Assessment of hemodynamic indices of conjunctival microvascular function in patients with coronary microvascular dysfunction

Microvascular Research

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--Manuscript Draft--

Manuscript Number:	MVR-D-22-00378R2
Article Type:	Research Paper
Keywords:	INOCA; microvascular angina; MICROVASCULAR DYSFUNCTION; conjunctiva; cardiovascular screening.
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Abstract:	Objective Coronary microvascular dysfunction (CMD) is a cause of ischaemia with non-obstructive coronary arteries (INOCA). It is notoriously underdiagnosed due to the need for invasive microvascular function testing. We hypothesised that systemic microvascular dysfunction could be demonstrated non-invasively in the microcirculation of the bulbar conjunctiva in patients with CMD. Methods Patients undergoing coronary angiography for the investigation of chest pain or dyspnoea, with physiologically insignificant epicardial disease (fractional flow reserve ≥0.80) were recruited. All patients underwent invasive coronary microvascular function testing. We compared a cohort of patients with evidence of CMD (IMR ≥25 or CFR <2.0); to a group of controls (IMR <25 and CFR ≥2.0). Conjunctival imaging was performed using a previously validated combination of a smartphone and slit-lamp biomicroscope. This technique allows measurement of vessel diameter and other indices of microvascular function by tracking erythrocyte motion. Results A total of 111 patients were included (43 CMD and 68 controls). There were no differences in baseline demographics, co-morbidities or epicardial coronary disease severity. The mean number of vessel segments analysed per patient was 21.0 ± 12.8 (3.2 ± 3.5 arterioles and 14.8 ± 10.8 venules). In the CMD cohort, significant reductions were observed in axial/cross-sectional velocity, blood flow, wall shear rate and stress. Conclusion The changes in microvascular function linked to CMD can be observed non-invasively in the bulbar conjunctiva. Conjunctival vascular imaging may have utility as a non-invasive tool to both diagnose CMD and augment conventional cardiovascular risk assessment.
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Editor-in-Chief
Microvascular Research

21st October 2022

Dear Editor-in-Chief,

We wish to submit an original research article entitled *"Assessment of indices of conjunctival microvascular function in patients with coronary microvascular dysfunction".*

I confirm on behalf of all authors that the article is original. All authors have participated in the work and have reviewed and agree with the content of the article. None of the article contents are under consideration for publication in any other journal or have been published in any journal. No portion of the text has been copied from other material in the literature (unless in quotation marks, with citation). I am aware that it is the author's responsibility to obtain permission for any figures or tables reproduced from any prior publications, and to cover fully any costs involved. Such permission must be obtained prior to final acceptance. I sign for and accept responsibility for releasing this material on behalf of any and all co-authors. All participants in the study have provided fully informed consent.

This article presents the findings from a pilot study to evaluate the ability for non-invasive conjunctival vascular screening to detect hemodynamic alterations in patients with coronary microvascular dysfunction.

We have no conflicts of interest to disclose. Please address all correspondence concerning this manuscript to: jonathan.mailey@belfasttrust.hscni.net.

Thank you for your consideration of this manuscript.

Sincerely,

Dr Jonathan A. Mailey

Reviewer Rebuttal

We appreciate the valuable feedback provided to improve the quality of the submitted manuscript. Below is a detailed response to all the points raised by the relevant reviewers. We hope that the revised manuscript suitably addresses all necessary points and greatly appreciate your consideration of our work moving forward in the publication process.

Reviewer #2:

1) Introduction, Last para: other groups that have also published results using conjunctival capillaroscopy after 2020, should be reported.

We have referenced the groups that have published on the evaluation of conjunctival capillaroscopy in different forms of CV disease. If there are other specific references that have been overlooked, we would happily include these in our manuscript.

2) Methods, Pages 9-11: references for the IMR and CFR formulas should be given.

These formulae have now been referenced (Page 11, lines 206 & 211)

3) Methods, Page 11, Lines 216-222: there is not a timing diagram presenting clearly, the timing of FFR, CFR, and IMR measurement and the timing of adenosine, heparin and nitroglycerine administration and at what doses.

It has been explained that FFR, CFR and IMR measurements were made following invasive coronary angiography (Page 9 lines 172-183). This procedure involves the simultaneous administration of adenosine, nitroglycerine and unfractionated heparin (standard clinical practice). It is specified on page 13, lines 249-253) that conjunctival imaging was delayed for 4 hours to ensure these medications that all have short half-lives were out of the patient's system. We don't believe it is particularly additive given limited space in the manuscript that a diagram needs to be included to demonstrate the timings of administration of these medications.

4) Intermediate stenoses of 50-70% are greater than 50% so it would be better to change " > 50% " with " > 70%".

The sentence in question explains that coronary stenoses were considered non-obstructive if the % stenosis did not exceed 50%, but if they were between 50 and 70% then the stenosis was interrogated with measurement of FFR. The suggested change would therefore be incorrect.

