22.7)	ng, 43.3	C-reactive protein, IL6	High
Anyfanti, P-2017	Greece (Rheumatoid arthritis)	133(75.2)	ng; 60.8	C-reactive protein	Low
Aissopou -2015	Greece (Systemic Sclerosis)	93 (86)	54.1	Erythrocyte sedimentation rate; C- reactive protein	Moderate
Beltrami M-2015	Brazil (Hypertension)	172(63.4)	27-80; 59.8	C-reactive protein	Moderate
Bachmayer, C-2013	Germany (Obesity)	92(ng)	ng, 43	C-reactive protein; tumour necrosis factor; IL-6	Low
Okada, M-2013	US (Rheumatoid arthritis)	248(72)	ng, 59.9	C-reactive protein	Low
Brazionis, L-2010	Australia (Diabetes type 2)	112(45)	44-83, 63.9	C-reactive protein, PAI-1, albumin	Moderate
Anne, M-2017	Poland (Hypertension)	78(36)	ng, 53.7	C-reactive protein, IL6, tumour necrosis factor alpha	Moderate

Abbreviation: ng, not given; Lp-PLA₂, lipoprotein-associated phospholipase A₂; PAI-1, plasminogen activator inhibitor-1

^a Inflammation balance score includes the relative contributions of markers (eg CRP, IFN, IL-6, Resistin, TNF, Adiponectin, IL-4, IL-10).

Study quality was assessed using the modified Newcastle-Ottawa Quality Assessment Scale for observational studies, which defined quality as high (10-9 stars), moderate (7-8 stars) or low (≤6 stars).

Table 2. Studies reported associations between C-reactive protein (CRP) or white cell count (WBC) with retinal vascular calibre

Study ID (Author-year)	Population group	Statistical analysis	Minimum level of adjustment	Evidence of associations estimates (confidence intervals or p values if available)		
				Arteriolar	venular	AVR
<i>C-reactive protein</i>						
Buttner, M-2019	General	Linear regression (Sβ)	Unadjusted	-0.01 (p =0.61)	0.02 (p =0.38)	Ng
Xinxin, L-2018	General	Spearman correlation (r', log CRP)	Unadjusted	0.03 (p =0.14)	0.05 (p =0.003)	-0.05 (p =0.002)
Gishti, O-2015	General	Pearson correlation (r)	Unadjusted	0.01 (p>0.05)	0.14 (p<0.05)	Ng
Daien, V-2013	General	Linear regression (Sβ, per 0.1 log)	Sex	0.04 (-0.03, 0.05)	0.13 (0.10, 0.16)	Ng
Myers, E-2012	General	Linear regression (Sβ)	Image focus, cataract status, refraction, and height	Ng	0.08 (0.05, 0.10)	Ng
Hanssen, H-2012	General	Linear regression (β, per unit)	Age and sex	2.12 (-4.46, 8.70)	11.83 (5.37, 18.29)	-0.03 (-0.06, -0.01)
Kawasaki, R-2012	General	ANOVA across retinal calibre tertiles	Unadjusted	p =0.12	P<0.001	Ng
Yim-Lui-2010	General	Linear regression across CRP quartiles	Age, sex, and race/ethnicity	p for trend =0.66	p for trend <0.001	Ng
De Jong, FJ-2007	General	Linear regression (Sβ)	Age and sex	0.02 (-0.00, 0.05)	0.10 (0.08, 0.13)	Ng
Tien, YW-2006	General	Linear regression (Sβ)	Age, sex, ethnicity and study centre	0.01 (p =0.64)	0.04 (p =0.002)	Ng
Klein, R-2006	General	Pearson correlation (r)	Unadjusted	0.02	0.01	Ng
Jabs, D-2019	Patients	Linear regression (Sβ, per log10 SD)	Age, sex, and race/ethnicity	0.08 (p =0.11)	0.16 (p =0.002)	Ng
Anne, M-2017	Patients	Spearman correlation (r')	Unadjusted	Ng	Ng	>0.05
Anyfanti, P-2017	Patients	Linear regression (Sβ)	Ng	-0.16 (-7.24, 1.44)	0.01 (-1.49, 1.58)	-0.23 (-6.20, -0.07)

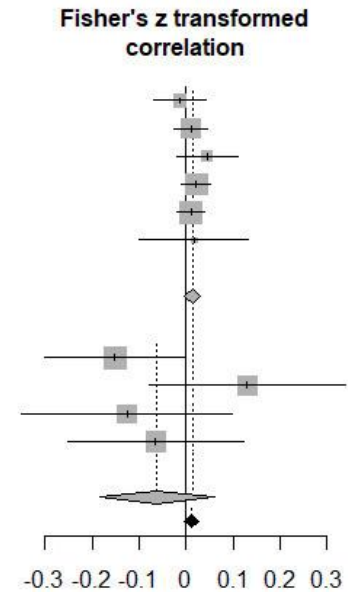
Study ID (Author-year)	Population group	Statistical analysis	Minimum level of adjustment	Evidence of associations estimates (confidence intervals or p values if available)		
				Arteriolar	venular	AVR
Aissopou -2015	Patients	ANOVA across systemic sclerosis groups	Ng	p =0.67	p =0.24	p =0.46
Beltrami M-2015	Patients	Pearson correlation (r)	Unadjusted	-0.15 (p =0.08)	-0.02 (p =0.8)	Ng
Bachmayer, C-2013	Patients	Pearson correlation (r)	Unadjusted	0.13 (p >0.05)	0.11 (p >0.05)	0.03 (p >0.05)
Okada, M-2013	Patients	Pearson correlation (r)	Unadjusted	0.13 (p >0.05)	0.03 (p >0.05)	Ng
Brazionis, L-2010	Patients	Pearson correlation (r)	Unadjusted	0.06 (p >0.05)	0.03 (p >0.05)	Ng
White blood cell count						
Buttner, M-2019	General	Linear regression (S β)	Unadjusted	-0.001 (p =0.96)	0.06 (p =0.03)	Ng
Liew, G-2014	General	Pearson correlation (r)	Age and sex	0.003 (p =0.04)	0.1 (p =0.01)	Ng
Myers, E-2012	General	Linear regression (S β)	Image focus, cataract status, refraction, and height	Ng	0.11 (0.09, 0.13)	Ng
Klein, B-2011	General	Linear regression (β in sex-specific WBC quintile)	Age and sex	-0.14 (p =0.34)	1.22 (p <0.001)	Ng
Liew, G-2008	General	Linear regression (β in each WBC interquartile range increase)	Age, sex, fellow retinal vascular calibre, blood pressure, HDL, LDL, CIMT, smoking	-0.2 (-0.6, 0.2)	1.2 (0.9 to 1.6)	Ng
Liew, G-2007	General	Linear regression (β for per SD change in retinal calibre)	Age, sex and fellow retinal vascular calibre	-0.03 (-0.11, 0.04)	0.26 (0.18, 0.33)	Ng
Klein, R-2006	General	Pearson correlation (r)	Unadjusted	0.10 (p <0.05)	0.17 (p <0.05)	Ng
Ikram, MK-2004	General	Linear regression (S β)	Age and sex	0.08 (0.06, 0.10)	0.14 (0.11, 0.17)	-0.05 (-0.08, -0.03)

Abbreviations: ng, not given; S β , standardised beta coefficient.

Figure 2. Associations of C-reactive protein and retinal vascular calibre

(A) Retinal arteriolar caliber

Study citation no.	Total	ZCOR (95% CI)	Weight
General population			
Buttner, M-2019	1682	0.02 (-0.03, 0.07)	12.3
Gishti.O-2015	4145	0.14 (0.11, 0.17)	14.8
Daien, V-2013	1224	0.13 (0.07, 0.19)	11.1
Myers, E-2012	4600	0.08 (0.05, 0.11)	15.0
De Jong, FJ-2007	5279	0.10 (0.07, 0.12)	15.2
Tien, YW-2006	5940	0.04 (0.01, 0.07)	15.4
Klein,R-2006	396	0.01 (-0.09, 0.11)	6.4
Random effects model	23266	0.08 (0.04, 0.11)	p < 0.001
Heterogeneity: $I^2 = 84\%$			
Patients			
Beltrami M-2015	172	-0.02 (-0.17, 0.13)	3.5
Bachmayer, C-2013	92	0.12 (-0.09, 0.33)	2.0
Okada, M-2013	79	0.03 (-0.20, 0.25)	1.8
Brazionis, L-2010	112	0.03 (-0.16, 0.21)	2.4
Random effects model	454	0.03 (-0.06, 0.12)	p = 0.55
Heterogeneity: $I^2 = 0\%$			
Overall Random effects model			
	23720	0.07 (0.04, 0.11)	p < 0.001
Heterogeneity: $I^2 = 75\%$			



(B) Retinal venular caliber

Study ID	Total	ZCOR (95% CI)	Weight
General population			
Buttner, M-2019	1682	-0.01 (-0.06, 0.04)	10.2
Gishti.O-2015	4145	0.01 (-0.02, 0.04)	21.8
Daien, V-2013	1224	0.04 (-0.02, 0.10)	7.6
De Jong, FJ-2007	5279	0.02 (-0.01, 0.05)	26.3
Tien, YW-2006	5940	0.01 (-0.02, 0.04)	28.6
Klein,R-2006	396	0.02 (-0.08, 0.11)	2.6
Random effects model	18666	0.01 (-0.00, 0.03)	p = 0.07
Heterogeneity: $I^2 = 0\%$			
Patients			
Beltrami M-2015	172	-0.15 (-0.30, 0.00)	1.1
Bachmayer, C-2013	92	0.13 (-0.08, 0.34)	0.6
Okada, M-2013	79	-0.13 (-0.35, 0.10)	0.5
Brazionis, L-2010	112	-0.06 (-0.25, 0.12)	0.7
Random effects model	455	-0.06 (-0.18, 0.06)	p = 0.33
Heterogeneity: $I^2 = 39\%$			
All participants Random effects model			
	19121	0.01 (-0.01, 0.03)	p = 0.19
Heterogeneity: $I^2 = 10\%$			

