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Complete List of Authors:	De Silva, Manikkuwadura; The University of Melbourne, Department of Optometry and Vision Sciences Zhang, Alexis; The University of Melbourne, Department of Optometry and Vision Sciences Karahalios, Emily; The University of Melbourne, Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health Chinnery, Holly; The University of Melbourne, Department of Optometry and Vision Sciences Downie, Laura; The University of Melbourne, Department of Optometry and Vision Sciences
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Laser-scanning *in vivo* confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters: protocol for a systematic review

Manikkuwadura Eranda Harshan De Silva,[1] Alexis Ceecee Zhang,[1] Amalia Karahalios,[2,3] Holly Rose Chinnery,[1] Laura Elizabeth Downie[1]

[1]The Department of Optometry and Vision Sciences, and [2]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Parkville, Victoria, Australia 3010; [3]School of Public Health and Preventive Medicine, Monash University, Melbourne, Australia, 3004.

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Corresponding author:

Dr Laura Downie

Department of Optometry and Vision Sciences

Level 4, Alice Hoy Building (Building 162)

The University of Melbourne, Parkville Victoria Australia 3010

Ph: +61 3 9035 3043, Fax: +61 3 9035 9905

Email: ldownie@unimelb.edu.au

ABSTRACT

Introduction: Laser scanning *in vivo* confocal microscopy (IVCM) enables non-invasive, high-resolution imaging of the cornea. In recent years, there has been a vast increase in researchers using laser-scanning IVCM to image and quantify corneal nerve parameters. However, a range of methodological approaches has been adopted. The primary aim of this systematic review is to critically appraise the reported method(s) of primary research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve plexus (SBNP) parameters in humans, and to examine corneal nerve parameters in healthy individuals.

Methods and Analysis: A systematic review of primary studies that have used laser-scanning IVCM to quantify SBNP parameters in humans will be conducted. Comprehensive electronic searches will be performed in OViD MedLine, Embase and the Cochrane Library. Two reviewers will independently assess titles and abstracts, and exclude studies not meeting the inclusion criteria. For studies judged eligible or potentially eligible, full-texts will be independently assessed by two reviewers to determine eligibility. A third reviewer will resolve any discrepancies in judgment. Risk of bias will be assessed using a custom tool, covering five methodological domains: participant selection, method of image capture, method of image analysis, data reporting and other sources of bias. A systematic narrative synthesis of findings will be provided. A multi-level random-effects meta-analysis will be performed for corneal nerve parameters derived from healthy participants. This review will be reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement.

Ethics and Dissemination: As this review considers published data, ethical approval is not required. We foresee that this synthesis will serve as a reference for future

studies, and can be used to inform best-practice standards for using IVCM in clinical research. A manuscript reporting the results of the review will be published and may also be presented at scientific conferences.

STRENGTHS AND LIMITATIONS OF THIS PROTOCOL

- This will be the first systematic review to consider the level of methodological rigour applied when using laser-scanning *in vivo* confocal microscopy (IVCM) for clinical research
- This systematic review will consider all primary research studies, irrespective of the study design, that have used laser-scanning IVCM to quantify corneal nerve parameters in human participants
- This systematic review protocol is reported in accordance with the Preferred Reporting Items for Systematic review and Meta-Analyses Protocols (PRISMA-P)
- We have developed a purpose-specific risk of bias tool for assessing IVCM methodological quality, which we consider will be a valuable guide for researchers using this technique, to consider potential sources of bias when developing IVCM protocols
- The review will not include unpublished studies or those published in a language other than English

INTRODUCTION

In vivo confocal microscopy (IVCM) is a non-invasive imaging method for visualising the structure of the living human cornea. IVCM provides high-resolution, morphological detail of the corneal architecture and can be applied to assess corneal parameters, in particular a range of metrics relating to corneal nerve integrity (e.g., density and branching characteristics). Several types of IVCM instruments are commercially available, including tandem-, slit- and laser-scanning devices.[1] Laser-scanning IVCM, which uses a red-wavelength diode laser source that poses no ocular safety hazard,[2] is currently considered the gold-standard device for clinical research. This technology provides a greater depth of focus, enhanced contrast and improved resolution compared with the alternative devices.[1]

While early studies using laser-scanning IVCM to examine corneal health were mostly qualitative in nature (e.g., for diagnosing corneal infection), the technique is now used to determine a range of quantitative clinical measures (e.g., corneal nerve density). Accurate quantification of corneal nerve parameters is important clinically for monitoring the potential effect of therapeutic interventions on corneal health, detecting corneal neuropathy, and acting as a surrogate biomarker for early stage diabetic peripheral neuropathy.[3, 4] Although a general method for examining the cornea and analysing corneal nerve parameters using laser-scanning IVCM has been described,[5] there is currently no gold-standard protocol for using laser-scanning IVCM for corneal nerve analysis available in the literature. As a result, a range of different approaches has been adopted.[3, 4, 6, 7]

Various factors, in particular the methods used for image capture and analysis, may introduce bias and thereby affect the accuracy of quantitative measures, when using IVCM to investigate corneal nerve parameters.[8] For example, as corneal nerve density varies with eccentricity (i.e., greater in the central versus peripheral cornea),[9] consideration should be given to the region of cornea imaged. In addition, factors such as the microscope field of view, depth of corneal imaging, image quality and post-capture image enhancements, may influence the visibility and/or clarity of nerves within the image field, thereby potentially impacting upon quantitative measurements.[8] The number of images analysed, per individual, also affects the confidence of quantitative estimates; it has been shown that at least eight images, with less than 20-percent image overlap between each image, should be analysed to obtain reliable estimates of corneal nerve density.[10] To avoid potential performance biases in studies involving different participant groups and/or clinical intervention studies, the confocal microscope operator and outcome assessor, should be masked to the participant's group allocation. In addition, the method for quantifying the sub-basal nerve parameters should be fully described by researchers, with preference given to the use of a validated, fully automated processing method (e.g., ACCMetrics[11]), to circumvent the potential bias induced by subjective judgment.

There has not been any previous research undertaken to consider the level of methodological rigour applied when using laser-scanning IVCM for clinical research. As researchers who are experienced with performing the technique, we have developed a purpose-specific risk of bias tool covering five key methodological domains that we consider important for minimising bias when using laser-scanning IVCM. The five domains are: participant selection, method of image capture, method

of image analysis, data reporting and other sources of bias (e.g., industry funding). We foresee the use of this purpose-specific risk of bias tool as a valuable guide for researchers, to consider potential sources of bias when developing their IVCM protocols. In this respect, the present paper has the capacity to contribute to significantly improving the quality of future research in the field.

The major aim of this systematic review is to critically appraise (i.e., assess the risk of bias in) the reported method(s) of primary research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve parameters in human participants. We will also determine key differences in methodology between studies and identify the specific methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the literature, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. We predict that there will be considerable variation in the image-capturing methodologies used by different investigators and between the studies, which may lead to potential biases and affect the reliability of reported data. For example, studies may have used an insufficient number of corneal images as a representative measure to quantify nerve density, potentially leading to sampling bias. Finally, a meta-analysis will be conducted on studies assessing corneal nerve fibre parameters in healthy individuals. As a result, this will help establish a more precise estimate of corneal nerve parameters for future research to use as a reference for identifying corneal nerve pathology.

Objectives

The primary objective of this systematic review is to critically appraise (i.e., assess the risk of bias in) the reported method(s) of primary research studies that have used laser-scanning *in vivo* confocal microscopy (IVCM) to quantify corneal sub-basal nerve parameters in human participants.