5) Is "physiologically" a preferred word for describing the FFR test?

Physiologically defined coronary stenosis severity is a standard term used to describe the severity of coronary stenosis and haemodynamic impact in the interventional cardiology community.

6) Lines 447-451 should move before "Conjunctival Microvascular Assessment".

This paragraph has been moved to page 13, lines 249-253 as suggested.

6) Methods, Page 15: The conversion factor should be given.

This conversion factor has now been provided (page 15, line 295)

7) Methods, Page 19, Line 375: It is not described how the vessel centerline is found.

This process is automated, whereby the software simply identifies the outer wall of the microvessel and centreline divides the vessel in 2, creating a radius measurement. We believe that the methods for hemodynamic parameter quantification have been suitably expanded in this revision.

8) Methods, Page 20, Line 392: this method does not follow the velocity in the cardiac cycle and there are also other limitations that are not reported. It should be described in detail a list of limitations of the STI imaging technique (based on wavelet transform of the STI space) among which is the inability of measuring blood pulsating velocity in the arterioles.

This has now been included as a limitation (page 42, lines 136 & 137)

9) Methods, Page 20, Line 401: "using the results" should change to "using the mathematical formulas".

This has been changed as suggested (page 20, line 403)

10) Methods, Page 23, Line 443: There are no references for the selected values of K parameters and their physical meaning.

A reference has now been added (page 23, line 445)

11) Methods, Page 23, Line 463: "Given the significant impact of diameter..", some references should be given.

The relationship of diameter to Q, WSR and WSS has been defined in the quoted formulae (page 21, lines 416-425). This highlights the exponential and inverse linear relationship between these measures.

12) Methods, Page 24, Lines 471-474: This is not clear. The sample size refers to each group separately.

We have clarified that our power calculation produced a study size of 50 patients in each group (page 24, lines 469-472).

Reviewer #3:

1. For the response to the prior reviewer's comment #1, I agree that both venular and arteriolar cross-sectional flow and wall shear stress are different in CMD vs control but it is not clear that these differences are greater in arterioles vs venules (statistical analysis does not address this) and I suggest that comparison not be included.

The statement that the most marked differences were observed in arterioles has been removed from the abstract. It has been clarified in the text that the differences in arteriole haemodynamics were numerically more pronounced than venules rather than being statistically significant (page 31, line 581).

2. In the text, referring to table 2, WSS did not differ between CMD and control; but further analysis of vessels between 10-25 microns and between 25-40 microns, both showed a significant reduction in WSS in CMD. Was this caused by a number of vessels larger than 40 microns included in the total arterial count or some other reason?

In the comparison of arterioles between groups, a significant difference in diameter was observed (as you suggest, due to a slightly larger number of >40 micron vessels in the controls). Controls therefore had on average larger diameter arterioles. WSR and WSS are inversely related to diameter and therefore the increase in diameter balanced the increase in velocity that was observed. When we compared vessels by diameter sub-groups this avoided comparing vessels of different sizes and therefore reflected the increased WSR and WSS observed in the controls.

3. Defining CMD as low CFR or IMR is not a wise idea. CFR may be lowered by a significant epicardial obstruction (diffuse or focal) or anemia or a hyperdynamic state giving a false + result. IMR already accounts for this and provides a more accurate assessment of CMD and should be the sole indicator of CMD.

We appreciate that CFR can be reduced by significant epicardial coronary artery disease but as discussed in the manuscript, we have included only participants with no significant obstructive epicardial CAD to mitigate this fact.

With respect, the widely accepted and guideline recommended definition of coronary microvascular dysfunction is based around the measurement of either a reduced CFR or elevated IMR. The pattern of CFR and IMR can then be used to infer whether the underlying pathophysiological mechanism is structural or functional microvascular dysfunction. We do not think it is correct to describe patients with an abnormal CFR and no epicardial CAD as not having coronary microvascular dysfunction.

4. It should be pointed out that while some differences are statistically significant, the difference is not functionally important (e.g. Table 2 cross-sectional velocity and axial velocity, Table S1 cross-sectional velocity in arterial and venular cross-sectional velocity).

In the discussion a paragraph has now been included to highlight that the numerical haemodynamic differences were small and that this might limit clinical significance (page 41, lines 123-126).

5. QCA is the optimal way to assess the % stenosis of a vessel. Even with moderate coronary disease 50% stenoses on single plain images can be read as 20-80% stenoses even by expert angiographers. This can be listed as a limitation.

This has now been listed as a limitation (page 42, lines 135-138).