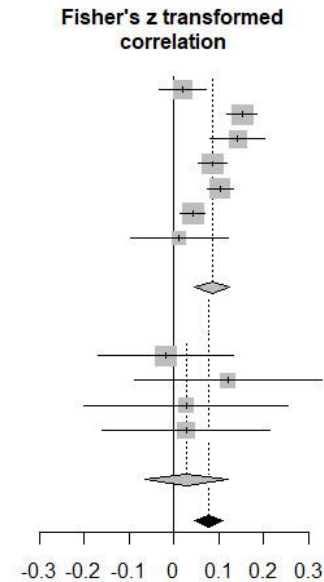
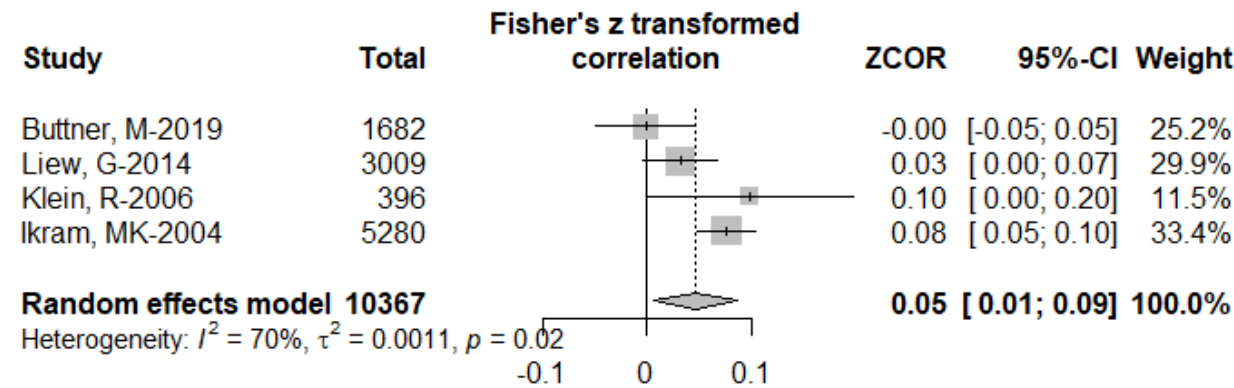
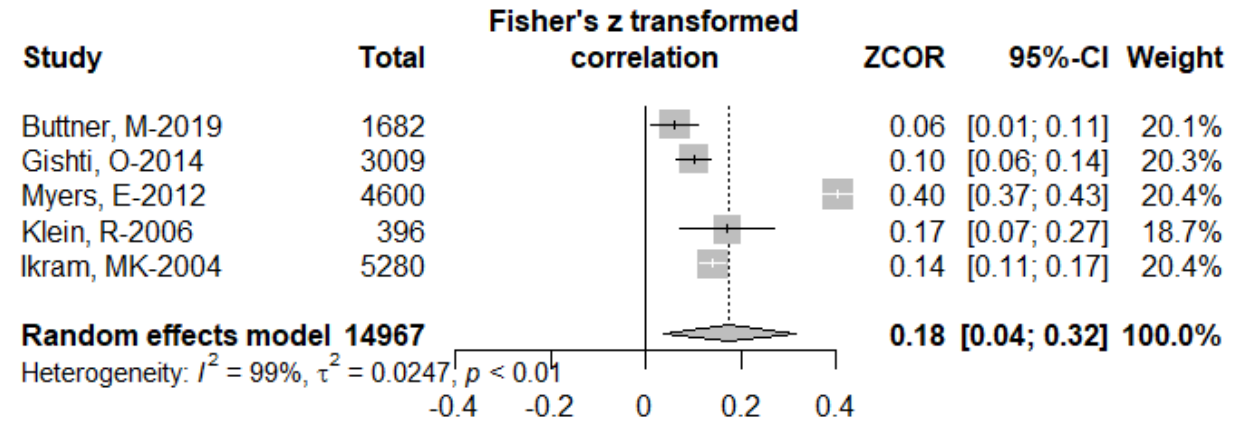


Figure 3. Associations of white blood cell count (WBC) and retinal vascular calibre; eligible studies were all based on general population.

(A) Retinal arteriolar caliber



(B) Retinal venular caliber



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Supplementary Table 1. Full search strategy developed in conjunction with institution librarian

Medline search (n = 602)
9. exp Retinal Vessels/ or exp Retinal Artery/ or exp Retinal Vein/ or retinal diseases/ or exp diabetic retinopathy/ or hypertensive retinopathy/ 10. Inflammation/ or Biomarkers/ or C-Reactive Protein/ or Intercellular Adhesion Molecule-1/ or Glycoproteins/ or Fibrinogen/ or Leukocyte Count/ or Interleukins/ or serum albumin/ or inflammation mediators/ or Tumor Necrosis Factor-alpha/ or serum Amyloid A protein/ or E-selectin/ or Plasminogen Inactivators/ or Orosomucoid/ 11. exp cohort studies/ or cross-sectional studies/ or follow-up studies/ or prospective studies/ or risk factors/ or Risk Assessment/ or case-control studies/ or Regression Analysis/ or Multivariate Analysis/ or Mass Screening/ 12. 1 and 2 and 3 13. Exp animals/ not human*.sh. 14. 4 not 5 15. Limit to (English or Chinese)
EMBASE search (n = 242)
9. Retinal blood vessel/ or Retinal Artery/ or Retinal Vein/ or retinal diseases/ 10. inflammation/ or biological marker/ or C reactive protein/ or intercellular adhesion molecule 1/ or glycoprotein/ or fibrinogen/ or leukocyte count/ or interleukin 6/ or serum albumin/ or tumor necrosis factor/ or serum amyloid A/ or endothelial leukocyte adhesion molecule 1/ or plasminogen activator inhibitor/ or orosomucoid/ 11. Cohort analysis/ or cross-sectional studies/ or observational study/ or prospective study/ or risk factor/ or longitudinal study/ or controlled studies/ or Regression Analysis/ or logistic regression analysis/ or risk assessment/ or epidemiology/ or priority journal or multiple regression or major clinical study/ or population research/ 12. 1 and 2 and 3 13. Exp animal/ not human*.sh. 14. 4 not 5 15. Limit to (English or Chinese)
Pubmed search (n = 1671)
7. inflammation OR inflammatory OR "C Reactive Protein" OR "intercellular adhesion molecule" OR glycoprotein OR fibrinogen OR "leukocyte count" OR "white cell count" OR interleukin OR albumin OR "tumor necrosis factor" OR "amyloid A" OR "leukocyte adhesion molecule" OR "E-selectin" OR "plasminogen activator inhibitor" OR Orosomucoid OR "Erythrocyte Sedimentation Rate" 8. "retinal vessel" OR "retinal vessels" OR "retinal artery" OR "retinal vein" OR Microvessel* OR "Hypertension retinopathy" OR "Hypertensive retinopathy" OR "Diabetic retinopathy" OR "retinal vascular calibre" OR "retinal vascular caliber" OR "retinal arteriolar calibre" OR "retinal arteriolar caliber" OR "retinal venular calibre" OR "retinal venular caliber" OR "retinal vascular changes" OR "retinal blood vessels" OR "retinal blood vessel" OR "retinal arteriolar narrowing" OR "retinal microcirculation" OR "retinal vasculature" 9. NOTNLM OR publisher[sb] OR inprocess[sb] OR pubmednotmedline[sb] OR indatereview[sb] OR pubstatusaheadofprint 10. animal* OR rat OR rats OR mice OR murine OR pig OR pigs OR cow OR cows OR bovine OR sheep 11. (1 and 2 and 3) not 4

Supplementary Table 2. Studies reported associations between other inflammatory biomarkers with retinal vascular calibre

Study ID (Author-year)	Population group	Markers examined	Statistical analysis	Level of adjustment	Evidence of associations estimates (confidence intervals or p values if available)		
					Arteriolar	venular	AVR
Siegrist, M-2014	General	IL-6	Linear regression ($S\beta$)	Age and sex	-0.01 (p =0.86)	-0.01 (p =0.77)	0.00 (p =0.99)
Bachmayer, C-2013	Patients	IL-6	Pearson correlation (r)	Unadjusted	0.09 (p >0.05)	0.04 (p >0.05)	0.06 (p >0.05)
		TNF α			0.04 (p >0.05)	0.03 (p >0.05)	0.06 (p >0.05)
Tien, YW-2006	General	IL-6	Linear regression (mean of each IL-6 or PAI-1 quartile)	Age, sex, ethnicity and study centre	p =0.34	P <0.001	ng
		PAI-1			p =0.44	P =0.05	ng
		IL-6	Pearson correlation (r)	Unadjusted	0.02 (p =0.66)	0.10 (p =0.06)	ng
Klein, R-2006	General	Amyloid A	Linear regression (mean of each marker's quartile)	Age, blood pressure, smoking, serum high- density lipoprotein cholesterol, and history of gout	p =0.01	p =0.02	ng
		TNF α			p =0.31	p =0.57	ng
		Albumin			p =0.29	p =0.63	ng
		Fibrinogen			Black Africans - 0.09 (p =0.49); white 0.10 (p =0.38)	Black Africans 0.29 (p =0.02); white 0.26 (p =0.02)	Black Africans - 0.28 (p =0.02); white -0.23 (p =0.04)
Lammertyn, L-2015	General	PAI-1	Pearson correlation (r)	Unadjusted	Black Africans - 0.001 (p =0.99); white 0.02 (p =0.85)	Black Africans 0.01 (p =0.91); white 0.01 (p =0.92)	Black Africans - 0.02 (p =0.86); white -0.01 (p =0.91)
		PAI-1	Pearson correlation (r)	Unadjusted	0.07 (p >0.05)	-0.07 (p >0.05)	0.14 (p <0.05)
Brazionis, L-2010	Patients	Albumin	Pearson correlation (r)	Unadjusted	-0.02 (p >0.05)	-0.14 (p <0.05)	0.09 (p >0.05)
Liew, G-2008	General	Fibrinogen	Linear regression (β in per Fibrinogen or albumin interquartile range increase)	Age, sex, fellow retinal vascular calibre, blood	0.09 (-0.3 to 0.5)	0.7 (0.3 to 1.1)	ng
Liew, G-2008	General	Albumin			1.5 (1.0 to 1.9)	-0.6 (-1.1 to -0.2)	ng

Study ID (Author-year)	Population group	Markers examined	Statistical analysis	Level of adjustment	Evidence of associations estimates (confidence intervals or p values if available)		
					Arteriolar	venular	AVR
De Jong, FJ-2007	General	Fibrinogen Lipoprotein- associated phospholipase A2	Linear regression (Sβ)	pressure, HDL, LDL, CIMT, smoking Age and sex	0.05 (p <0.05)	0.09 (p <0.05)	ng
			Linear regression (Sβ)	Age and sex	0.08 (p <0.05)	0.11 (p <0.05)	ng
Tien, YW-2006	General	Fibrinogen	Linear regression (mean in each IL-6 quartile increase)	Age, sex, ethnicity and study centre	p =0.02	p <0.001	ng
Yau, PL-2017	General	Inflammation balance score	Pearson correlation (r)	Age, sex and mean arterial blood pressure	p =0.02	p <0.001	ng
Aissopou -2015	Patients	Erythrocyte sedimentation rate	Mean difference across patients with limited and diffuse systemic sclerosis	Unadjusted	p =0.12	p =0.82	p =0.54
Daien, V-2013	General	Orosomucoid Erythrocyte sedimentation rate	Linear regression (Sβ)	Sex	0.01 (p =0.78)	0.06 (p =0.02)	ng
Ikram, MK-2004	General	Erythrocyte sedimentation rate	Linear regression (Sβ)	Age and sex	0.05 (p <0.05)	0.04 (p <0.05)	0.02 (p >0.05)
Jabs, D-2019	AIDS	IL-6	Linear regression (Sβ)	Age, sex, race, hyperlipidaemia and cardiovascular disease	ng	5.3 (<0.001)	

Abbreviations: PAI-1, Plasminogen activator inhibitor-1; Sβ, standardised beta coefficient; ng, not given; TNFα, tumour necrosis factor α.