The secondary objectives are:

(i) to identify the methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the included studies, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. As shown in Table 1, the five main methodological domains that will be assessed are: participant selection, method of image capture, method of image analysis, data reporting and other sources of bias;

(ii) to determine normative values for corneal sub-basal nerve plexus parameters by pooling the estimates from available studies.

METHODS AND ANALYSIS

The proposed systematic review and meta-analysis will be undertaken using the approach recommended by the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) statement.[12]

Eligibility criteria

All studies published in English will be included, from the date of database inception until 17 May 2017. In cases where multiple publications of the same data exist, the study reporting on the largest number of human participants will be included.

As well, studies will be selected according to the following criteria:

i. Study designs

We will include all primary, empirical research studies that have used a laser-scanning confocal microscope to perform corneal confocal microscopy on at least one human participant, where corneal sub-basal nerve plexus parameters were quantified. We will include studies from across the spectrum of clinical research questions defined by the National Health and Medical Research Council (2009)[13] (e.g., intervention, diagnostic-test accuracy, etiology, prognosis and screening intervention) and study designs (e.g., randomised controlled trial (RCT), pseudo-RCT, non-RCT, cohort, case-control, interrupted time series, case series, case study), to enable the comparison of methodological quality across study types.

Studies reporting only on aspects of corneal architecture other than sub-basal nerve parameters (e.g., epithelial thickness, endothelial cell count/morphology, corneal haze, etc.) will be excluded. We will exclude review papers (including systematic reviews), conference abstracts and studies reporting methods for analysing laser-scanning IVCM images, where human participants were not recruited. We will also exclude studies that have used alternative types of confocal microscopes for image

capture (e.g., tandem scanning and slit scanning), as the type of confocal microscope affects the quantitation of corneal sub-basal nerve parameters.[1, 14]

ii. Participants

We will include all studies that report corneal sub-basal nerve plexus findings for at least one human participant. There will be no restriction on participant health status for the systematic review (although restrictions will apply for the meta-analysis, which will only include data from healthy adults), thus included studies may involve healthy individuals, as well as those with ocular and/or systemic conditions.

Information sources

A comprehensive search, to identify all relevant studies, will be undertaken in the following electronic databases: Ovid MEDLINE(R) (Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) 1946 to May 2017), Ovid EMBASE (Embase Classic+Embase, 1947 to 2017) and the Cochrane Library.

To ensure literature saturation, we will scan the reference lists of included studies, or relevant reviews, identified by our search. We will also search the authors' personal bibliographic reference files to ensure that all relevant studies are captured. We will also circulate a list of the included articles to our review team.

Search strategies

The search strategies, detailed below, were formulated with the assistance of an experienced systematic review librarian.

i. MedLine(OViD) and Embase

1	exp cornea/
2	cornea*.tw.
3	1 or 2
4	exp ophthalmic nerve/
5	nerve*.tw.
6	subbasal.tw.
7	sub-basal.tw.
8	mm?mm.tw.
9	neuropath*.tw.
10	plex*.tw.
11	4 or 5 or 6 or 7 or 8 or 9 or 10
12	confocal.tw.
13	microscop*.tw.
14	"in?vivo".tw.
15	12 or 13 or 14
16	3 and 11 and 15

ii. The Cochrane library

1	cornea
2	nerve*
3	innervat*
4	subbasal
5	sub-basal
6	mm?mm

7	neuropath*
8	plex*
9	confocal
10	microscop*
11	"in?vivo"
12	2 or 3 or 4 or 5 or 6 or 7 or 8
13	9 or 10 or 11
14	1 and 12 and 13

Study Records

i. Data management

The systematic search will be carried out by the review team, using the previously-defined search strategies, and guided by Items 9 and 10 of the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) statement.[15] After performing the search strategies separately in each electronic database, the researchers will import the results from each search into an EndNote library. As the same article may be located in more than one database, duplicate entries will be identified and removed.

ii. Study selection

We will use Covidence systematic review software,[16] an online program that facilitates collaboration between reviewers for systematic reviews, for the study screening process. Two reviewers (MEHDS and ACZ) will independently assess the

titles and abstracts of all unique studies, identified from the electronic search strategies, and exclude those that do not meet the inclusion criteria. For studies judged to be eligible or potentially eligible for inclusion, the full text articles will be sourced and independently assessed by the same reviewers (MEHDS and ACZ), to clarify their eligibility for inclusion. Any discrepancies in classification that arise during this process will be resolved by consensus between the two reviewers and a third reviewer (LED). For studies that progress to the full-text screening stage, we will record the reason that studies were excluded.

If there are cases where it is unclear whether the inclusion criterion are met, we will attempt to contact the study corresponding author for clarification; if no response is received within four weeks of the request, or the requested information is not provided, the information within the full-text article will be used to decide upon the eligibility of the study.

A diagram will be created to report the flow of studies through the systematic review.

iii. Data collection

Relevant data, from eligible studies, will be independently extracted by two reviewers in Covidence, using a standardised data extraction form. Extracted data will be summarised in tables. If any data extraction discrepancies arise, these will be resolved by discussion and consensus amongst the review team.

Data items

Extracted data from each included study will include:

(i) article details: year of publication, journal of publication;

(ii) study details: type of research question (i.e., intervention, diagnostic-test accuracy, aetiology, prognosis, screening intervention), setting, location, study design (e.g., RCT, pseudo-RCT, non-RCT, cohort, case-control, interrupted time series, case series, case study), number of participants, health status of the participant population(s) (e.g., healthy, diabetes, etc.), participant characteristics (age, gender), population eligibility criteria (inclusion and exclusion criteria);

(iii) methodological details: unit of analysis (one eye (right or left), both eyes or average of both eyes, as applicable), corneal sub-basal nerve parameters assessed (see 'Outcomes' section for further details), IVCM image capture field of view (i.e., 300µm or 400µm), IVCM mode of image capture (e.g., volume, sequence or section scan), whether a representative IVCM sub-basal nerve plexus image is provided in the paper (dichotomous);

(iv) other details: source of funding statement (dichotomous: present or absent), actual source of funding (e.g., industry or Government funding), conflict of interest statement (dichotomous: present or absent), conflict of interest type (e.g., employee of company conducting study);

(v) quantitative measures: data (i.e., mean (SD) or median (IQR)) for the following four key central, corneal sub-basal nerve plexus parameters: CNFL, CNFD, CNBD and CTBD, as defined in the 'Outcomes' section. Where data are provided for both eyes, we will also extract the correlation coefficient. If longitudinal data are reported, we will use baseline data in

our analyses. As all of the sub-basal nerve plexus parameters are continuous outcomes, we will extract data on the means and standard deviation (SD) for each parameter, or similar measures of central tendency and variability.

Outcomes

The primary outcome will be the methodological quality of included research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve parameters in human participants.

The secondary outcomes are as follows:

(i) identification of the methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the included studies, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies;

(ii) meta-analysis of mean normative values (i.e., from healthy individuals) for corneal sub-basal nerve plexus parameters, quantified from the central cornea (as defined by the study authors), and using these definitions for the analysis:

- Corneal Nerve Fibre Length (CNFL), defined as the total length of all nerve fibres in the image capture frame (mm/mm^2).[17, 18] If an alternative definition is used, such as limiting the quantification of fibres to those of a certain minimum length, these data will be excluded;

- Corneal Nerve Fibre Density (CNFD), defined as the total number of the main fibres divided by the area of the image frame (fibres/mm^2);[18]
- Corneal Nerve Branch Density (CNBD), defined as the total number of main nerve branches, being branches that stem from a nerve fibre, divided by the area of the image frame (branches on main fibre/mm^2);[18]
- Corneal nerve Total Branch Density (CTBD), defined as the total number of branches within the area of the image frame (total branches/mm^2).