Highlights

- Coronary microvascular dysfunction is highly prevalent and associated with an adverse long-term cardiovascular prognosis
- This is the first study to demonstrate alterations in systemic microvascular function in a cohort of patients with coronary microvascular disease
- The non-invasive demonstration of microvascular disease may have utility cardiovascular risk assessment and screening

Graphical Abstract

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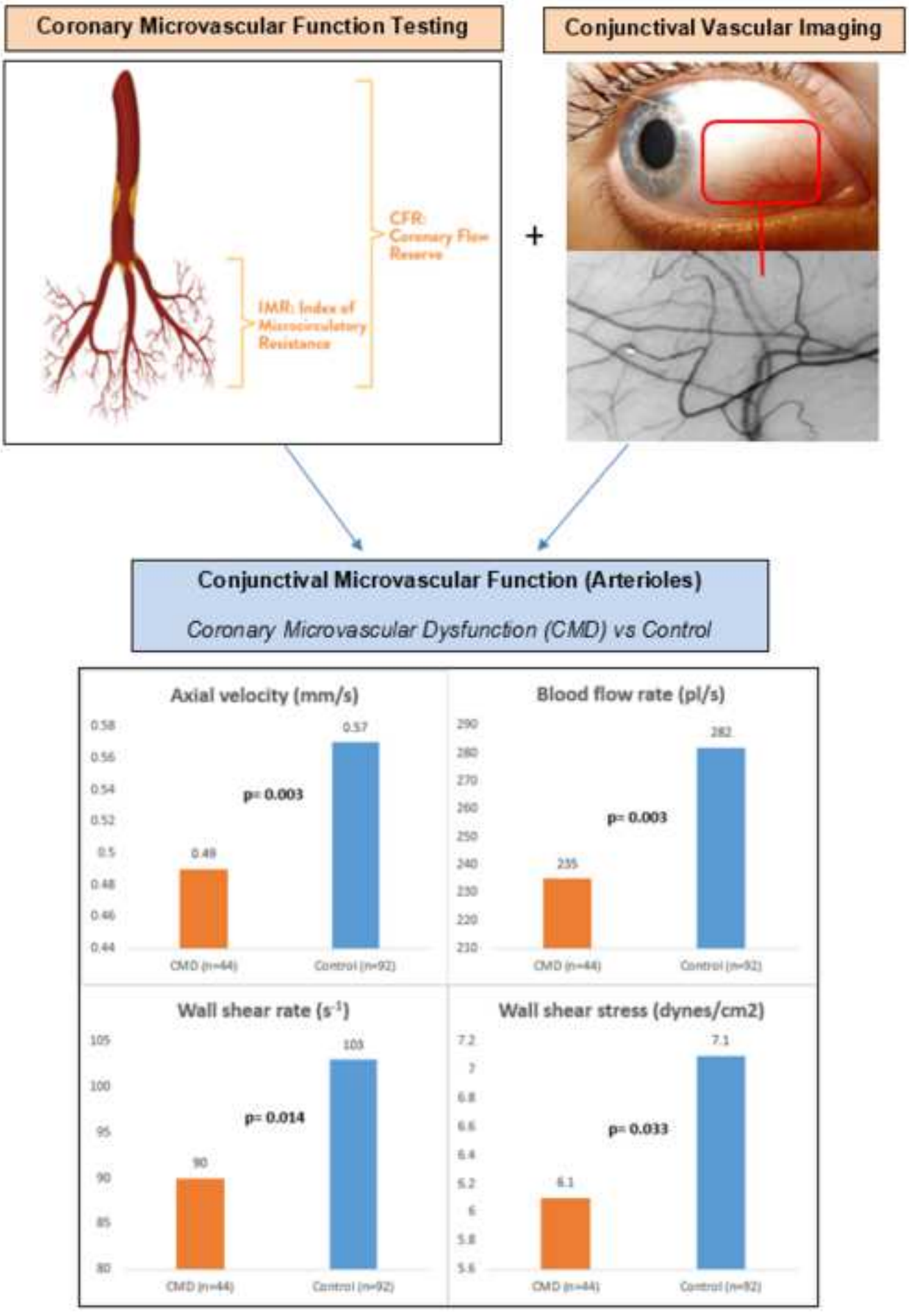