BMJ Open Retinal microvasculature: population epidemiology and concordance in Australian children aged 11–12 years and their parents

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ABSTRACT

Objectives To describe distributions and concordance of retinal microvasculature measurements in a population-based sample of Australian parent–child dyads at child age 11–12 years.

Design Cross-sectional Child Health CheckPoint study, between waves 6 and 7 of the national population-based Longitudinal Study of Australian Children (LSAC).

Setting Assessment centres in seven Australian cities, February 2015–March 2016.

Participants Of the 1874 participating families, 1288 children (51% girls) and 1264 parents (87% mothers, mean age 43.7) were analysed. Diabetic participants and non-biological pairs were excluded from concordance analyses.

Outcome measures Retinal photographs were taken by non-mydratric fundus camera. Trained graders scored vascular calibre using semi-automated software, yielding estimates of central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE) and arteriolar–venular ratio (AVR). Pearson's correlation coefficients and multivariable linear regression models assessed parent–child concordance. Survey weights and methods accounted for LSAC's complex sampling, stratification and clustering within postcodes.

Results Mean (SD) of CRAE and CRVE were larger in children (159.5 (11.8) and 231.1 (16.5) μ m, respectively) than parents (151.5 (14.0) and 220.6 (19.0) μ m), yielding similar AVR (children 0.69 (0.05), parents 0.69 (0.06)). Correlation coefficients for parent–child pairs were 0.22 (95% CI 0.16 to 0.27) for CRAE, 0.23 (95% CI 0.17 to 0.28) for CRVE and 0.18 (95% CI 0.13 to 0.24) for AVR. Mother–child and father–child values were similar (0.20 and 0.32 for CRAE, 0.22 and 0.29 for CRVE, respectively). Relationships attenuated slightly on adjustment for age, sex, blood pressure, diabetes and body mass index. Percentiles and concordance are presented for the whole sample and by sex.

Conclusions Arteriolar and venular calibre were similar to previously documented measures in midlife adult and late childhood populations. Population parent–child concordance values align with moderate polygenic heritability reported in smaller studies.

INTRODUCTION

The role of the microvasculature (ie, small vessels) is increasingly recognised in the

Strengths and limitations of this study

- This is the largest Australian population-based study to investigate the concordance of microvascular structural measurements.
- Our adult sample provides novel data regarding the relatively neglected midlife phase, with most other adult population samples being elderly.
- We used gold-standard methodology and demonstrated high inter-rater and intra-rater reliability.
- Most of the participating parents were mothers, resulting in less precise descriptive and concordance estimates for fathers.

pathogenesis of cardiovascular disease.^{1–3} Microvascular abnormalities already present in early life are thought to predispose to both cardiovascular risk factors and outcomes (eg, stroke, myocardial infarction).^{4–6}

The retina offers a fast, non-invasive platform to study microvascular health *in vivo*, with similar pathological changes associated with abnormalities and diseases in the cerebral, coronary and renal microvasculatures.^{7–10} The most commonly assessed retinal microvasculature measure is the vessel calibre (diameter),¹⁰ summarised as the central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE) and its ratio, the arteriolar–venular ratio (AVR). Meta-analyses have demonstrated statistically significant correlations of smaller retinal arteriolar and wider retinal venular vessel calibre with subsequent cardiovascular disease, including stroke, obesity and coronary heart disease.^{11–14} Cardiovascular risk factors (such as hypertension, diabetes mellitus and obesity) have been similarly associated with smaller retinal arterioles and larger retinal venules via both shared and unique underlying pathophysiology.^{6 15 16} Thus, the retinal vasculature has

been suggested to be a robust biomarker of cardio-metabolic diseases and allows studying the natural course of small vessel changes over life and its relationship to cardiovascular outcomes in later years.^{10 15}

However, an important gap in the literature is that the natural course and determinants of the retinal vascular calibre are not fully documented. There is some evidence for distinct but differing curvilinear relationships of arteriolar and venular calibres with age.^{10 15} Thus, from disparate cross-sectional studies, Ikram's 2012 narrative review reported that both arteriolar and venular calibre increased from birth (85.5 and 130.0 µm respectively) to age 6 years (165.6 and 232.0 µm). Arteriolar calibre increased further by midlife (202.3 µm) while venular calibre remained static, with calibres of both arterioles and venules reduced from midlife to old age.¹⁰ However, limitations to this literature include differing protocols for imaging and scoring and a marked under-representation of population studies, with few data from young and mid-adulthood. These limitations preclude robust conclusions regarding reference ranges and predictive cutpoints across the lifespan.

While heritability of retinal vascular calibres appears substantial from small community-based samples using a variety of protocols,¹⁵ large studies of parent-child pairs drawn from the general population are lacking. Parent-child correlations for CRAE and CRVE respectively were 0.24 and 0.27 in 341 American adult pairs (aged 43–86 years) in the Beaver Dam Eye Study¹⁷; 0.13 and 0.20 for 174 adult pairs from the Flemish Study on Environment, Genes and Health Outcomes (aged 20+ years)¹⁸ and 0.12 and 0.31 in 304 pairs from the strabismus, amblyopia and refractive error in Singaporean Children study (mean ages: 40 years for parents, 9 years for children).¹⁹ In all three studies, spousal concordance was negligible for both metrics. In contrast, monozygotic twins have shown much higher concordance (CRAE 0.60 to 0.80, CRVE 0.63 to 0.88) than dizygotic pairs (CRAE 0.12 to 0.50 and CRVE 0.13 to 0.35),^{20–24} with higher heritability estimates in twin (CRAE 0.56 to 0.70, CRVE 0.64 to 0.83) than parent-child pairs (CRAE 0.25 to 0.54, CRVE 0.28 to 0.72).^{17–23} Genome-wide studies have identified several promising loci associated with retinal vessel calibre.^{23 25} However, it remains unclear whether these values remain constant across the lifecycle or what opportunities these offer for preventive health.

Thus, understanding the population epidemiology of retinal calibre in larger studies of healthy midlife adults and children would inform age-specific and sex-specific reference values and facilitate secular trend analyses, international comparisons and, potentially, early risk stratification. The Child Health CheckPoint, a cross-sectional biophysical assessment nested within the Longitudinal Study of Australian Children (LSAC), provided an opportunity to address this issue/question in a national population-based sample of Australian parent-child dyads at child age 11–12 years. We aimed to (1) assess the distribution of retinal vessel calibre in both age groups

and (2) investigate parent-child concordance of these measures.

METHODS

Study design and participants

Details of the initial LSAC study design and recruitment are outlined elsewhere.^{26 27} Briefly, LSAC recruited a nationally representative birth cohort (B cohort) of 5107 infants using a 2-stage clustered design. First, 10% of Australian postcodes (stratified by state and urban/rural locations) were randomly selected, then in-age children (born between March 2003 and February 2004) within those enrolled in the Medicare Australia database (Australia's universal healthcare system into which 98% of children are enrolled by their first birthday) were selected. Study participants were then followed up in biennial 'waves' of data collection up to 2015.²⁷ The initial recruitment rate in 2004 was 57.2%, of whom 73.7% (n=3764) were retained to LSAC wave 6 in 2014. At the wave 6 visit, all contactable and consenting families (n=3513) were invited to consent to their contact details being shared with the Child Health CheckPoint team. In 2015, families that consented to contact were sent an information pack via post and received an information and recruitment phone call. The CheckPoint's detailed cross-sectional biophysical assessment, nested between LSAC waves 6 and 7 at child age 11–12 years, took place between February 2015 and March 2016. A total of 1874 families participated. A more detailed description of the CheckPoint study design is available elsewhere.^{28 29}

Consent

The attending parents/caregivers provided written informed consent for themselves and their children to participate in the study.

Procedure

Retinal photographs were taken during each participant's 3.5-hour visit to the CheckPoint assessment centres in 7 large cities (mainly state capitals) around Australia. During this visit, each child and parent rotated sequentially and separately through a number of 15 min assessment stations.²⁸ At the 'See Here' station, participants sat in front of a fundus camera (EOS 60D SLR) while the procedure was explained; the room was then darkened while the participant rested chin and forehead on the head rest of the fundus camera for several minutes. Two digital photographs (one each centring on the optic disc and macula) were taken for each eye using standard protocols from the Centre for Eye Research Australia. Because CheckPoint was unable to transport its single retinal camera to smaller regional centres or home visits, 518 CheckPoint families did not contribute to retinal vessel calibre data (figure 1).

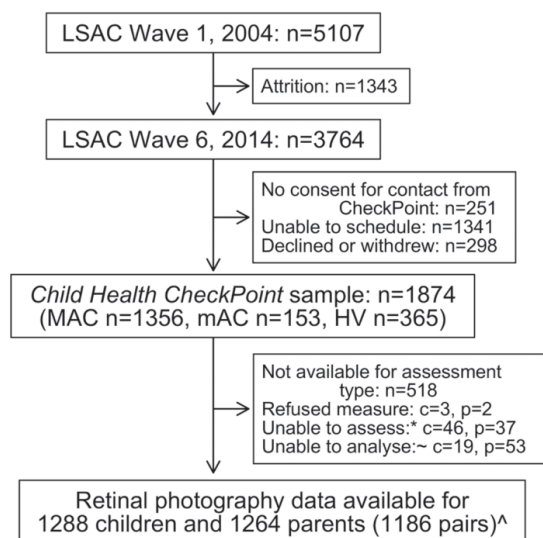


Figure 1 Participant flow through Child Health CheckPoint. *Unable to collect image due to equipment failure or time constraints. ~Data excluded for images that did not meet quality criteria for 'Big 6' analysis. ^Data from 10 non-biological child-parent pairs and 29 diabetic participants excluded from concordance analyses. c, number of children; HV, home visit assessment; LSAC, Longitudinal Study of Australian Children; MAC, main assessment centre; mAC, mini assessment centre; n, number of families; p, number of attending adults

Retinal image scoring

Right eye images were selected as first choice for scoring. Reasonable correlations ($r > 0.71$) of retinal vascular diameters between right and left eyes have previously been reported,^{30 31} and this allowed harmonisation with other vascular measures obtained in CheckPoint, such as pulse wave velocity and blood pressure, that also assessed the right-side circulation. Left eye images were used where right eye images were deemed ungradable. Issues preventing grading of images included poor focus (potentially blurring vessel edges), dark images (increasing the difficulty for graders to visually validate the vessel trace), and confounding pathology (which can obscure the vessels). Images were evaluated at the Zhongshan Ophthalmic Centre in Guangdong, China (77%) and the Centre for Eye Research Australia in Melbourne, Australia (23%), separately. In total, 2624 images were graded, including 1307 from children and 1317 from parents (87% and 92% from the right eye for children and parents, respectively). Nineteen child and 53 parent images did not meet the quality criteria for use in analyses.