Assessment of risk of bias in included studies

To facilitate the assessment of methodological quality in each of the included studies, as per the objective of this review, we developed a purpose-specific, 19-item risk of bias tool (Table 1) to assess internal validity, encompassing five main domains:

- participant selection (including selection bias)
- method of image capture (including performance bias, sampling bias)
- method of image analysis (including performance bias)
- data reporting (selective reporting of outcomes, attrition bias)
- other sources of bias (funding source, conflicts of interest)

This risk of bias tool was developed by the review team (MEHDS, ACZ, HRC, LED), who possess expertise in using IVCM for corneal nerve analyses, for this review and was framed using the Cochrane Assessing Risk of Bias in included studies (Chapter 8 in the Cochrane Handbook of Systematic Reviews of Interventions).[19]

Table 1 - Risk of bias table for assessing the methodological quality of studies using laser-scanning *in vivo* confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters

	Risk of Bias		
Item	**Low**	**Unclear**	**High**
Participant selection			
Participant inclusion and exclusion criteria (selection bias)	Eligibility criteria are stated, with clear specifications to define participant status (e.g., HbA1c level for participants with diabetes).	Eligibility criteria are stated without clear specifications to define participant status (e.g., diabetes stated, without further details verifying the diagnosis).	No eligibility criteria are stated. (Participants may have been included with conditions that could confound the measurement of nerve parameters)
Method of image capture			
Masking of confocal operator (performance bias)	Clearly states that the confocal operator was masked to participant/group allocation OR that all personnel were masked throughout the study OR not applicable (if a single study population was examined).	General statement regarding masking (e.g., "double-masked" study), without further information about whether this applies to the corneal image capture (if multiple outcomes were measured) AND where this factor is considered relevant (e.g., RCT).	No information provided in relation to masking of personnel where masking is considered relevant (e.g., intervention study); we assume that in the absence of reporting, the operator was not masked.
Specification of participant fixation target	States that a consistent fixation target was used for all participants.	States that a fixation target was used, but no other relevant information is provided.	Fixation target not mentioned (bias potentially introduced with eye movements).
Location of cornea imaged	Quantitative description of location of cornea imaged (e.g., within a 2mm radius of the corneal apex).	Qualitative description of the location of cornea imaged (e.g., central or peripheral).	Location of corneal imaging is not specified.
Specification of corneal depth	Quantitative description of the corneal depth imaged is included (e.g., imaged at a depth range of 10-15µm below the basal epithelium).	Qualitative description of the corneal depth imaged is included (e.g., imaged just below basal epithelial cell layer)	Depth of cornea imaged is not stated.
Illumination setting on confocal microscope	States that images were acquired using fixed illumination intensity	Confocal illumination settings were not reported.	States that confocal images were acquired using "automated

	for all participants.[20]		brightness" settings that were optimised for each participant.
Selection of eye (left or right or both)	Clearly specifies which eye was assessed, with a sound method of selection (e.g., random, all right eyes, average of right and left).	Specifies which eye was assessed without sound justification for the method of selection (e.g., potential inconsistent use of data from one or both eyes).	No mention of which eye was assessed OR data from both eyes was used without appropriate statistical adjustment.
Method of image analysis			
Masking of outcome assessor (performance bias)	Clearly states that the assessor of the corneal images was masked to the participant/group allocation, OR not applicable (if a single study population was examined).	General statement regarding masking (e.g., "double-masked"), without specifying whether this applies to the corneal image analysis (if multiple outcomes were measured) AND where this factor is considered relevant (e.g., RCT).	No information provided in relation to masking of the outcome assessor where masking is considered relevant (e.g., intervention study); we assume that in the absence of reporting, the assessor was not masked.
Image selection – quality (sampling bias)	Clearly states that image quality was assessed AND that images where the imaging depth was inconsistent or the image was blurred were removed from the analysis sample AND representative images are provided within the manuscript that confirm the images meet these criteria.	States that image quality was assessed, but does not specify the criteria for excluding images from the analysis OR there are no representative images provided within the manuscript.	No information is provided in relation to the assessment of image quality OR there are representative images within the manuscript that appear blurred or not within a fixed plane of focus.
Image selection - number and sampling (sampling bias)	Number of images analysed is clearly stated, and at least eight images were analysed per region with <20% overlap between images.[10]	Number of images analysed is clearly stated but either does not state level of overlap of images was <20% or 5-7 images were analysed.[10]	Number of images analysed is not stated or <5 images were analysed.
Image selection – method of randomisation and sequence generation (sampling bias)	Images described to be randomly selected for analysis and method for random selection is reported (e.g., computer generated list, random	Images described to be randomly selected for analysis but with no further details as to how the randomisation schedule was generated.	Image selection is not reported or image selection method is based upon subjective judgement (e.g., "most representative" or "best

	table, other method of generating random list).		image selected").
Image processing - order of analysis	Method involved an automated processing method (e.g., ACCMetrics,[11] or similar) or not applicable (if only single timepoint measured per participant, e.g., cross-sectional)	Order of image analysis was not reported, when a manual method was used for quantification and this is considered relevant to the outcomes.	Images were analysed consecutively (per participant) using a manual method (e.g., subjective quantification) and this is considered relevant to the outcomes.
Post-capture image enhancements	Clearly states that there were no post-capture image enhancements (i.e., brightness, sharpening, etc.) performed, or a uniform enhancement was applied across all images.	No statement regarding whether post-capture image enhancements were performed.	Post-capture image enhancements were performed to optimise image contrast/brightness (or some other parameter) individually for each image.
Definition of sub-basal nerve parameters	The nerve parameters being evaluated (e.g., CNFL, CNFD, CNBD) are clearly defined, either in the paper itself or by referencing a previously validated method.	The nerve parameters being evaluated are stated but not are sufficiently well defined to allow reproduction of the method.	The nerve parameters being evaluated are stated but without definition (e.g., "CNFL was measured", with no information given on how the parameter was defined).
Method for quantifying sub-basal nerve parameters	A validated, fully automated processing method (e.g., ACCMetrics,[11] or similar) was used.	A semi-automated processing method (e.g., CCMetrics)[11] was used.	A fully manual processing method used.
Repeatability of sub-basal nerve parameter quantification (intra- and inter-observer variability)	A validated, fully automated processing method (e.g., ACCMetrics, or similar) was used.	Repeatability testing was performed and reported for a semi-automated or fully manual method.	No repeatability testing was undertaken for a semi-automated or fully manual method.
Data reporting			
Thoroughness of reporting of nerve parameters – selective reporting of outcomes	Data relating to all quantified nerve parameters (as mentioned in the study methods) are reported	Data relating to all quantified nerve parameters (as mentioned in the study methods) are reported	Selective reporting of nerve parameters (i.e., mismatch between parameters described in results and those

	in the results with both point measures and measures of variability.	in the results with a qualitative descriptor only (with or without reporting of p-values).	mentioned in methods).
Completeness of nerve parameter data (population level) – attrition bias	Missing data for less than 20% of recruited participants, and if multiple study groups or a longitudinal study, then there is an equal degree of missing data in both groups at follow-up, with no obvious reason why absence of data is related to study group or time point respectively.	Completeness of data is not reported, or if multiple study groups or a longitudinal study, the degree of missing data is for >20% of participants but occurs to an equal degree in both study groups.	If multiple study groups, there is an unequal degree of missing data between study groups, and/or the absence of the data appears to be related to the study outcome.
Other			
Other sources of bias	No other apparent sources of bias.	Source of funding not reported.	Industry-funded study.