Figure 1

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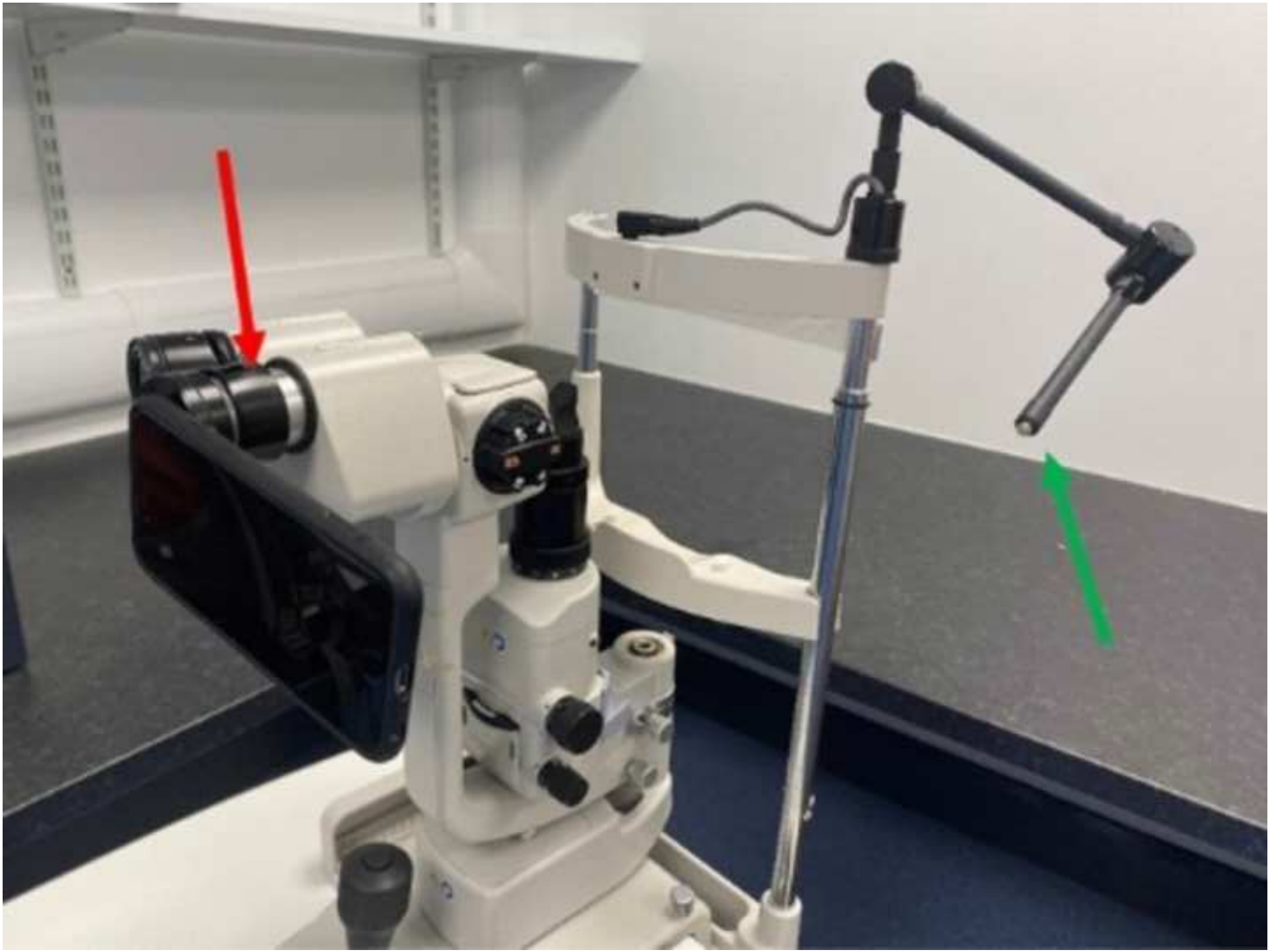