Each image was graded by one of four experienced graders (two from China and two from Australia) masked to participant characteristics who measured the diameters of retinal vessels using the software programme

Integrative Vessel Analysis (IVAN, University of Wisconsin, Madison, USA). Retinal vessels were identified as arterioles or venules from a specific area (one-half to one disc diameter from the margin of the optic disc). Each grader then selected a segment of each vessel within this area for measurement. Diameters of all the selected segments were measured automatically by the IVAN software. For each participant, summary estimates of the average retinal vascular calibre were calculated according to the Big6 (revised Knudston-Parr) formula,³⁰ which combines measurements of the six largest arterioles or venules, represented as the CRAE and CRVE. The AVR was calculated as CRAE divided by the CRVE.

Other sample characteristics

Measures of potential confounding variables were included as detailed elsewhere.²⁸ Briefly, age was calculated to the nearest week using the date of birth, either imported from Medicare Australia's database at the time of LSAC enrolment (child) or self-reported (parent), and date of assessment. Sex was self-reported.

Body mass index (BMI) was calculated as kg/m^2 , using measured height and weight.²⁸ For children, age-adjusted and sex-adjusted BMI z-scores were calculated using the US Centers for Disease Control and Prevention growth reference charts.³²

Brachial blood pressure was measured using the SphygmoCor XCEL (AtCor Medical Pty, West Ryde, NSW, Australia), following 7 min rest. Mean arterial blood pressure was calculated from the systolic and diastolic measures.

In Australia, Socio-Economic Indexes for Areas provide standardised scores for socio-economic position by geographical area (postcode of family domicile) compiled from 2011 Australian census data. We used the Socio-Economic Indexes for Areas index of Relative Socio-economic Disadvantage (Disadvantage Index) which numerically summarises the social and economic conditions of Australian neighbourhoods (national mean of 1000 and SD of 100, where higher values represent less disadvantage).³³ Parents reported child and parent medical histories via iPad-administered questionnaires at the assessment centre.

Child and parent participants who attended the CheckPoint assessment centres also completed a visual acuity assessment (not conducted in home visits). As part of this assessment, they were asked if they 'usually wear glasses or contact lenses'. Staff members recorded their response as yes or no; the strength of prescription was not captured.

Statistical analyses

All analyses were performed using Stata V.14.2.

Continuous descriptive variables were summarised using weighted means and SD; categorical variables were summarised by number and weighted percentage for children and adults separately and by sex. Survey weights were calculated taking into account the selection

probability of each child, and were adjusted for non-response, loss to follow-up and benchmarked to population numbers in major (post stratification) categories of the population of children born in 2004. Standard errors were calculated taking into account the complex design and survey weights.³⁴ More details on the calculation of survey weights is provided elsewhere.^{28 35}

Concordance between parents and children was assessed by (1) Pearson's correlation coefficient (CCs) with 95% CIs; (2) linear regression with child variable as dependent variable and parent variable as independent variable adjusted for parent and child age, BMI, mean arterial blood pressure, Disadvantage Index score and sex where appropriate and (3) partial correlation coefficients adjusted for the same covariates. The Pearson's correlation and linear regression analyses were repeated using weighted multi-level survey analyses; as these yielded similar results, unweighted results are displayed. Twenty nine diabetic participants, and 10

non-biological child-parent pairs were excluded from concordance analyses.

Reliability of retinal vessel calibre measurements

Inter-grader reliability was assessed by three of the four graders reanalysing a subset of 50 randomly chosen images. To assess intra-grader reliability, the same three graders each re-graded 25 of the 50 randomly chosen images. Hence, an assessment was made as to reproducibility of grading made by different graders and repeatability of gradings made by the same grader. Two-way mixed-effects intraclass correlation coefficients were used for the reliability analysis.

Patient and public involvement

Because LSAC is a population-based longitudinal study, no patient groups were involved in its design or conduct. To our knowledge, the public was not involved in the study design, recruitment or conduct of the LSAC study

Table 1 Sample characteristics and retinal vessel calibre measures, stratified by sex, of children and parents

Sample characteristics	Mean (SD)*		
	All	Male	Female
Children†	(n=1288)	(n=633)	(n=655)
Age, years	11.96 (0.4)	11.96 (0.4)	11.96 (0.4)
BMI, kg/m ²	19.4 (3.6)	19.2 (3.6)	19.6 (3.6)
BMI z-score	0.37 (1.00)	0.37 (1.02)	0.38 (0.99)
Systolic blood pressure, mm Hg	108.7 (8.2)	108.3 (8.4)	109.1 (7.9)
Diastolic blood pressure, mm Hg	63.3 (5.7)	63.0 (6.1)	63.6 (5.2)
Neighbourhood Disadvantage Index	1011.2 (61.0)	1009.6 (63.2)	1012.8 (58.8)
Diabetes (%)	0.4	0.2	0.6
Eye condition or glasses/contact lenses (%)	20.9	17.9	23.8
Retinal vessel measures			
CRAE, µm	159.5 (11.8)	156.8 (11.8)	162.1 (11.2)
CRVE, µm	231.1 (16.5)	227.8 (15.6)	234.3 (16.8)
AVR	0.69 (0.05)	0.69 (0.05)	0.69 (0.05)
Parents‡	(n=1264)	(n=169)	(n=1095)
Age, years	43.7 (5.6)	46.2 (6.5)	43.4 (5.3)
BMI, kg/m ²	28.4 (6.5)	28.7 (4.2)	28.3 (6.7)
Systolic blood pressure, mm Hg	120.5 (12.9)	128.4 (11.6)	119.3 (12.7)
Diastolic blood pressure, mm Hg	73.8 (8.8)	78.3 (8.4)	73.1 (8.6)
Diabetes (%)	2.9	5.3	2.6
Eye condition or glasses/contact lenses (%)	53.2	47.0	54.12
Retinal vessel measures			
CRAE, µm	151.5 (14.0)	148.4 (14.3)	152.0 (13.8)
CRVE, µm	220.6 (19.0)	217.9 (21.3)	221.0 (18.6)
AVR	0.69 (0.06)	0.68 (0.06)	0.69 (0.06)

*Mean (SD), unless otherwise specified. †n for each child variable ranges from 1213 to 1288.

‡n for each parent variable ranges from 1169 to 1264.

AVR, arteriolar-venular ratio; BMI, body mass index; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent.

Table 2 Retinal vessel calibre percentiles

Retinal calibre	Child							Parent						
	P5	P10	P25	P50	P75	P90	P95	P5	P10	P25	P50	P75	P90	P95
Central retinal arteriolar equivalent, μm														
Male	137.1	141.5	148.0	158.0	165.1	170.7	174.2	123.6	128.7	139.9	148.3	158.2	167.2	171.6
Female	143.8	147.3	153.8	162.5	169.9	175.3	179.3	128.8	134.8	143.3	152.1	162.3	169.1	174.0
All	139.8	144.0	151.3	160.2	167.6	173.1	178.5	128.1	134.1	143.0	151.4	161.7	169.0	173.7
Central retinal venular equivalent, μm														
Male	203.3	208.9	217.7	226.7	237.4	247.4	252.7	186.8	190.9	202.1	215.4	231.5	247.6	261.5
Female	208.8	213.3	222.6	234.0	245.4	256.8	262.8	192.3	197.5	208.4	220.8	233.8	244.8	253.0
All	206.1	210.8	220.4	230.4	241.4	252.1	259.3	190.9	196.8	207.2	220.2	233.7	245.7	253.0
Arteriolar-venular ratio														
Male	0.61	0.63	0.66	0.69	0.72	0.76	0.78	0.60	0.61	0.64	0.67	0.73	0.78	0.79
Female	0.61	0.62	0.66	0.69	0.73	0.76	0.78	0.59	0.61	0.65	0.69	0.73	0.77	0.78
All	0.61	0.63	0.66	0.69	0.72	0.76	0.78	0.59	0.61	0.65	0.69	0.73	0.77	0.78

P, percentile.

or its CheckPoint module. Parents received a summary health report for their child and themselves at or soon after the assessment visit. They consented to take part knowing that they would not otherwise receive individual results about themselves or their child.

RESULTS

Sample characteristics

A total of 2552 participants (1288 children and 1264 adults) were included in the descriptive aim 1 analyses (figure 1). This represents 95% of the 1356 pairs who attended CheckPoint assessment centres (where retinal photography was offered). Reasons for participants not having retinal photography images and data are attendance at smaller assessment centres without a retinal camera or home visit (n=518 participants), participant

refusal (n=160 participants), and image not taken (eg, the camera required repair) or the quality was too poor (n=5 participants, figure 1). A total of 1186 parent-child pairs were included in aim 2 analyses; 10 non-biologic adult-child pairs and 29 diabetic participants were excluded from the concordance assessments (figure 1).

Table 1 shows sample characteristics of children and parents stratified by sex. In children, sex was evenly represented (51% girls), while mean age-specific and sex-specific BMI z-scores were higher than Center for Disease Control historical norms (0.37). The parent sample largely comprised mothers (86.6%). Participants on average were slightly more advantaged (Disadvantage Index mean score 1011, SD 61) than the national Australian population (mean 1000, SD 100).³³

Descriptive epidemiology of retinal vessel calibre

Summary statistics for parent and child retinal values are displayed in table 1, and broken down for reference into 5th to 95th percentiles in table 2. The corresponding distribution graphs (figure 2) demonstrate approximately normal distribution in all measures for both children and adults. Mean CRAE and CRVE were around 0.6 SD larger in children (159.5 (11.8) and 231.1 (16.5) μm , respectively) than parents (151.5 (14.0) and 220.6 (19.0) μm), yielding similar AVR (children 0.69 (0.05), parents 0.69 (0.06)), table 1). Boys' CRAE and CRVE mean values were marginally smaller than girls' values (CRAE 156.8 μm vs 162.1 μm , CRVE 227.8 μm vs 234.3 μm), and fathers similarly showed smaller mean values than mothers (CRAE 148.4 μm vs 152.0 μm , CRVE 217.9 μm vs 221.0 μm). Mean AVR was strikingly similar in parents and children and between all four groups.