Two reviewers (MEHDS and ACZ) will judge the risk of each type of bias (19 items in total) in each included study as either: (i) low risk, (ii) unclear risk (due to either lack of information or uncertainty about the potential for bias), or (iii) high risk. Review authors will resolve any disagreements in bias assessment by consensus with a third reviewer (LED). Reviewers will not be masked to the journal of publication or the study author name when undertaking the risk of bias assessment.

Wherever possible, we will justify each risk of bias assessment with direct quotations from the study. If there are cases where further information is considered necessary to determine the risk of bias in a particular domain, we will attempt to contact the study corresponding author for this information. If no response is received within four

weeks of the request, or the requested information is not provided, the information within the full-text article will be used to inform the risk of bias assessment.

Data synthesis

For outcomes related to methodological quality, a systematic narrative synthesis will be provided, with relevant information summarised in text and tables.

If there are at least three relevant studies, we will undertake meta-analyses of the quantitative data for the specified corneal sub-basal nerve plexus parameters (i.e. mean (SD)). Data from male and female participants will be pooled, as studies have shown that gender has no significant effect on corneal sub-basal nerve plexus parameters.[21, 22]

We will convert non-parametric data to means (SD), using an established approach.[23] We will fit a multi-level random-effects model to pool the estimates. We will include estimates from male and female participants, as corneal sub-basal nerve plexus parameters do not vary by gender.[21, 22] The multi-level model will take into account the correlation between estimates from the same study that are presented separately for each sex and/or estimates presented separately for the left and right eyes. Next, we will fit a meta-regression model to assess how much of the between-study variation is explained by the following characteristics: (i) participant age (as this factor is potentially important relation to sub-basal nerve plexus parameters,[24] and (ii) study design (e.g., RCT, cohort (including pseudo-RCT and non-RCT) and other (including cross-sectional, case series/study)).

Statistical analyses will be carried out using the *metafor* package in R.[25, 26]

Meta-bias(es)

As there are no limitations on the potential study designs eligible for inclusion in this review, we expect that we will not be able to compare the outcomes reported in published reports with study protocols, unless the included study is a RCT, to assess for selective outcome reporting or selective analysis reporting. Furthermore, as our meta-analysis is being undertaken to determine values for a normative parameter (i.e., corneal sub-basal nerve plexus parameters), rather than the effect of an intervention, we do not expect meta-biases (such as publication bias, delayed publication, etc.) to be a factor for this analysis.

Sensitivity analyses

Provided there are a sufficient number of studies included in the review, sensitivity analyses will be performed for the CNFL outcome measure, to assess for the effect of excluding studies that: (i) were appraised as having a high risk of bias in the domains of image selection – number and sampling, or method for quantifying sub-basal nerve parameters, (ii) included contact lens wearers (i.e., contact lens wear was not listed as an eligibility exclusion criterion), and (iii) were lower order levels of evidence (e.g., case reports, case series, interrupted time series).

Confidence in cumulative evidence

If appropriate, we will present a 'Summary of Findings' table for the quantitative outcomes. The quality and strength of the body of evidence will be assessed using an

approach based upon the Grading of Recommendations Assessment, Development and Evaluation (GRADE).[27]

CONCLUSIONS

In recent years, an increasing number of research studies have adopted non-invasive, laser-scanning IVCM to quantify corneal sub-basal nerve plexus parameters. However, there has not been any research to formally consider the quality of the methods used in these investigations. This systematic review will provide insight into the quality of the methods reported in clinical studies using laser-scanning IVCM to quantify corneal nerve parameters. The review will also identify specific methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the literature, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. Furthermore, by researchers considering the elements of the purpose-specific risk of bias tool as a guide when developing their IVCM protocols, this review has the capacity to significantly improve the quality of future research in the field. By undertaking a meta-analysis, we will also determine mean normative values (i.e., from healthy individuals) for central corneal sub-basal nerve plexus parameters. These data will be of significant value for future studies, as reference normative values, building upon a previous pooled analysis of data derived from multiple laser-scanning IVCM testing centres.[28]

ETHICS AND DISSEMINATION

This systematic review and meta-analysis will analyse published data and therefore does not require ethics approval. A manuscript reporting the results of the systematic review will be published in a peer-reviewed journal and may also be presented at scientific conferences.

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CONTRIBUTIONS OF AUTHORS

MEHDS and LED drafted the initial protocol, with input, discussion and editing from ACZ and HRC. AK contributed to the 'Data synthesis', 'Meta-bias(es)', and 'Sensitivity analyses' sections, and the protocol components for undertaking the meta-analysis. All authors provided final approval of the paper.

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COMPETING INTERESTS STATEMENT

All authors have no conflicts of interest.

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PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist: recommended items to address in a systematic review protocol*

Section and topic	Item No	Checklist item	Reported in manuscript (page number + section details)
ADMINISTRATIVE INFORMATION			
Title:			
Identification	1a	Identify the report as a protocol of a systematic review	1
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	Not applicable
Registration	2	If registered, provide the name of the registry (such as PROSPERO) and registration number	Methodological review (not eligible for registration on PROSPERO)
Authors:			
Contact	3a	Provide name, institutional affiliation, e-mail address of all protocol authors; provide physical mailing address of corresponding author	1
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	23
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	Not applicable
Support:			
Sources	5a	Indicate sources of financial or other support for the review	23
Sponsor	5b	Provide name for the review funder and/or sponsor	Not applicable
Role of sponsor or funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	Not applicable
INTRODUCTION			
Rationale	6	Describe the rationale for the review in the context of what is already known	4
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	7
METHODS			
Eligibility criteria	8	Specify the study characteristics (such as PICO, study design, setting, time frame) and report characteristics (such as years considered, language, publication status) to be used as criteria for eligibility for the review	8
Information sources	9	Describe all intended information sources (such as electronic databases, contact with study	9

		authors, trial registers or other grey literature sources) with planned dates of coverage	
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	9
Study records:			
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	11
Selection process	11b	State the process that will be used for selecting studies (such as two independent reviewers) through each phase of the review (that is, screening, eligibility and inclusion in meta-analysis)	11
Data collection process	11c	Describe planned method of extracting data from reports (such as piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	12
Data items	12	List and define all variables for which data will be sought (such as PICO items, funding sources), any pre-planned data assumptions and simplifications	13
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	14
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	15
Data synthesis	15a	Describe criteria under which study data will be quantitatively synthesised	20
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data and methods of combining data from studies, including any planned exploration of consistency (such as I^2, Kendall's τ)	20
	15c	Describe any proposed additional analyses (such as sensitivity or subgroup analyses, meta-regression)	21
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (such as publication bias across studies, selective reporting within studies)	21
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (such as GRADE)	21

*** It is strongly recommended that this checklist be read in conjunction with the PRISMA-P Explanation and Elaboration (cite when available) for important clarification on the items. Amendments to a review protocol should be tracked and dated. The copyright for PRISMA-P (including checklist) is held by the PRISMA-P Group and is distributed under a Creative Commons Attribution Licence 4.0.**

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Laser-scanning in vivo confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters: protocol for a systematic review

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Laser-scanning *in vivo* confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters: protocol for a systematic review

Manikkuwadura Eranda Harshan De Silva,[1] Alexis Ceecee Zhang,[1] Amalia Karahalios,[2,3] Holly Rose Chinnery,[1] Laura Elizabeth Downie[1]

[1]The Department of Optometry and Vision Sciences, and [2]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Parkville, Victoria, Australia 3010; [3]School of Public Health and Preventive Medicine, Monash University, Melbourne, Australia, 3004.