Figure 2

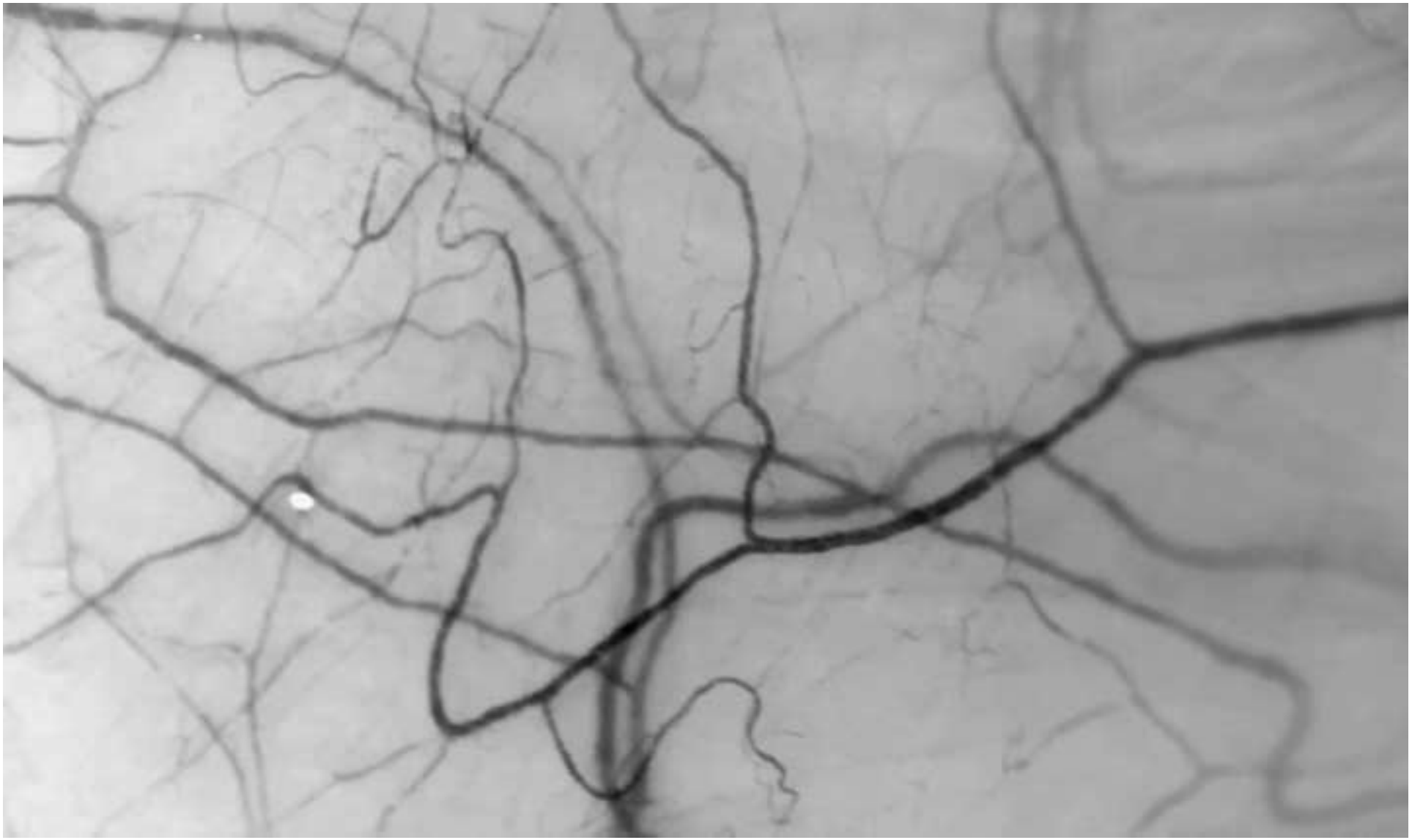

Figure 3

Click here to access/download;Figure;Figure 3 (1).tif

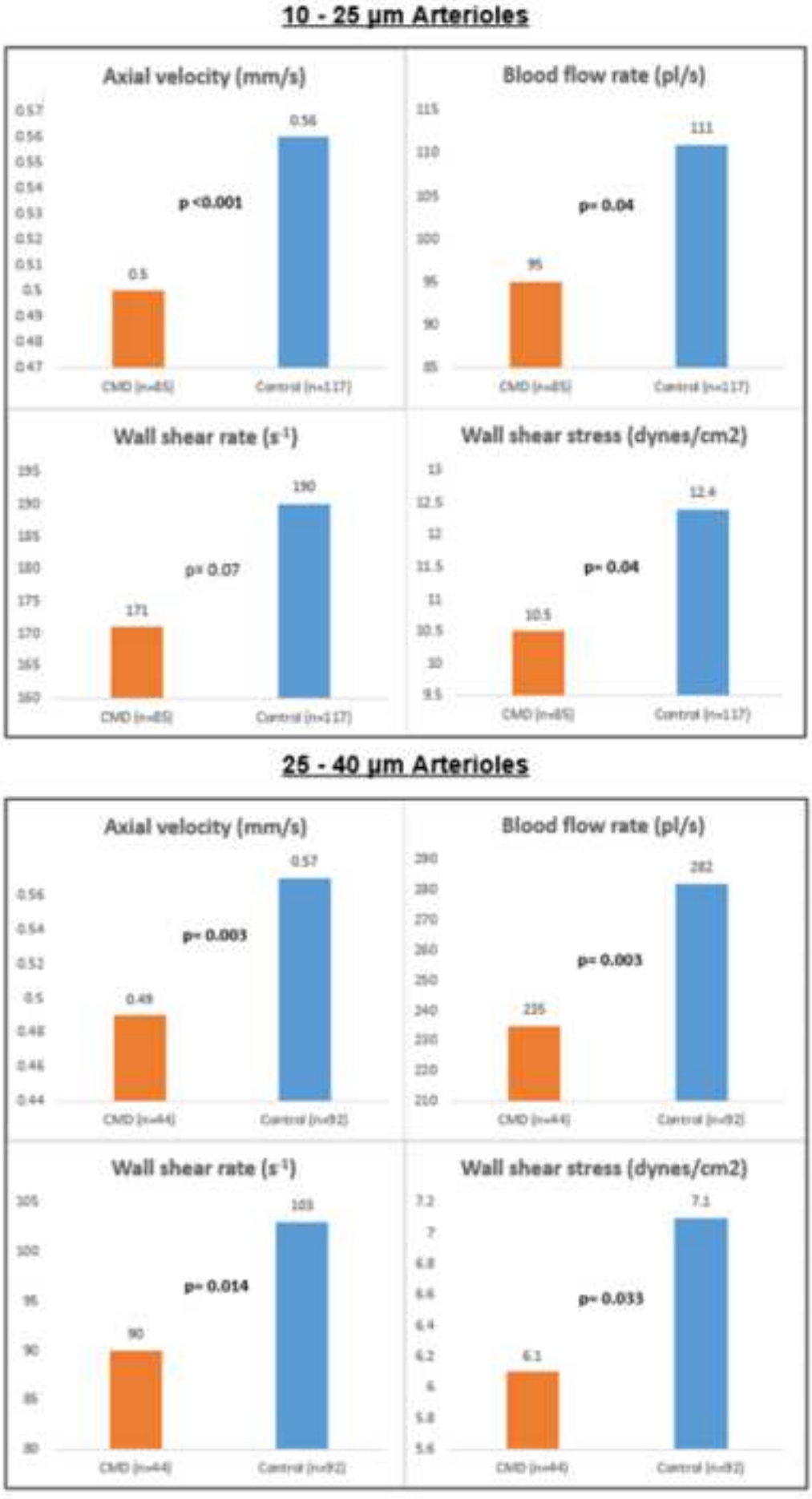

Figure 4

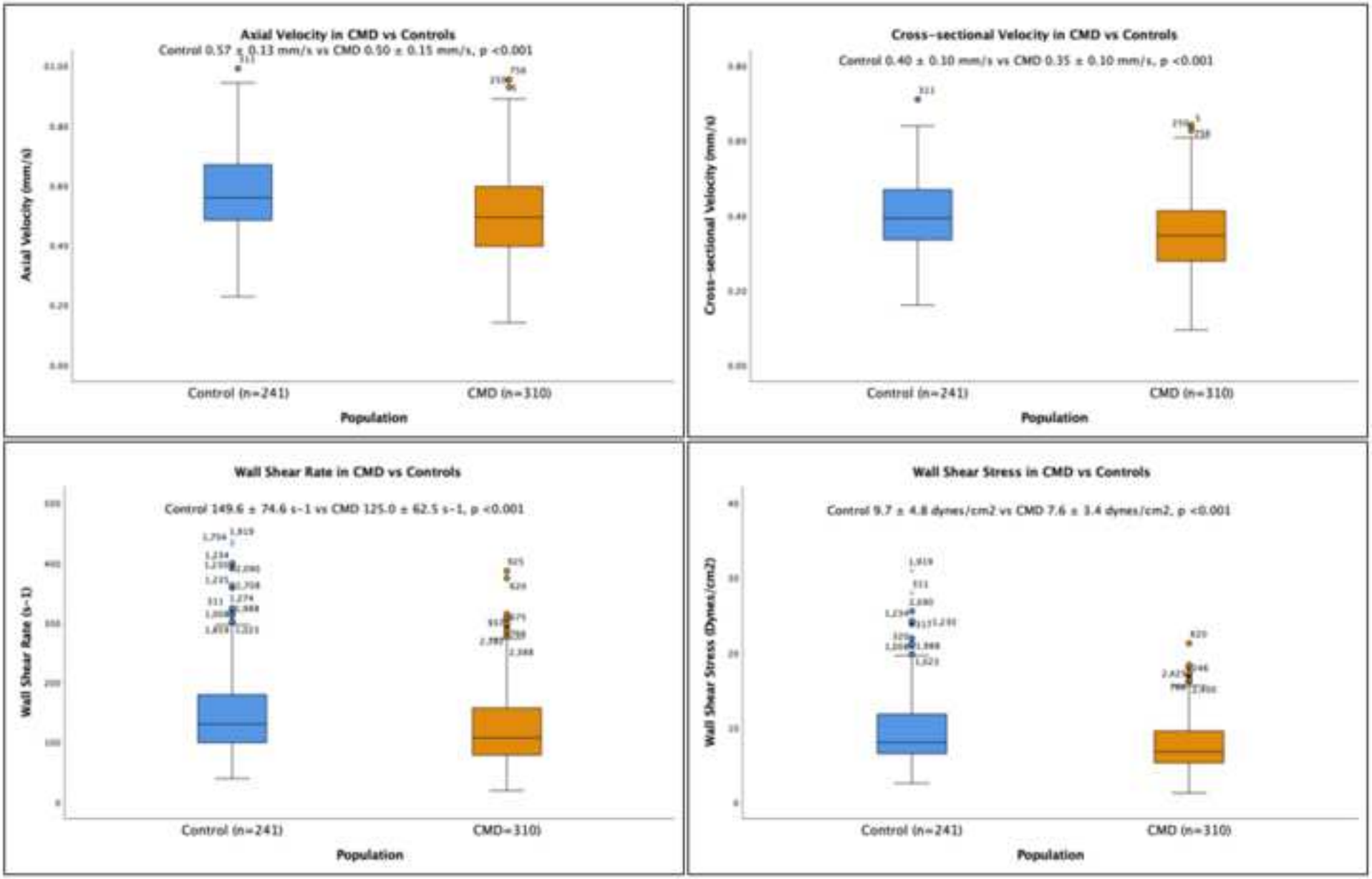

Table 2. Comparison of conjunctival microcirculatory parameters in all vessels

Parameter	**CMD (n=975)**	**Control (n=1320)**	**p-value**
Diameter *(µm)*	24.9 ± 8.4	24.8 ± 7.9	0.88
Axial velocity *(mm/s)*	0.52 ± 0.15	0.55 ± 0.14	**<0.001**
Cross-sectional velocity *(mm/s)*	0.36 ± 0.10	0.38 ± 0.10	**<0.001**
Blood flow rate *(pl/s)*	193.6 ± 132.8	200.5 ± 131.4	0.06
Wall shear rate *(s^{-1})*	136.4 ± 75.5	142.3 ± 74.6	**0.03**
Wall shear stress (*dynes/cm^2*)	8.8 ± 4.5	9.6 ± 5.0	**<0.001**

Table 3. Comparison of conjunctival haemodynamics in arterioles and venules (excluding subjects with a previous history of PCI, MI, diabetes mellitus or systemic hypertension)

Arterioles			
Parameter	**CMD (n=50)**	**Control (n=37)**	**p-value**
Diameter *(μm)*	21.4 ± 6.8	22.8 ± 7.4	0.36
Axial velocity *(mm/s)*	0.48 ± 0.12	0.56 ± 0.14	**0.002**
Cross-sectional velocity *(mm/s)*	0.34 ± 0.09	0.40 ± 0.10	**0.004**