Parent-child concordance

Correlations for the 1186 parent-child pairs are displayed in table 3. CCs for parent-child dyads were 0.22 for CRAE

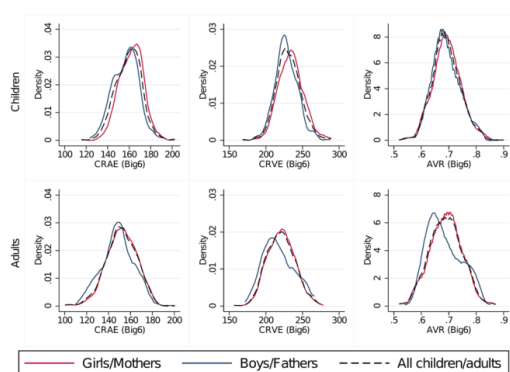


Figure 2 Density plots for retinal vessel calibre measures. AVR, arteriolar-venular ratio; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; BIG6, revised Knudston-Parr formula for calculating CRAE and CRVE using IVAN software.³⁰

Table 3 Parent-child concordance

Pearson's correlation coefficient	Parent-child			Mother-child			Father-child		
	n	CC	95% CI	n	CC	95% CI	n	CC	95% CI
CRAE, μm	1186	0.22	0.16 to 0.27	1029	0.20	0.14 to 0.26	157	0.32	0.17 to 0.45
CRVE, μm	1186	0.23	0.17 to 0.28	1029	0.22	0.16 to 0.28	157	0.29	0.14 to 0.42
AVR	1186	0.18	0.13 to 0.24	1029	0.18	0.12 to 0.24	157	0.20	0.04 to 0.35
Adjusted linear regression	n	RC	P value	n	RC	P value	n	RC	P value
CRAE, μm	998	0.18	<0.001	867	0.18	<0.001	131	0.21	0.004
CRVE, μm	998	0.20	<0.001	867	0.20	<0.001	131	0.22	0.003
AVR	998	0.16	<0.001	867	0.16	<0.001	131	0.15	0.06

Non-biological caregivers (n=10) and diabetic participants (n=29) were excluded from these analyses. Covariates in adjusted linear regression models include parent and child ages, Body mass index and Disadvantage Index, mean arterial blood pressure and parent and child sex in models including both sexes.

AVR, arteriolar-venular ratio; CC, estimated Pearson's correlation coefficient; n, number of biological child-parent pairs with this measure; RC, estimated regression coefficient.

and 0.23 for CRVE, and (as expected for a derived ratio value) slightly smaller for AVR (0.18). Mother-child and father-child correlations are presented for reference but not compared statistically; noting slightly higher values for father-child dyads but wider CIs in keeping with the much smaller numbers. In linear regression adjusting for all covariates, estimates attenuated only marginally and all associations remained strong (estimated regression coefficients for parent-child dyads: CRAE 0.18, CRVE 0.20, AVR 0.16) with the exception of father-child AVR. Similarly, partial correlations adjusted for covariates attenuated slightly (correlation coefficient decreased by 0.01 to 0.06, data not shown).

Reliability analysis

Within-grader intraclass correlations were very high for all three graders for CRAE (0.90 to 0.99, 95% CI 0.76 to 0.99) and CRVE (0.92 to 0.98, 95% CI 0.82 to 0.99). This equated to a greater within-grader range for the derived AVR variable (0.69 to 0.97, 95% CI 0.28 to 0.99). Between-grader interclass correlations were also high at 0.79 (95% CI 0.47 to 0.91), 0.92 (95% CI 0.77 to 0.96) and 0.75 (95% CI 0.58 to 0.86) for CRAE, CRVE and AVR respectively.

Unplanned post-hoc analysis

As noted above, both CRAE and CRVE were around 0.6 SD smaller in the midlife parents than the 11–12-year olds. This contrasts with Ikram's 2012 review, from which we had expected that CRAE would be substantially larger by midlife, but that CRVE would remain static (prior to both reducing in old age).¹⁰ Because these results were surprising, we therefore conducted some unplanned post-hoc analyses to determine how our findings fit within the existing literature. These should be considered as exploratory and hypothesis-generating only.

We plotted mean values for CRAE and CRVE by mean age (figure 3) for 15 community-based studies published since 2004 with participants aged from 6 years to old age.^{11 22 36–47} We included all adult studies (summarised

in online supplementary table 1) in Sun *et al*'s systematic review¹⁵ that had a community or population-based sample of >1000 individuals^{40–47}; the Norwegian Tromsø Eye Study was identified through cited reference searches.³⁷ Sun's review also identified the child population in the Singapore Cohort Study of the Risk Factors for Myopia (SCORM),³⁶ and we searched the SCORM study's bibliography and other published reviews for additional children's cohorts.^{11 22 38 39} We superimposed the

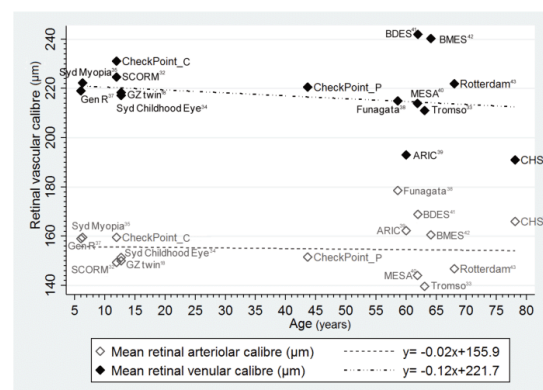


Figure 3 Epidemiology of retinal vascular calibre by age. Mean retinal arteriolar and venular calibre by age from CheckPoint and published community-based studies with n>1000. Each symbol represents the mean value for a single study. Each study is summarised in online supplementary table 1, and the paper from which that study's data are drawn is provided in the list of references. ARIC, Atherosclerosis Risk in Communities Study; BDES, Beaver Dame Eye Study; BMES, Blue Mountains Eye Study; CHS, Cardiovascular Health Study; CheckPoint_C, Child Health CheckPoint data of children; CheckPoint_P, Child Health CheckPoint data of parents; MESA, Multi-Ethnic Study of Atherosclerosis; SCORM, Singapore Cohort Study of the Risk Factors for Myopia.

results from CheckPoint, separately for adults and children. As visual inspection did not suggest a curvilinear relationship, we then fitted two exploratory linear regression models, with mean age for each study (CheckPoint children and parents considered separately) as the independent variable and with calibre size (arteriolar and venular) as the two dependent variables. It can be seen that venular calibre appears to narrow slightly (though not significantly differently from 0) at a rate of approximately $-1.2\ \mu\text{m}$ per decade from childhood through adulthood (β coefficient -0.12 , 95% CI -0.37 to 0.14 , $R^2=0.03$). However, there was no obvious trend for arteriolar calibre (β coefficient -0.02 , 95% CI -0.21 to 0.17 , $R^2=0.002$). Two striking features of the figure are the dearth of published values between childhood and late life, and the marked spread of mean values in the elderly.

DISCUSSION

Principal findings

We provide the first national population-based percentile values for the pattern and distributions of retinal vessel calibre in Australian 11–12-year old children and midlife adults. Surprisingly, in light of previous literature, mean scores for both CRAE and CRVE were around 0.6 SD smaller in parents than in children. Parent–child correlations were similar for all retinal vessel calibre metrics (CRAE 0.22, CRVE 0.23, AVR 0.18) to studies whose offspring probands were already adults, suggesting that the strong element of heritability in microvascular anatomical structure is largely phenotypically overt within the first decade of life.

Strengths and weaknesses

Strengths include the national recruitment basis for this large study, providing a reference point for microvascular measurement for future Australian studies and also filling a significant international gap in population-based studies of these age groups. Results were largely consistent with previous studies and should generalise to the wider Australian child population.

Limitations include our under-representation of the most disadvantaged sector (reflecting social biases in both recruitment and retention, issues common to large longitudinal studies) and the small number of fathers in the parent sample. We adjusted only for a limited range of potential confounders. While the distributions of parent retinal vessel calibre might have changed slightly had we further adjusted for smoking status, sedentary lifestyle and diet (all previously associated with altered retinal vessel calibre⁴⁸), their impact on concordance would likely be small because these factors are all strongly socially patterned. By 2019 this cohort will also be able to consider genome-wide association (GWAS) data, potentially shedding further light on the roles of genetic and shared environmental factors. We were not able to measure refractive errors in this study. Retinal vascular calibre measurements may be influenced by refractive

errors and refraction is different between children and adults.^{49 50} However, we would expect these effects to be small, particularly as other sources of systematic bias were minimised (measured on the same day with the same equipment by the same person who was blind to dyadic membership).

Meaning and implications for clinicians and policy makers

Findings for all outcomes are consistent with the previous literature. We showed a larger SD in the parent than the child group, indicating a greater spread of variation in retinal calibre with age. This would be in keeping with greater physiological dysregulation for some individuals with age in response to genetic and risk exposure (eg, higher blood pressure, obesity etc) over multiple decades. Direct comparisons of CRAE and CRVE across the lifecourse and between ethnicities remain difficult due to differences in equipment, software and analysis formulae for CRAE and CRVE. Such factors could well account for the differences between our own and three international studies at mean age 11.1–12.7 years: smaller mean CRAE and CRVE in Chinese twins (CRAE $150.1\ \mu\text{m}$, CRVE $218.4\ \mu\text{m}$) and Singapore Chinese children (CRAE $149.3\ \mu\text{m}$, CRVE $224.6\ \mu\text{m}$), but much larger values in a smaller study of German children (CRAE $208\ \mu\text{m}$, CRVE $236.2\ \mu\text{m}$).^{22 36 51} These discrepancies highlight the need for a uniform approach to measurement of retinal parameters in future epidemiological investigations. Our post-hoc analysis of published literature suggests both a dearth of population studies between childhood and old age and a likely fall in venular calibre from childhood through midlife. An individual participant meta-analysis would be the next step to more precisely determine how retinal calibre varies by age, incorporating any additional large-scale studies that now exist.

The moderate parent–child correlations of this Australian population are very similar to those in previous studies with much older adult offspring, suggesting that in terms of microvasculature, children do not become more phenotypically similar to their parents between childhood and midlife. Although this is the first study to report intergenerational concordance by parent sex, our marginally stronger father–child than mother–child correlations must be taken with caution due to the small sample of fathers. Like all other parent–child and twin studies, except the Beaver Dam Eye study, our CRVE correlations were marginally larger than those for CRAE.^{17–23} Previously, Li *et al* have postulated that the retinal arteriolar phenotype may be more sensitive to environmental influences than the venular phenotype, despite having a significant, moderate degree of inheritance.¹⁹ This is further supported by GWAS studies, where only CRVE showed significant associations with the identified gene loci.^{23 25}

Unanswered questions and future research

We provide normative values for retinal vessel calibre for Australian children aged 11–12 years and midlife adults using standardised protocols. Our findings make

explicit the need for reliable age-specific normative reference values across the life course. Ideally, this would be extended to large long-running cohort studies with access to clinical outcomes if retinal calibre is to realise the potential as a population screening and risk stratification tool for cardiovascular disease, with or without consideration of other retinal vascular features such as branching angles, tortuosity and fractal dimension, polygenic risk scores and macrovascular risk.