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Corresponding author:

Dr Laura Downie

Department of Optometry and Vision Sciences

Level 4, Alice Hoy Building (Building 162)

The University of Melbourne, Parkville Victoria Australia 3010

Ph: +61 3 9035 3043, Fax: +61 3 9035 9905

Email: ldownie@unimelb.edu.au

ABSTRACT

Introduction: Laser scanning *in vivo* confocal microscopy (IVCM) enables non-invasive, high-resolution imaging of the cornea. In recent years, there has been a vast increase in researchers using laser-scanning IVCM to image and quantify corneal nerve parameters. However, a range of methodological approaches has been adopted. The primary aim of this systematic review is to critically appraise the reported method(s) of primary research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve plexus (SBNP) parameters in humans, and to examine corneal nerve parameters in healthy individuals.

Methods and Analysis: A systematic review of primary studies that have used laser-scanning IVCM to quantify SBNP parameters in humans will be conducted. Comprehensive electronic searches will be performed in OViD MedLine, Embase and the Cochrane Library. Two reviewers will independently assess titles and abstracts, and exclude studies not meeting the inclusion criteria. For studies judged eligible or potentially eligible, full-texts will be independently assessed by two reviewers to determine eligibility. A third reviewer will resolve any discrepancies in judgment. Risk of bias will be assessed using a custom tool, covering five methodological domains: participant selection, method of image capture, method of image analysis, data reporting and other sources of bias. A systematic narrative synthesis of findings will be provided. A multi-level random-effects meta-analysis will be performed for corneal nerve parameters derived from healthy participants. This review will be reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement.

Ethics and Dissemination: As this review considers published data, ethical approval is not required. We foresee that this synthesis will serve as a reference for future

studies, and can be used to inform best-practice standards for using IVCM in clinical research. A manuscript reporting the results of the review will be published and may also be presented at scientific conferences.

STRENGTHS AND LIMITATIONS OF THIS PROTOCOL

- This will be the first systematic review to consider the level of methodological rigour applied when using laser-scanning *in vivo* confocal microscopy (IVCM) for clinical research
- This systematic review will consider all primary research studies, irrespective of the study design, that have used laser-scanning IVCM to quantify corneal nerve parameters in human participants
- This systematic review protocol is reported in accordance with the Preferred Reporting Items for Systematic review and Meta-Analyses Protocols (PRISMA-P)
- We have developed a purpose-specific risk of bias tool for assessing IVCM methodological quality, which we consider will be a valuable guide for researchers using this technique, to consider potential sources of bias when developing IVCM protocols
- The review will not include unpublished studies or those published in a language other than English

INTRODUCTION

In vivo confocal microscopy (IVCM) is a non-invasive imaging method for visualising the structure of the living human cornea. IVCM provides high-resolution, morphological detail of the corneal architecture and can be applied to assess corneal parameters, in particular a range of metrics relating to corneal nerve integrity (e.g., density and branching characteristics). Several types of IVCM instruments are commercially available, including tandem-, slit- and laser-scanning devices.[1] Laser-scanning IVCM, which uses a red-wavelength diode laser source that poses no ocular safety hazard,[2] is currently considered the gold-standard device for clinical research. This technology provides a greater depth of focus, enhanced contrast and improved resolution compared with the alternative devices.[1]

While early studies using laser-scanning IVCM to examine corneal health were mostly qualitative in nature (e.g., for diagnosing corneal infection), the technique is now used to determine a range of quantitative clinical measures (e.g., corneal nerve density). Accurate quantification of corneal nerve parameters is important clinically for monitoring the potential effect of therapeutic interventions on corneal health, detecting corneal neuropathy, and acting as a surrogate biomarker for early stage diabetic peripheral neuropathy.[3, 4] Although a general method for examining the cornea and analysing corneal nerve parameters using laser-scanning IVCM has been described,[5] there is currently no gold-standard protocol for using laser-scanning IVCM for corneal nerve analysis available in the literature. As a result, a range of different approaches has been adopted.[3, 4, 6, 7]

Various factors, in particular the methods used for image capture and analysis, may introduce bias and thereby affect the accuracy of quantitative measures, when using IVCM to investigate corneal nerve parameters.[8] For example, as corneal nerve density varies with eccentricity (i.e., greater in the central versus peripheral cornea),[9] consideration should be given to the region of cornea imaged. In addition, factors such as the microscope field of view, depth of corneal imaging, image quality and post-capture image enhancements, may influence the visibility and/or clarity of nerves within the image field, thereby potentially impacting upon quantitative measurements.[8] The number of images analysed, per individual, also affects the confidence of quantitative estimates; it has been shown that at least eight images, with less than 20-percent image overlap between each image, should be analysed to obtain reliable estimates of corneal nerve density.[10] To avoid potential performance biases in studies involving different participant groups and/or clinical intervention studies, the confocal microscope operator and outcome assessor, should be masked to the participant's group allocation. In addition, the method for quantifying the sub-basal nerve parameters should be fully described by researchers, with preference given to the use of a validated, fully automated processing method (e.g., ACCMetrics[11]), to circumvent the potential bias induced by subjective judgment.

There has not been any previous research undertaken to consider the level of methodological rigour applied when using laser-scanning IVCM for clinical research. As researchers who are experienced with performing the technique, we have developed a purpose-specific risk of bias tool covering five key methodological domains that we consider important for minimising bias when using laser-scanning IVCM. The five domains are: participant selection, method of image capture, method

of image analysis, data reporting and other sources of bias (e.g., industry funding). We foresee the use of this purpose-specific risk of bias tool as a valuable guide for researchers, to consider potential sources of bias when developing their IVCM protocols. In this respect, the present paper has the capacity to contribute to significantly improving the quality of future research in the field.

The major aim of this systematic review is to critically appraise (i.e., assess the risk of bias in) the reported method(s) of primary research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve parameters in human participants. We will also determine key differences in methodology between studies and identify the specific methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the literature, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. We predict that there will be considerable variation in the image-capturing methodologies used by different investigators and between the studies, which may lead to potential biases and affect the reliability of reported data. For example, studies may have used an insufficient number of corneal images as a representative measure to quantify nerve density, potentially leading to sampling bias. Finally, a meta-analysis will be conducted on studies assessing corneal nerve fibre parameters in healthy individuals. As a result, this will help establish a more precise estimate of corneal nerve parameters for future research to use as a reference for identifying corneal nerve pathology.

Objectives

The primary objective of this systematic review is to critically appraise (i.e., assess the risk of bias in) the reported method(s) of primary research studies that have used laser-scanning *in vivo* confocal microscopy (IVCM) to quantify corneal sub-basal nerve parameters in human participants.

The secondary objectives are:

(i) to identify the methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the included studies, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. As shown in Table 1, the five main methodological domains that will be assessed are: participant selection, method of image capture, method of image analysis, data reporting and other sources of bias;

(ii) to determine normative values for corneal sub-basal nerve plexus parameters by pooling the estimates from available studies.