Blood flow rate *(pl/s)*	129.9 ± 94.8	169.5 ± 100.9	**0.03**
Wall shear rate *(s^{-1})*	144.6 ± 78.4	161.7 ± 88.5	0.25
Wall shear stress (*dynes/cm^2*)	8.2 ± 3.8	10.4 ± 5.5	0.06
Venules			
Parameter	**CMD (n=221)**	**Control (n=163)**	**p-value**
Diameter *(μm)*	26.2 ± 7.6	24.4 ± 7.4	**0.02**
Axial velocity *(mm/s)*	0.51 ± 0.15	0.57 ± 0.13	**<0.001**
Cross-sectional velocity *(mm/s)*	0.35 ± 0.11	0.40 ± 0.10	**<0.001**
Blood flow rate *(pl/s)*	201.7 ± 121.1	200.2 ± 124.6	0.88
Wall shear rate *(s^{-1})*	120.8 ± 59.8	148.4 ± 74.3	**<0.001**
Wall shear stress (*dynes/cm^2*)	7.4 ± 3.3	9.6 ± 4.6	**<0.001**

Table 4. Comparison of baseline pharmacological therapies between groups

Medication	**CMD (n=43)**	**Control (n=68)**	**p-value**
Antiplatelet- *n (%)*			
• Aspirin	29 (67.4)	41 (60.3)	0.45
• P2Y12 inhibitor	11 (25.6)	20 (29.4)	0.66
Anti-hypertensive- *n (%)*			
• ACE inhibitor	20 (46.5)	29 (42.6)	0.69
• Angiotensin-2 receptor blocker	10 (23.3)	5 (7.4)	**0.02**
• Mineralocorticoid receptor antagonist	1 (2.3)	1 (1.5)	1.0
• Calcium channel blocker	14 (32.6)	15 (22.1)	0.22
• Thiazide diuretic	5 (11.6)	5 (7.4)	0.51