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Data sharing statement The Longitudinal Study of Australian Children datasets and technical documents are available to researchers at no cost via a licence agreement. Data access requests are co-ordinated by the National Centre for Longitudinal Data. More information is available at <https://dataverse.ada.edu.au/dataverse/lsac>.

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Associations of Retinal Vessel Caliber With Hearing Status in Childhood and Midlife

A Cross-Generational Population-Based Study

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[+ Supplemental content](#)

IMPORTANCE Microvascular phenotypes, which can be assessed using retinal imaging, may be informative about the life course pathogenesis of hearing loss.

OBJECTIVE To investigate whether differences in retinal vessel caliber (specifically wider venules and narrower arterioles) are associated with hearing threshold and hearing loss in mid-childhood and midlife.

DESIGN, SETTING, AND PARTICIPANTS A population-based cross-sectional study (Child Health CheckPoint) was nested within the Longitudinal Study of Australian Children. A total of 1281 children and 1255 attending parents were assessed using retinal microvasculature and air conduction audiometry data at a main assessment center in 7 large cities in Australia.

MAIN OUTCOMES AND MEASURES Air conduction audiometry was used to calculate the high Fletcher index (mean threshold of 1, 2, and 4 kHz), and bilateral hearing loss was defined as a high Fletcher index greater than 15 dB hearing level in the better-hearing ear. Retinal arteriolar and venular caliber were measured from fundus photographs using validated computer-based software. Linear and logistic regression quantified the associations of retinal vessel caliber with hearing threshold and hearing loss, respectively.

RESULTS Of the 1281 included children (mean age, 11.4 years; 49.1% boys), the mean (SD) high Fletcher index was 7.9 (5.8) dB hearing level. Of the 1255 included adults (mean age, 43.8 years; 86.6% women), the mean (SD) high Fletcher index was 13.0 (6.8) dB hearing level; 109 of 1281 children (8.5%) and 328 of 1255 adults (26.1%) had hearing loss. In adults, each 1-SD (18.6- μ m) wider retinal venular caliber (worse) was associated with higher (worse) hearing threshold at lower individual frequencies (eg, 2 kHz: β = 0.63; 95% CI, 0.10-1.17) and overall high Fletcher index (eg, 2 kHz: β = 0.52; 95% CI, 0.07-0.96), as well as a 1.20-fold (95% CI, 1.03-1.40) higher odds of hearing loss. In children, patterns of venular associations were similar but smaller and less certain. Narrower retinal arteriolar caliber (worse) was associated with a 1.16-fold (95% CI, 1.00-1.37) higher odds of hearing loss in adults (per 1-SD [14.0- μ m] narrower arteriolar caliber) but not in children.

CONCLUSIONS AND RELEVANCE Adverse retinal microvascular characteristics are associated with hearing loss by midlife, with venular associations possibly emerging by age 11 to 12 years. Microvascular health may contribute to the pathogenesis of hearing loss across the life course, warranting replication and mechanistic studies to inform causal inference and prevention efforts.

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Hearing loss is the most prevalent sensory disability, with its prevalence increasing over time and with advancing age.¹⁻³ Like other noncommunicable diseases, hearing loss may have early life origins,^{4,5} and the development of timely interventions (eg, therapy for cytomegalovirus infection in the early weeks of life) may have important benefits.⁶ This requires population-based data for an individual over time or across generations to understand when risk factors for hearing loss begin to emerge during the life course.⁷ To our knowledge, such studies are lacking, and although genetics, ototoxic drugs, and noise exposure are the most studied risk factors for hearing loss, the biological mechanisms underlying most hearing loss are still poorly understood.

A growing body of evidence suggests that microvascular changes may contribute to hearing loss. The stria vascularis, a highly vascularized region located in the cochlear microcirculation, is the metabolic energy source underlying cochlear function.⁸ Histopathologic studies show that patients with age-related hearing loss have increased thickness of vessel walls of the stria capillaries and decreased numbers of stria vessels compared with those with normal hearing.⁹ This is thought to augment stria atrophy and cause degeneration of hair cells by alteration of the endolymph composition.¹⁰

The ability to measure many aspects of the microvasculature now provides opportunities to study its associations with hearing status. Retinal photography is a noninvasive method of investigating the retinal microvasculature. Because the retinal microvasculature shares similar anatomic and physiologic properties with the microvasculature elsewhere in the body,¹¹ it is a widely used surrogate marker for systemic microcirculation in population-based studies.^{12,13} Many studies have shown that adverse retinal microvascular characteristics (eg, narrower arterioles, wider venules) are associated with cardiovascular risk factors (eg, obesity, hypertension, type 2 diabetes),¹⁴⁻¹⁶ which are also risk factors for hearing loss.^{17,18} A cohort study of 10 470 adults aged 45 to 64 years¹⁴ showed that participants with wider retinal venules or narrower retinal arterioles were more likely to have higher systolic blood pressure, total cholesterol level, and body mass index over a mean follow-up period of 16 years. A participant-level meta-analysis of 5 cohort studies¹⁹ showed that wider retinal vessel caliber was associated with risk of type 2 diabetes (per 1-SD wider venular caliber; pooled hazard ratio, 1.09; 95% CI, 1.02-1.15). Furthermore, some genes (eg, *Norrin* and *Frizzled-4*)²⁰ are known to play a central role in the vascular development of both the retina and stria vascularis. These studies support the hypothesis that abnormalities in retinal microcirculation may be correlated with abnormalities in cochlear microcirculation.²⁰

To our knowledge, only 1 population-based study has reported associations of retinal microvasculature with hearing loss. In the Blue Mountains Hearing Study,²¹ older adults (older than 54 years) with retinopathy (a sign of retinal microvascular damage) were twice as likely as those without to have lower-frequency hearing loss greater than 40 dB hearing level (HL) across frequencies of 0.5, 1, and 2 kHz after excluding persons with type 2 diabetes. To our knowledge, there are no analogous observational studies of participants in childhood or midlife that would be informative regarding when this as-

Key Points

Question Are differences in retinal vessel caliber associated with hearing threshold and hearing loss in mid-childhood and midlife?

Findings In this population-based cross-sectional study of 1281 children and 1255 adults, wider retinal venules and narrower retinal arterioles were associated with hearing loss by midlife, with venular associations possibly emerging by age 11 to 12 years.

Meaning Microvascular health may contribute to the pathogenesis of hearing loss across the life course, warranting replication and mechanistic studies to inform causal inference and prevention efforts.

sociation emerges and whether it progresses with age. Last, little is known about the association of more subtle microvascular differences (eg, retinal vessel caliber) with hearing loss, which may be valuable for understanding the pathogenesis of early hearing loss.

The Australian population-based cross-sectional Child Health CheckPoint study²² provides a unique opportunity to address these evidence gaps in parent-child pairs with children aged 11 to 12 years. Therefore, we aimed to determine if retinal arteriolar and venular calibers were associated with (1) the range of hearing thresholds and (2) hearing loss in mid-childhood and midlife.

Methods

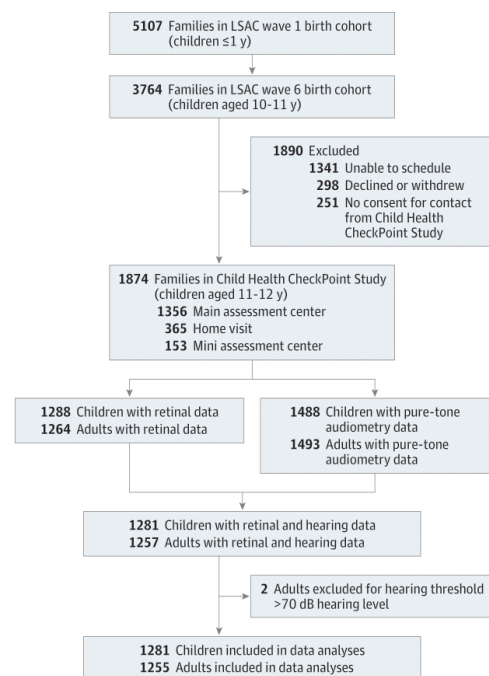
Study Design and Participants

The Child Health CheckPoint study was a cross-sectional population-based substudy nested within the Longitudinal Study of Australian Children (LSAC).^{23,24} In 2004, LSAC used a 2-stage random-sampling framework clustered by state, urban/rural split, and postcode to recruit a nationally representative sample of 5107 infants 1 year and younger (birth cohort).²³ Since then, LSAC has conducted follow-up of children and their families biennially. The response rate to the initial mailed invitation in 2004 was 57.2% (5107 of 8921), of whom 73.7% (3764 of 5107) were retained for wave 6 in 2014.²⁵ The LSAC interviewers obtained written informed consent at the wave 6 interview to give contact details to CheckPoint, which was open to all retained birth cohort children aged 11 to 12 years and 1 attending parent for each child between LSAC's wave 6 (2014) and wave 7 (2016). The Royal Children's Hospital Human Research Ethics Committee and the Australian Institute of Family Studies Ethics Committee approved the study. Parents provided written consent, and children gave verbal assent. Although we did not complete a checklist for the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines, all STROBE elements are contained within this article.

Procedures

Starting in December 2014, CheckPoint contacted families to ascertain interest and booked a single appointment for parent-child pairs. The CheckPoint assessment center conducted a visit in each Australian state sequentially between February 3, 2015, and

Figure 1. Participant Flow Diagram



LSAC indicates Longitudinal Study of Australian Children.²³

March 25, 2016.^{22,26} Ultimately, 1874 families participated, equating to 36.7% and 49.8% of those originally recruited (1874 of 5107) and retained for wave 6 (1874 of 3764) of LSAC (Figure 1). The attending parent was determined by the family; in practice, this was usually the mother. CheckPoint offered a specialized 3.5-hour visit to a main assessment center in 7 large Australian cities. Of the 1874 families, 518 took part in a shorter assessment at a mini assessment center or home visit and did not have retinal photographs, so they were not included in our analyses. At the main assessment center, the child and parent rotated separately through multiple 15-minute assessment stations targeting health measures relevant to noncommunicable diseases. In this article, we report data from retinal photographs, audiometry, and tympanometry.