METHODS AND ANALYSIS

The proposed systematic review and meta-analysis will be undertaken using the approach recommended by the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) statement.[12]

Eligibility criteria

All studies published in English will be included, from the date of database inception until 17 May 2017. In cases where multiple publications of the same data exist, the study reporting on the largest number of human participants will be included.

As well, studies will be selected according to the following criteria:

i. Study designs

We will include all primary, empirical research studies that have used a laser-scanning confocal microscope to perform corneal confocal microscopy on at least one human participant, where corneal sub-basal nerve plexus parameters were quantified. We will include studies from across the spectrum of clinical research questions defined by the National Health and Medical Research Council (2009)[13] (e.g., intervention, diagnostic-test accuracy, aetiology, prognosis and screening intervention) and study designs (e.g., randomised controlled trial (RCT), pseudo-RCT, non-RCT, cohort, case-control, cross-sectional, interrupted time series, case series, case study), to enable the comparison of methodological quality across study types.

Studies reporting only on aspects of corneal architecture other than sub-basal nerve parameters (e.g., epithelial thickness, endothelial cell count/morphology, corneal haze, etc.) will be excluded. We will exclude review papers (including systematic reviews), conference abstracts and studies reporting methods for analysing laser-scanning IVCM images, where human participants were not recruited. We will also exclude studies that have used alternative types of confocal microscopes for image

capture (e.g., tandem scanning and slit scanning), as the type of confocal microscope affects the quantitation of corneal sub-basal nerve parameters.[1, 14]

ii. Participants

We will include all studies that report corneal sub-basal nerve plexus findings for at least one human participant. There will be no restriction on participant health status for the systematic review (although restrictions will apply for the meta-analysis, which will only include data from healthy adults), thus included studies may involve healthy individuals, as well as those with ocular and/or systemic conditions.

Information sources

A comprehensive search, to identify all relevant studies, will be undertaken in the following electronic databases: Ovid MEDLINE(R) (Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) 1946 to May 2017), Ovid EMBASE (Embase Classic+Embase, 1947 to May 2017) and the Cochrane Library.

To ensure literature saturation, we will scan the reference lists of included studies, or relevant reviews, identified by our search. We will also search the authors' personal bibliographic reference files to ensure that all relevant studies are captured. We will also circulate a list of the included articles to our review team.

Search strategies

The search strategies are provided as Supplementary Material.

Study Records

i. Data management

The systematic search will be carried out by the review team, using the previously-defined search strategies, and guided by Items 9 and 10 of the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) statement.[15] After performing the search strategies separately in each electronic database, the researchers will import the results from each search into an EndNote library. As the same article may be located in more than one database, duplicate entries will be identified and removed.

ii. Study selection

We will use Covidence systematic review software,[16] an online program that facilitates collaboration between reviewers for systematic reviews, for the study screening process. Two reviewers (MEHDS and ACZ) will independently assess the titles and abstracts of all unique studies, identified from the electronic search strategies, and exclude those that do not meet the inclusion criteria. For studies judged to be eligible or potentially eligible for inclusion, the full text articles will be sourced and independently assessed by the two reviewers, to clarify their eligibility for inclusion. Any discrepancies in classification that arise during this process will be resolved by consensus between the two reviewers and a third reviewer. For studies that progress to the full-text screening stage, we will record the reason that studies were excluded.

If there are cases where it is unclear whether the inclusion criterion are met, we will attempt to contact the study corresponding author for clarification; if no response is received within four weeks of the request, or the requested information is not provided, the information within the full-text article will be used to decide upon the eligibility of the study.

A diagram will be created to report the flow of studies through the systematic review.

iii. Data collection

Relevant data, from eligible studies, will be independently extracted by two reviewers in Covidence, using a standardised data extraction form. Extracted data will be summarised in tables. If any data extraction discrepancies arise, these will be resolved by discussion and consensus amongst the review team.

Data items

Extracted data from each included study will include:

(i) article details: year of publication, journal of publication;

(ii) study details: type of research question (i.e., intervention, diagnostic-test accuracy, aetiology, prognosis, screening intervention), setting, location, study design (e.g., RCT, pseudo-RCT, non-RCT, cohort, case-control, cross-sectional, interrupted time series, case series, case study), number of participants, health status of the participant population(s) (e.g., healthy, diabetes, etc.), participant characteristics (age, gender), population eligibility criteria (inclusion and exclusion criteria);

(iii) methodological details: unit of analysis (one eye (right or left), both eyes or average of both eyes, as applicable), corneal sub-basal nerve parameters assessed (see 'Outcomes' section for further details), IVCM image capture field of view (i.e., 300µm or 400µm), IVCM mode of image capture (e.g., volume, sequence or section scan), whether a representative IVCM sub-basal nerve plexus image is provided in the paper (dichotomous);

(iv) other details: source of funding statement (dichotomous: present or absent), actual source of funding (e.g., industry or Government funding), conflict of interest statement (dichotomous: present or absent), conflict of interest type (e.g., employee of company conducting study);

(v) quantitative measures: data (i.e., mean (SD) or median (IQR)) for the following four key central, corneal sub-basal nerve plexus parameters: CNFL, CNFD, CNBD and CTBD, as defined in the 'Outcomes' section. Where data are provided for both eyes, we will also extract the correlation coefficient. If longitudinal data are reported, we will use baseline data in our analyses. As all of the sub-basal nerve plexus parameters are continuous outcomes, we will extract data on the means and standard deviation (SD) for each parameter, or similar measures of central tendency and variability.

Outcomes

The primary outcome will be the methodological quality of included research studies that have used laser-scanning IVCM to quantify corneal sub-basal nerve parameters in human participants.

The secondary outcomes are as follows:

(i) identification of the methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the included studies, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies;

(ii) meta-analysis of mean normative values (i.e., from healthy individuals) for corneal sub-basal nerve plexus parameters, quantified from the central cornea (as defined by the study authors), and using these definitions for the analysis:

- Corneal Nerve Fibre Length (CNFL), defined as the total length of all nerve fibres in the image capture frame (mm/mm^2).[17, 18] If an alternative definition is used, such as limiting the quantification of fibres to those of a certain minimum length, these data will be excluded;
- Corneal Nerve Fibre Density (CNFD), defined as the total number of main fibres divided by the area of the image frame (fibres/mm^2);[18]
- Corneal Nerve Branch Density (CNBD), defined as the total number of main nerve branches, being branches that stem from a nerve fibre, divided by the area of the image frame (branches on main fibre/mm^2);[18]
- Corneal nerve Total Branch Density (CTBD), defined as the total number of branches within the area of the image frame (total branches/mm^2).