SGLT-2 inhibitor- *n (%)*	7 (16.3)	4 (5.9)	0.10
Anti-anginal- *n (%)*			
• Beta blocker	31 (72.1)	41 (60.3)	0.21
• Ranolazine	8 (18.6)	5 (7.4)	0.07
• Nicorandil	4 (9.3)	3 (4.4)	0.43
• Long-acting nitrate	18 (41.9)	25 (36.8)	0.59
Statin- *n (%)*	37 (86.0)	55 (80.9)	0.48

Table 1. Baseline Characteristics

Characteristic	CMD (n=43)	Control (n=68)	p-value
Age- *yrs ± SD*	66.0 ± 9.8	63.1 ± 9.2	0.08
Male sex- *n (%)*	21 (48.8)	42 (61.8)	0.18
Body mass index- *kg/m^2 ± SD*	29.4 ± 5.7	30.9 ± 6.8	0.13
Systolic BP- *mmHg ± SD*	124.6 ± 17.0	125.2 ± 15.8	0.58
Diastolic BP- *mmHg ± SD*	70.5 ± 9.6	72.4 ± 10.7	0.64
Smoking history- *n (%)*	23 (53.5)	35 (51.5)	0.84
Hypertension- *n (%)*	22 (51.2)	36 (52.9)	0.86
Diabetes mellitus- *n (%)*	13 (30.2)	21 (30.9)	0.94
Hypercholesterolaemia- *n (%)*	37 (86.0)	51 (75.0)	0.16
Ischaemic heart disease- *n (%)* • Previous myocardial infarction • Previous percutaneous coronary intervention	13 (30.2) 10 (23.3) 13 (30.2)	26 (38.2) 16 (23.5) 25 (36.8)	0.39 0.97 0.48
Stroke- *n (%)*	4 (9.3)	6 (8.8)	1.0
Peripheral vascular disease- *n (%)*	3 (7.0)	1 (1.5)	0.30
Chronic kidney disease- *n (%)*	7 (16.3)	9 (13.2)	0.66