Measures

Retinal Vessel Caliber

Two optic disc-centered digital photographs (Figure 2) from each eye were taken using a fundus camera (EOS 60D DSLR; Canon). Details of measurement procedures have been described elsewhere.²⁸ Each image was graded by 1 of 4 graders, masked to participant characteristics, using the software program Integrative Vessel Analysis (IVAN; University of Wisconsin). Right eye images were selected as the first choice for scoring, and left eye images were used when right eye images were deemed ungrad-

Figure 2. Retinal Fundus Photograph



Photograph was assessed quantitatively using Integrative Vessel Analysis software (IVAN; University of Wisconsin). The venules (blue marks) and arterioles (red marks) are identified. Green circles indicate optic disc diameters from the disc center; yellow line, center of optic disc. For each participant, a segment of each vessel within the specific area (0.5-1.0 disc diameter from the margin of the optic disc [white 2-headed arrow]) was selected by the grader for measurement. The IVAN software then estimates summary values for the mean retinal vascular caliber according to the Big-Six (revised Knudtson-Parr²⁷) formula, which combines measurements of the 6 largest arterioles or venules.

able. Retinal vessels were identified by the software as arterioles or venules from a specific area (0.5-1.0 disc diameter from the margin of the optic disc). Each grader then selected a segment of each vessel within this area for measurement. Diameters of all selected segments were measured automatically using IVAN software. For each participant, summary estimates of the mean retinal vessel caliber were calculated according to the revised Knudtson-Parr formula,²⁷ which combines measurements of the 6 largest arterioles or venules. Our intergrader and intragrader reliability ranged from 0.79 to 0.99 (95% CI, 0.47-0.99).

Audiometry

Parent and child protocols were identical, with methods reported in detail elsewhere.²⁹ Trained examiners performed air conduction pure-tone audiometry using a computer-based audiometer (Oscilla USB-330, version 3.3.4; Oscilla Power) with headphones (Oscilla Power), following a standardized modified Hughson-Westlake audiometric technique.³⁰ Testing of the first frequency (1 kHz) began at 30 dB HL; if within normal limits, testing for other frequencies began at 20 dB HL. Participants with known hearing loss removed hearing aids and/or cochlear implant speech processors (2 children and 3 adults); testing of the first frequency then began at 60 dB HL and of successive frequencies at 20 dB HL higher than the previous frequency. For the first 143 parent-child pairs, only 3 frequencies were tested (1, 2, and 4 kHz) for each ear across an intensity range of -10 to 120 dB HL. As CheckPoint systems became faster and additional funding was sourced, testing

at 8 kHz (1342 pairs) and tympanometry (1090 pairs) were successively added. We gave parents written feedback about their own and their child's hearing results; those with thresholds greater than 20 dB HL in at least 1 ear at 2 or more frequencies were advised to consider clinical audiologic assessment.

Tympanometry (Middle Ear Function)

A combined audiometer and tympanometer (TSM500; Oscilla) automatically calculated ear canal volume, middle ear pressure, and compliance during an air pressure sweep. The resulting tympanogram curve represents the movement of the middle ear system while the pressure delivered to the tympanic membrane is varied. Tympanogram results were classified as type A (normal compliance, reflecting normal middle ear function), B (no or negligible compliance), or C (normal compliance, negative middle ear pressure), with criteria and interpretation detailed elsewhere.¹ Hearing loss in combination with a type A tympanogram result was considered sensorineural. Hearing loss in combination with a type B or C tympanogram result was considered to be of uncertain origin (ie, could reflect sensorineural and/or middle ear abnormalities).

Potential confounding factors included age, sex, gestational age (in weeks), birth weight, socioeconomic position (SEP), smoking, and preexisting conditions (including any type of diabetes and hypertension); all have been associated with both hearing loss and retinal microvasculature.³¹⁻³⁴ Children's date of birth, sex, gestational age, and birth weight were obtained from LSAC records. In adults, self-reported date of birth and sex were acquired at the time of assessment. The SEP included parent-reported combined household income, current or most recent occupation of each parent, and highest achieved educational qualification for each parent. Each component of the score was scaled, and an unweighted mean was calculated using 3 values in a single-parent household or 5 values in a dual-parent household. The unweighted mean variable for each LSAC wave was then standardized within the wave to a mean (SD) of 0 (1). Higher scores indicate less disadvantage. In this study, we used SEP from wave 6. For adults, smoking was determined by their answer to the question, "Are you currently smoking?" For children, we created a binary variable for passive smoking exposure if the parent questionnaire recorded any smokers at home during any LSAC wave when the child was 11 years or younger. Preexisting conditions were self-reported (yes/no) by questionnaire at the Check-Point study assessment, including diabetes and receiving medication for hypertension.

Data Management

We calculated high Fletcher index (hFI) (mean hearing threshold across 1, 2, and 4 kHz) because its range of frequencies maps most closely to the range of important speech sound frequencies.³⁵ We defined hearing loss as an hFI exceeding 15 dB HL in the better-hearing ear, in line with the American Speech-Language-Hearing Association criteria for slight or worse bilateral hearing loss.³⁶ Slight or worse hearing loss was the primary outcome measure because we were interested in early associations of retinal microvasculature with hearing loss, in keeping with a life course approach.

Statistical Analysis

We conducted linear regression analyses to examine associations of retinal vessel caliber with hearing threshold at each frequency and hFI as well as logistic regression to examine associations of retinal vessel caliber with the binary variable of hearing loss outcome (hearing threshold >15 dB HL vs <15 dB HL). For children, we adjusted analyses for age, sex, gestational age, birth weight, and SEP. For adults, we adjusted analyses for age, sex, and SEP. In sensitivity analyses, we (1) repeated all analyses for participants with type A tympanogram results only (normal middle ear function); (2) repeated adult analyses using the American Speech-Language-Hearing Association cutoff point for mild hearing loss of greater than 25 dB HL³⁶; and (3) further adjusted for smoking and preexisting conditions and for parent global region of birth and indigenous (Aboriginal/Torres Strait Islander) status. All analyses were performed using Stata statistical software version 14.0 (StataCorp), with separate analyses for children and parents.

Results

Sample Characteristics

Figure 1 presents the number of participants from wave 1 of LSAC onward; 1281 children (mean [SD] age, 11.4 [0.5] years; 49.1% [630 of 1281] boys) and 1255 attending parents (mean [SD] age, 43.8 [5.0] years; 86.6% [168 of 1255] women) were included in the analyses. The sample mean SEP was 0.26 SD higher than the mean SEP for all families in LSAC wave 6 and 0.40 SD higher than the mean SEP for all 5017 families enrolled in LSAC wave 1. Mean (SD) hFI was 7.9 (5.8) dB HL for children and 13.0 (6.8) dB HL for adults, with 8.5% (109 of 1281) and 26.1% (328 of 1255) showing hearing loss greater than 15 dB HL (Table 1). In adults, 56 of 1255 (4.5%) had mild or worse hearing loss. The means (SDs) for retinal arteriolar and venular caliber were 159.1 (11.9) μ m and 230.7 (16.6) μ m, respectively, in children and 151.1 (14.0) μ m and 218.9 (18.6) μ m in adults.

Associations of Retinal Vessel Caliber With Hearing Acuity

In adults, each 1-SD (18.6- μ m) wider retinal venular caliber was associated with higher (worse) hearing threshold (dB HL) at lower individual frequencies (eg, 2 kHz: β = 0.63; 95% CI, 0.10-1.17) and overall hFI (eg, 2 kHz: β = 0.52; 95% CI, 0.07-0.96) but not with higher individual frequencies (Figure 3 and Table 2). Similar but attenuated patterns were seen for children's hearing threshold, with greatest clarity at 1 kHz per 1-SD (16.6- μ m) wider venular caliber (β = 0.47; 95% CI, 0.03-0.92). Each 1-SD (18.6- μ m) wider venular caliber also had a 1.20-fold (95% CI, 1.03-1.40) higher odds of adult hearing loss as measured by the hFI, mainly driven by hearing loss at lower frequencies (1 kHz: odds ratio [OR] = 1.20; 95% CI, 1.03-1.41; 2 kHz: OR = 1.23; 95% CI, 1.04-1.45). Again, similar but smaller and less precise associations were noted for children's hearing loss (Table 2).

In adults, each 1-SD (14.0- μ m) narrower retinal arteriolar caliber was associated with a higher odds of hearing loss at lower individual frequencies (1 kHz: OR = 1.19; 95% CI, 1.02-1.39; 2 kHz: OR = 1.18; 95% CI, 1.00-1.39) and overall hFI

Table 1. Participant Characteristics

Characteristic	Children (n = 1281)	Adults (n = 1255)
Mean (SD) age, y	11.4 (0.5)	43.8 (5.0)
Male sex, No. (%)	630 (49.1)	168 (13.4)
Mean (SD) birth weight, kg	3.4 (0.6)	NA
Gestational age, wk	39.2 (2.0)	NA
<38, No. (%)	147 (11.5)	NA
38 to <42, No. (%)	1065 (83.1)	NA
≥42, No. (%)	60 (4.7)	NA
Family socioeconomic position	0.26 (0.97)	0.26 (0.97)
Smoking, No. (%)		
Current	NA	104 (8.3)
Passive	183 (14.3)	NA
Preexisting condition, No. (%)		
Diabetes	3 (0.2)	29 (2.3)
Hypertension (while receiving medication)	NA	65 (5.2)
Mean (SD) hearing threshold, dB HL ^a		
1 kHz	10.4 (7.0)	13.7 (7.4)
2 kHz	7.7 (7.1)	12.8 (8.1)
4 kHz	5.6 (8.0)	12.7 (10.0)
8 kHz	5.0 (9.1)	14.4 (14.0)
High Fletcher index (mean of 1, 2, and 4 kHz)	7.9 (5.8)	13.0 (6.8)
Hearing loss, No. (%)		
>15 dB HL ^b	109 (8.5)	328 (26.1)
>25 dB HL ^c	10 (0.8)	56 (4.5)
Mean (SD) retinal caliber, μm		
Arteriolar	159.1 (11.9)	151.1 (14.0)
Venular	230.7 (16.6)	218.9 (18.6)

Abbreviations: HL, hearing level; NA, not applicable.

^a In the better-hearing ear.^b Defined as high Fletcher index >15 dB HL in the better-hearing ear.^c Defined as high Fletcher index >25 dB HL in the better-hearing ear.

(OR = 1.16; 95% CI, 1.00-1.37). However, retinal arteriolar caliber was not associated with adult thresholds or with child hearing loss or thresholds.

Sensitivity Analyses

Similar patterns of associations were found for participants with type A tympanogram results only (eTable 1 in the [Supplement](#)) and for adults with hearing loss greater than 25 dB HL (eTable 2 in the [Supplement](#)). These similar patterns were also found when further adjusting for smoking and preexisting conditions (eTable 3 in the [Supplement](#)) and for parent global region of birth and indigenous status (data available on request).