Assessment of risk of bias in included studies

To facilitate the assessment of methodological quality in each of the included studies, as per the objective of this review, we developed a purpose-specific, 18-item risk of bias tool (Table 1) to assess internal validity, encompassing five main domains:

- participant selection (including selection bias)
- method of image capture (including performance bias and sampling bias)
- method of image analysis (including performance bias)
- data reporting (selective reporting of outcomes and attrition bias)
- other sources of bias (funding source and conflicts of interest)

The risk of bias tool was developed by the review team (MEHDS, ACZ, HRC, LED), who possess expertise in using IVCM for corneal nerve analyses, for this review and was framed using the Cochrane Assessing Risk of Bias in included studies (Chapter 8 in the Cochrane Handbook of Systematic Reviews of Interventions).[19]

Table 1 - Risk of bias table for assessing the methodological quality of studies using laser-scanning *in vivo* confocal microscopy (IVCM) for evaluating human corneal sub-basal nerve plexus parameters

	Risk of Bias		
Item	**Low**	**Unclear**	**High**
Participant selection			
Participant inclusion and exclusion criteria (selection bias)	Eligibility criteria are stated, with clear specifications to define participant status (e.g., HbA1c level for participants with diabetes).	Eligibility criteria are stated without clear specifications to define participant status (e.g., diabetes stated, without further details verifying the diagnosis).	No eligibility criteria are stated. Participants may have been included with conditions that could confound the measurement of nerve parameters.
Method of image capture			
Masking of confocal operator (performance bias)	Clearly states that the confocal operator was masked to participant/group allocation OR that all personnel were masked throughout the study OR not applicable (if a single study population was examined).	A general statement regarding masking (e.g., "double-masked" study) is included, without further information about whether this applies to the corneal image capture (if multiple outcomes were measured) and where this factor is considered relevant (e.g., a RCT).	No information is provided in relation to the masking of personnel, where masking is considered relevant (e.g., intervention study); we assume that in the absence of reporting, the operator was not masked.
Specification of participant fixation target	States that a consistent fixation target was used for all participants.	States that a fixation target was used, but no other relevant information is provided.	Use of a fixation target is not mentioned (bias potentially introduced with eye movements).
Location of cornea imaged	Quantitative description of location of cornea imaged (e.g., within a 2mm radius of the corneal apex).	Qualitative description of the location of cornea imaged (e.g., central or peripheral).	Location of corneal imaging is not specified.
Specification of corneal depth	Quantitative description of the corneal depth imaged is included (e.g., imaged at a depth range of 10-15µm below the basal epithelium or a method is used to project nerves imaged different depths onto a single plane	Qualitative description of the corneal depth imaged is included (e.g., imaged just below basal epithelial cell layer)	Depth of cornea imaged is not stated.

	[20]).		
Illumination setting on confocal microscope	States that images were acquired using fixed illumination intensity for all participants.[21]	Confocal illumination settings were not reported.	States that confocal images were acquired using "automated brightness" settings that were optimised for each participant.
Selection of eye (left or right or both)	Clearly specifies which eye was assessed, with a sound method of selection (e.g., random, all right eyes, average of right and left eyes).	Specifies which eye was assessed without sound justification for the method of selection (e.g., potential inconsistent use of data from one or both eyes).	No mention of which eye was assessed, or data from both eyes was used without appropriate statistical adjustment.
Method of image analysis			
Masking of outcome assessor (performance bias)	Clearly states that the assessor of the corneal images was masked to the participant/group allocation, or this bias domain is not applicable (if a single study population was studied).	A general statement regarding masking is included (e.g., "double-masked"), without specifying whether this applies to the corneal image analysis (if multiple outcomes were measured) and where this factor is considered relevant (e.g., a RCT).	No information is provided in relation to masking of the outcome assessor, where masking is considered relevant (e.g., intervention study); we assume that in the absence of reporting, the assessor was not masked.
Image selection – quality (sampling bias)	Clearly states that image quality was assessed AND that images where the imaging depth was inconsistent or the image was blurred were removed from the analysis sample AND representative images are provided within the manuscript that confirm the images meet these criteria.	States that image quality was assessed, but does not specify the criteria for excluding images from the analysis OR there are no representative images provided within the manuscript.	No information is provided in relation to the assessment of image quality OR there are representative images within the manuscript that appear blurred or not within a fixed plane of focus.
Image selection - number and sampling (sampling bias)	Number of images analysed is clearly stated, and at least eight images were analysed per region with <20% overlap between images.[10]	Number of images analysed is clearly stated but either does not state level of overlap of images was <20% or 5-7 images were analysed.[10]	Number of images analysed is not stated or <5 images were analysed.
Image selection – method	Images described to be	Images described to be	Image selection is not

of randomisation and sequence generation (sampling bias)	randomly selected for analysis and method for random selection is reported (e.g., computer generated list, random table, other method of generating random list).	randomly selected for analysis but with no further details as to how the randomisation schedule was generated.	reported or image selection method is based upon subjective judgement (e.g., "most representative" or "best image selected").
Image processing - order of analysis	Method involved an automated processing method (e.g., ACCMetrics,(11) or similar) or not applicable (if only a single timepoint was measured per participant, e.g., cross-sectional study)	Order of image analysis was not reported, when a manual method was used for quantification and this is considered relevant to the outcome(s).	Images were analysed consecutively (per participant) using a manual method (e.g., subjective quantification) and this is considered relevant to the outcome(s).
Post-capture image enhancements	Clearly states that there were no post-capture image enhancements (i.e., brightness, sharpening, etc.) performed, OR a uniform enhancement was applied across all images.	No statement regarding whether post-capture image enhancements were performed.	Post-capture image enhancements were performed to optimise image contrast/brightness (or some other parameter) individually for each image.
Definition of sub-basal nerve parameters	The nerve parameters being evaluated (e.g., CNFL, CNFD, CNBD) are clearly defined, either in the paper itself or by referencing a previously validated method.	The nerve parameters being evaluated are stated but are not sufficiently well defined to allow full reproduction of the method.	The nerve parameters being evaluated are stated but without any definition (e.g., "CNFL was measured", with no information given on how the parameter was defined).
Method and repeatability of sub-basal nerve parameter quantification (intra- and inter-observer variability)	Use of a validated, fully automated processing method (e.g., ACCMetrics, [11] or similar [22, 23]) .	Use of a semi-automated or fully manual method, where repeatability testing was performed and reported.	Use of a semi-automated or fully manual method, where no repeatability testing was reported.
Data reporting			
Thoroughness of reporting of nerve parameters – selective reporting of outcomes	Data relating to all quantified nerve parameters (as mentioned in the study methods) are reported in the results with both point measures and measures of variability.	Data relating to all quantified nerve parameters (as mentioned in the study methods) are reported in the results with a qualitative descriptor only (with or without	Selective reporting of nerve parameters (i.e., mismatch between parameters described in results and those mentioned in methods).

		reporting of p-values).	
Completeness of nerve parameter data (population level) – attrition bias	Missing data for less than 20% of recruited participants, and if multiple study groups or a longitudinal study, then there is an equal degree of missing data in both groups at follow-up, with no obvious reason why absence of data is related to study group or time point respectively.	Completeness of data is not reported, or if multiple study groups or a longitudinal study, the degree of missing data is for >20% of participants but occurs to an equal degree in both study groups.	If multiple study groups, there is an unequal degree of missing data between study groups, and/or the absence of the data appears to be related to the study outcome.
Other			
Other sources of bias	No other apparent sources of bias.	Source of funding not reported.	Industry-funded study.

Two reviewers will judge the risk of each type of bias (18 items in total) in each included study as either: (i) low risk, (ii) unclear risk (due to either lack of information or uncertainty about the potential for bias), or (iii) high risk. Review authors will resolve any disagreements in bias assessment by consensus with a third reviewer. Reviewers will not be masked to the journal of publication or the study author name when undertaking the risk of bias assessment.

Wherever possible, we will justify each risk of bias assessment with direct quotations from the study. If there are cases where further information is considered necessary to determine the risk of bias in a particular domain, we will attempt to contact the study corresponding author for this information. If no response is received within four weeks of the request, or the requested information is not provided, the information within the full-text article will be used to inform the risk of bias assessment.

Data synthesis

For outcomes related to methodological quality, a systematic narrative synthesis will be provided, with relevant information summarised in text and tables.