Discussion

Principal Findings

In contemporaneous population-based samples, we found that wider retinal venules and narrower retinal arterioles—both considered adverse microvascular changes—were associated with poorer hearing by midlife for lower frequencies and hFI (composite threshold most relevant to speech frequencies)

but not for higher frequencies. The association of retinal venular caliber with hearing acuity may start to emerge by mid-childhood, with 1-SD wider venular caliber increasing the odds of hearing loss by 20% for adults.

Interpretation in Light of Other Studies

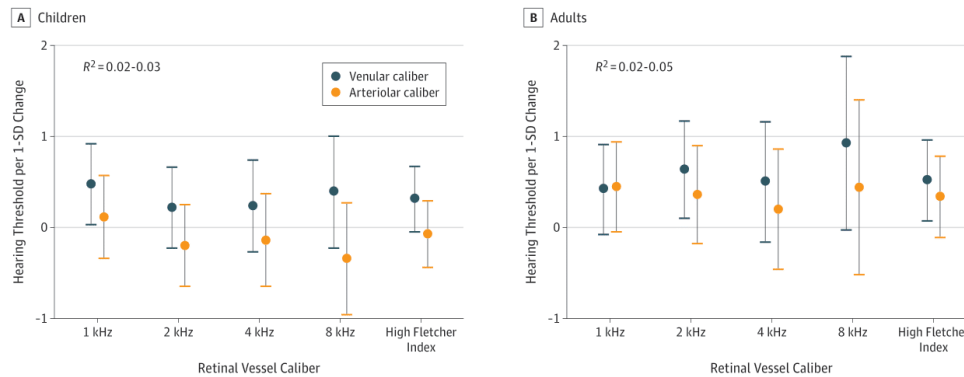
Our early and midlife findings are congruent with the larger associations among older adults in the Blue Mountains Hearing Study.²¹ Clinically evident retinopathy was associated with more than double the rate of low-frequency hearing loss greater than 40 dB HL (OR = 2.10; 95% CI, 1.09-4.06) among adults 54 years and older,²¹ contrasting with our smaller effect sizes (eg, per 1-SD wider retinal venular caliber: OR = 1.20; 95% CI, 1.03-1.40). This is not surprising because retinopathy is a sign of advanced microvascular damage. There is also indirect supporting evidence from studies examining associations of cardiovascular diseases and risk factors with hearing loss.^{17,37,38} For example, in the Framingham cohort,¹⁷ cardiovascular diseases (eg, stroke, coronary artery disease, intermittent claudication) were associated with low-frequency hearing loss (ORs ranged from 1.75 to 4.39) more than than high-frequency hearing loss (ORs ranged from 1.49 to 1.97). Similarly, the US National Health and Nutrition Examination Survey found that obesity was associated only with unilateral low-frequency hearing loss (OR = 1.9; 95% CI, 1.1-3.1) among 12- to 19-year-olds.³⁷ These associations in lower-frequency hearing loss are plausible, since lower-frequency sounds are transduced at the apex of the cochlea, where the blood supply is provided by distal capillaries.¹⁷ In contrast, high-frequency hearing is believed to be most influenced by noise exposure, which is known to initially damage the outer hair cells associated with the perception of high-frequency sounds (3-kHz to 6-kHz range)^{39,40} via the accumulation of reactive oxygen species and the active stimulation of intracellular stress pathways.⁴¹

Implications

Our findings support the theory of a life course microvascular contribution to the pathogenesis of hearing loss, especially in lower-frequency ranges. It will be valuable to conduct patient follow-up over time to establish the causal relationships of changes in microvasculature with age at hearing loss onset, rate of decline, and hearing loss trajectories.

Second, retinal venular but not arteriolar caliber was associated with hearing acuity in mid-childhood, suggesting that venular circulation may be more central to early-onset hearing loss than arteriolar circulation. To our knowledge, studies to date have suggested that retinal arteriolar and venular caliber reflect different vascular pathophysiologic processes.⁴² Smaller arteriolar caliber is closely associated with elevated blood pressure. Therefore, the lack of association between retinal arteriolar caliber and child hearing acuity may reflect the low prevalence of hypertension in children. Studies of other advanced arteriolar damage signs (eg, focal arteriolar narrowing, arteriovenous nicking) could help researchers better understand the role of arterioles in child hearing loss. In contrast, larger venular caliber appears to arise from inflammation and endothelial dysfunction.⁴³ The associations we found between venular caliber and hearing acuity may indicate that the earliest stages of hearing decline have

Figure 3. Associations of Retinal Vessel Caliber With Hearing Thresholds



A, Associations of retinal vessel caliber with hearing thresholds per 1-SD change in children, adjusted for age, sex, gestational age, birth weight, and family socioeconomic position. B, Associations of retinal vessel caliber with hearing thresholds per 1-SD change in adults, adjusted for age, sex, and family socioeconomic position. Associations were calculated using linear regression. The regression coefficient (β) represents the change in hearing threshold (dB hearing level [HL]) per 1-SD wider retinal venular caliber (or per 1-SD narrower retinal arteriolar caliber). All individual frequencies and high Fletcher index thresholds are for the better-hearing ear.

Table 2. Associations of Retinal Vessel Caliber per 1-SD Change With Hearing Acuity

Hearing Acuity	Retinal Vessel Caliber, Measure (95% CI) ^a	
	Venular per 1-SD Wider	Arteriolar per 1-SD Narrower
Linear regression ^b		
Child threshold ^c		
1 kHz	0.47 (0.03 to 0.92)	0.12 (−0.34 to 0.57)
2 kHz	0.21 (−0.23 to 0.66)	−0.20 (−0.65 to 0.25)
4 kHz	0.23 (−0.27 to 0.74)	−0.14 (−0.65 to 0.37)
8 kHz	0.39 (−0.23 to 1.00)	−0.34 (−0.96 to 0.27)
High Fletcher index	0.31 (−0.05 to 0.67)	−0.07 (−0.44 to 0.29)
Adult threshold ^d		
1 kHz	0.42 (−0.08 to 0.91)	0.45 (−0.05 to 0.94)
2 kHz	0.63 (0.10 to 1.17)	0.36 (−0.18 to 0.90)
4 kHz	0.50 (−0.16 to 1.16)	0.20 (−0.46 to 0.86)
8 kHz	0.92 (−0.03 to 1.88)	0.44 (−0.52 to 1.40)
High Fletcher index	0.52 (0.07 to 0.96)	0.34 (−0.11 to 0.78)
Logistic regression ^e		
Child hearing loss >15 dB HL ^c		
1 kHz	1.06 (0.89 to 1.27)	0.97 (0.81 to 1.16)
2 kHz	1.11 (0.87 to 1.42)	0.89 (0.69 to 1.15)
4 kHz	1.21 (0.93 to 1.57)	0.89 (0.68 to 1.19)
8 kHz	1.14 (0.90 to 1.46)	0.93 (0.71 to 1.19)
High Fletcher index	1.12 (0.89 to 1.41)	0.96 (0.76 to 1.22)
Adult hearing loss >15 dB HL ^d		
1 kHz	1.20 (1.03 to 1.41)	1.19 (1.02 to 1.39)
2 kHz	1.23 (1.04 to 1.45)	1.18 (1.00 to 1.39)
4 kHz	1.11 (0.95 to 1.30)	1.12 (0.96 to 1.32)
8 kHz	1.08 (0.92 to 1.26)	0.99 (0.96 to 1.32)
High Fletcher index	1.20 (1.03 to 1.40)	1.16 (1.00 to 1.37)

Abbreviations: HL, hearing level; OR, odds ratio.

^a The regression coefficient (β) represents the change in hearing threshold (dB HL) per 1-SD change in retinal vessel caliber. The OR is calculated for bilateral hearing loss greater than 15 dB HL per 1-SD change in retinal vessel caliber. All individual frequencies and high Fletcher index thresholds were for the better-hearing ear.

^b Linear regression is presented as β (95% CI) per 1-SD wider venular caliber or narrower arteriolar caliber.

^c Adjusted for age, sex, birth weight, gestational age, and family socioeconomic position.

^d Adjusted for age, sex, and family socioeconomic position.

^e Logistic regression is presented as OR (95% CI) per 1-SD wider venular caliber or narrower arteriolar caliber.

inflammatory origins.⁴⁴ In animal studies, inflammation stimulates the recruitment of circulating immunocytes to the cochlea.⁴⁵ These cells enter the cochlea through the postcapillary venules in the spiral ligament, whose fibrocytes function to recirculate

potassium and maintain the endocochlear potential. Future population-based studies should investigate whether and how much retinal venular caliber mediates the association of inflammation with hearing loss.

Third, there is increasing interest in targeting microcirculation in the prevention and treatment of cardiovascular diseases. For example, angiotensin-converting enzyme inhibitors and calcium channel blockers may have direct beneficial associations with microvascular structure and preventing cardiovascular morbidity.⁴⁶ In light of the findings from our study, we advocate that population-based trials of these agents in cardiovascular diseases add hearing loss as an outcome measure. Fast, high-quality hearing apps now make this much more feasible at the population level.

Strengths and Limitations

To our knowledge, we are the first to investigate with a high degree of measurement comparability the same associations in a large, population-based sample of children and adults. Assessments of the 2 generations were conducted on the same day, by the same staff, using the same equipment and protocols, and in the same order for all participants. These methodologic considerations avoid many of the potential sources of bias and unknown confounding seen in cross-generational studies. Children aged 11 to 12 years had good compliance with testing protocols^{47,48} and accurate ascertainment of even slight hearing loss. Retinal vessel caliber was quantified using a previously validated computer-based technique with comparable intergrader and intragrader reliability.⁴⁹ In addition, our study goes further than previous studies by investigating the patterns (frequency-specific effects) of the associations of subtle microvascular differences with hearing loss and when in the life course these associations may emerge.

There were also limitations. First, as in other population-based studies,^{37,50,51} we lacked bone conduction audiometry data owing to the strict time constraints of a whole-participant assessment, with hearing just 1 of multiple health domains measured within CheckPoint. However, our sensitivity analyses indicated that middle ear function likely had little influence on our results. Second, as with all longitudinal cohort studies, there has been significant attrition over time. Our findings may not generalize to more disadvantaged families, although adjusting for family SEP may have partly mitigated this problem. Third, retinal and audiometric data were collected at 1 time point, so we are unable to draw any inferences regarding causality. Fourth, the effect sizes of these associations were small (accounting for 3% to 9% of variance in hearing outcomes). Residual confounding from race/ethnicity, occupational noise exposure,⁵² and genetic risk factors could have contributed to the associations seen.²⁰ However, small associations may still be important at the population level.

Conclusions

Adverse retinal microvascular characteristics are associated with hearing loss by midlife, with retinal venular associations possibly emerging by age 11 to 12 years. Microvascular health may contribute to the pathogenesis of hearing loss across the life course, warranting replication and mechanistic studies to inform causal inference and prevention efforts.

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Author Contributions: Drs Wake and Wang had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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