If there are at least three relevant studies, we will undertake meta-analyses of the quantitative data for the specified corneal sub-basal nerve plexus parameters (i.e. mean (SD)). Data from male and female participants will be pooled, as studies have shown that gender has no significant effect on corneal sub-basal nerve plexus parameters.[24, 25]

We will convert non-parametric data to means (SD), using an established approach.[26] We will fit a multi-level random-effects model to pool the estimates. We will include estimates from male and female participants, as corneal sub-basal nerve plexus parameters do not vary by gender.[24, 25] The multi-level model will take into account the correlation between estimates from the same study that are presented separately for each sex and/or estimates presented separately for the left and right eyes. Next, we will fit a meta-regression model to assess how much of the between-study variation is explained by the following characteristics: (i) participant age (as this factor is potentially important relation to sub-basal nerve plexus parameters,[27] and (ii) study design (e.g., RCT, cohort (including pseudo-RCT and non-RCT) and other (including cross-sectional, case series/study)).

Statistical analyses will be carried out using the *metafor* package in R.[28, 29]

Meta-bias(es)

As there are no limitations on the potential study designs eligible for inclusion in this review, we expect that we will not be able to compare the outcomes reported in published reports with study protocols, unless the included study is a RCT, to assess for selective outcome reporting or selective analysis reporting. Furthermore, as our meta-analysis is being undertaken to determine values for normative parameters (i.e., corneal sub-basal nerve plexus parameters), rather than the effect of an intervention, we do not expect meta-biases (such as publication bias, delayed publication, etc.) to be a significant factor for this analysis.

Sensitivity analyses

Provided there are a sufficient number of studies included in the review, sensitivity analyses will be performed for the CNFL outcome, to assess for the effect of excluding studies that: (i) were appraised as having a high risk of bias in the domains of image selection – number and sampling, or method for quantifying sub-basal nerve parameters, (ii) included contact lens wearers (i.e., contact lens wear was not listed as an eligibility exclusion criterion), (iii) were lower order levels of evidence (e.g., case reports, case series, interrupted time series), and (iv) are from the same corresponding/senior author, in the event that at least 50 percent of the included papers are from the research laboratory of the same corresponding author.

Confidence in cumulative evidence

If appropriate, we will present a 'Summary of Findings' table for the quantitative outcomes. The quality and strength of the body of evidence will be assessed using an approach based upon the Grading of Recommendations Assessment, Development and Evaluation (GRADE).[30]

CONCLUSIONS

In recent years, an increasing number of research studies have adopted non-invasive, laser-scanning IVCM to quantify corneal sub-basal nerve plexus parameters. However, there has not been any research to formally consider the quality of the methods used in these investigations. This systematic review will provide insight into the quality of the methods reported in clinical studies using laser-scanning IVCM to quantify corneal nerve parameters. The review will also identify specific methodological domains that are least well performed and/or reported (i.e., are judged as having the highest risk of bias) in the literature, as a basis for informing laser-scanning IVCM methods and their robust reporting, in future clinical studies. Furthermore, by researchers considering the elements of the purpose-specific risk of bias tool as a guide when developing their IVCM protocols, this review has the capacity to significantly improve the quality of future research in the field. By undertaking a meta-analysis, we will also determine mean normative values (i.e., from healthy individuals) for central corneal sub-basal nerve plexus parameters. These data will be of significant value for future studies, as reference normative values, building upon a previous pooled analysis of data derived from multiple laser-scanning IVCM testing centres.[31]

ETHICS AND DISSEMINATION

This systematic review and meta-analysis will analyse published data and therefore does not require ethics approval. A manuscript reporting the results of the systematic review will be published in a peer-reviewed journal and may also be presented at scientific conferences.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Tania Celeste, Biosciences Librarian at the University of Melbourne, who assisted with developing the search strategies for this review.

CONTRIBUTIONS OF AUTHORS

MEHDS and LED drafted the initial protocol, with input, discussion and editing from ACZ and HRC. AK contributed to the 'Data synthesis', 'Meta-bias(es)', and 'Sensitivity analyses' sections, and the protocol components for undertaking the meta-analysis. All authors provided final approval of the paper.

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COMPETING INTERESTS STATEMENT

All authors have no conflicts of interest.

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Search strategies

i. MedLine(OViD) and Embase

1	exp cornea/
2	cornea*.tw.
3	1 or 2
4	exp ophthalmic nerve/
5	nerve*.tw.
6	subbasal.tw.
7	sub-basal.tw.
8	mm?mm.tw.
9	neuropath*.tw.
10	plex*.tw.
11	4 or 5 or 6 or 7 or 8 or 9 or 10
12	confocal.tw.
13	microscop*.tw.
14	"in?vivo".tw.
15	12 or 13 or 14
16	3 and 11 and 15

ii. The Cochrane library

1	cornea
2	nerve*
3	innervat*
4	subbasal
5	sub-basal
6	mm?mm
7	neuropath*
8	plex*
9	confocal
10	microscop*
11	"in?vivo"
12	2 or 3 or 4 or 5 or 6 or 7 or 8
13	9 or 10 or 11
14	1 and 12 and 13

PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist: recommended items to address in a systematic review protocol*

Section and topic	Item No	Checklist item	Reported in manuscript (page number + section details)
ADMINISTRATIVE INFORMATION			
Title:			
Identification	1a	Identify the report as a protocol of a systematic review	1
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	Not applicable
Registration	2	If registered, provide the name of the registry (such as PROSPERO) and registration number	Methodological review (not eligible for registration on PROSPERO)
Authors:			
Contact	3a	Provide name, institutional affiliation, e-mail address of all protocol authors; provide physical mailing address of corresponding author	1
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	23
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	Not applicable
Support:			
Sources	5a	Indicate sources of financial or other support for the review	23
Sponsor	5b	Provide name for the review funder and/or sponsor	Not applicable
Role of sponsor or funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	Not applicable
INTRODUCTION			
Rationale	6	Describe the rationale for the review in the context of what is already known	4
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	7
METHODS			
Eligibility criteria	8	Specify the study characteristics (such as PICO, study design, setting, time frame) and report characteristics (such as years considered, language, publication status) to be used as criteria for eligibility for the review	8
Information sources	9	Describe all intended information sources (such as electronic databases, contact with study	9

		authors, trial registers or other grey literature sources) with planned dates of coverage	
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	9
Study records:			
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	11
Selection process	11b	State the process that will be used for selecting studies (such as two independent reviewers) through each phase of the review (that is, screening, eligibility and inclusion in meta-analysis)	11
Data collection process	11c	Describe planned method of extracting data from reports (such as piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	12
Data items	12	List and define all variables for which data will be sought (such as PICO items, funding sources), any pre-planned data assumptions and simplifications	13
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	14
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	15
Data synthesis	15a	Describe criteria under which study data will be quantitatively synthesised	20
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data and methods of combining data from studies, including any planned exploration of consistency (such as I^2, Kendall's τ)	20
	15c	Describe any proposed additional analyses (such as sensitivity or subgroup analyses, meta-regression)	21
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (such as publication bias across studies, selective reporting within studies)	21
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (such as GRADE)	21

*** It is strongly recommended that this checklist be read in conjunction with the PRISMA-P Explanation and Elaboration (cite when available) for important clarification on the items. Amendments to a review protocol should be tracked and dated. The copyright for PRISMA-P (including checklist) is held by the PRISMA-P Group and is distributed under a Creative Commons Attribution Licence 4.0.**

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