• eGFR >60 • eGFR 45-59 • eGFR 30-44	36 (83.7) 6 (14.0) 1 (2.3)	59 (86.8) 8 (11.8) 1 (1.5)	
Chronic lung disease- *n (%)*	8 (18.6)	4 (5.9)	0.06
Biomarkers/Blood tests			
HbA1c *(mmol/mol)*	43.7 ± 15.8	44.2 ± 12.8	0.38
Creatinine *(µmol/L)*	79.9 ± 23.7	84.3 ± 15.5	0.057
Creatinine Clearance *(ml/min)*	99.1 ± 30.6	104.6 ± 39.7	0.73
Haemoglobin *(g/L)*	137.1 ± 12.6	138.9 ± 13.6	0.47
Haematocrit *(l/l)*	0.41 ± 0.03	0.41 ± 0.04	0.57
Platelets *(10^9/L)*	258.9 ± 65.5	244.9 ± 59.4	0.36
NT-proBNP *(ng/L)*	910.0 ± 3000	199.4 ± 290.6	**0.01**
Cholesterol *(mmol/L)*	3.7 ± 0.9	3.8 ± 1.1	0.75
Triglycerides *(mmol/L)*	1.65 ± 1.51	1.79 ± 0.88	**0.046**
High Density Lipoprotein *(mmol/L)*	1.32 ± 0.34	1.19 ± 0.31	**0.042**
Low Density Lipoprotein *(mmol/L)*	1.71 ± 0.76	1.86 ± 0.96	0.95
Urate *(mmol/L)*	0.33 ± 0.08	0.33 ± 0.07	0.78
C-reactive protein *(mg/L)*	3.6 ± 5.0	2.8 ± 3.3	0.60

Dr Jonathan A. Mailey
Royal Victoria Hospital
274 Grosvenor Road
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Northern Ireland

Editor-in-Chief

Microvascular Research

5th December 2022

Dear Editor-in-Chief,

I confirm on behalf of all authors that the article is original. All authors have participated in the work and have reviewed and agree with the content of the article. None of the article contents are under consideration for publication in any other journal or have been published in any journal. No portion of the text has been copied from other material in the literature (unless in quotation marks, with citation). I am aware that it is the author's responsibility to obtain permission for any figures or tables reproduced from any prior publications, and to cover fully any costs involved. Such permission must be obtained prior to final acceptance. I sign for and accept responsibility for releasing this material on behalf of any and all co-authors. All participants in the study have provided fully informed consent.

Sincerely,

Dr Jonathan A. Mailey

Assessment of hemodynamic indices of conjunctival microvascular function in patients with coronary microvascular dysfunction

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**Joint senior authors*

Short Title- INOCA affects more than the coronaries

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Highlights

- Coronary microvascular dysfunction is highly prevalent and associated with an adverse long-term cardiovascular prognosis
- This is the first study to demonstrate alterations in systemic microvascular function in a cohort of patients with coronary microvascular disease
- The non-invasive demonstration of microvascular disease may have utility cardiovascular risk assessment and screening

ABSTRACT

Objective

Coronary microvascular dysfunction (CMD) is a cause of ischaemia with non-obstructive coronary arteries (INOCA). It is notoriously underdiagnosed due to the need for invasive microvascular function testing. We hypothesised that systemic microvascular dysfunction could be demonstrated non-invasively in the microcirculation of the bulbar conjunctiva in patients with CMD.

Methods

Patients undergoing coronary angiography for the investigation of chest pain or dyspnoea, with physiologically insignificant epicardial disease (fractional flow reserve ≥0.80) were recruited. All patients underwent invasive coronary microvascular function testing. We compared a cohort of patients with evidence of CMD (IMR ≥25 or CFR <2.0); to a group of controls (IMR <25 and CFR ≥2.0). Conjunctival imaging was performed using a previously validated combination of a smartphone and slit-lamp biomicroscope. This technique allows measurement of vessel diameter and other indices of microvascular function by tracking erythrocyte motion.

Results

A total of 111 patients were included (43 CMD and 68 controls). There were no differences in baseline demographics, co-morbidities or epicardial coronary disease severity. The mean number of vessel segments analysed per patient was 21.0 ±

12.8 (3.2 ± 3.5 arterioles and 14.8 ± 10.8 venules). In the CMD cohort, significant reductions were observed in axial/cross-sectional velocity, blood flow, wall shear rate and stress.

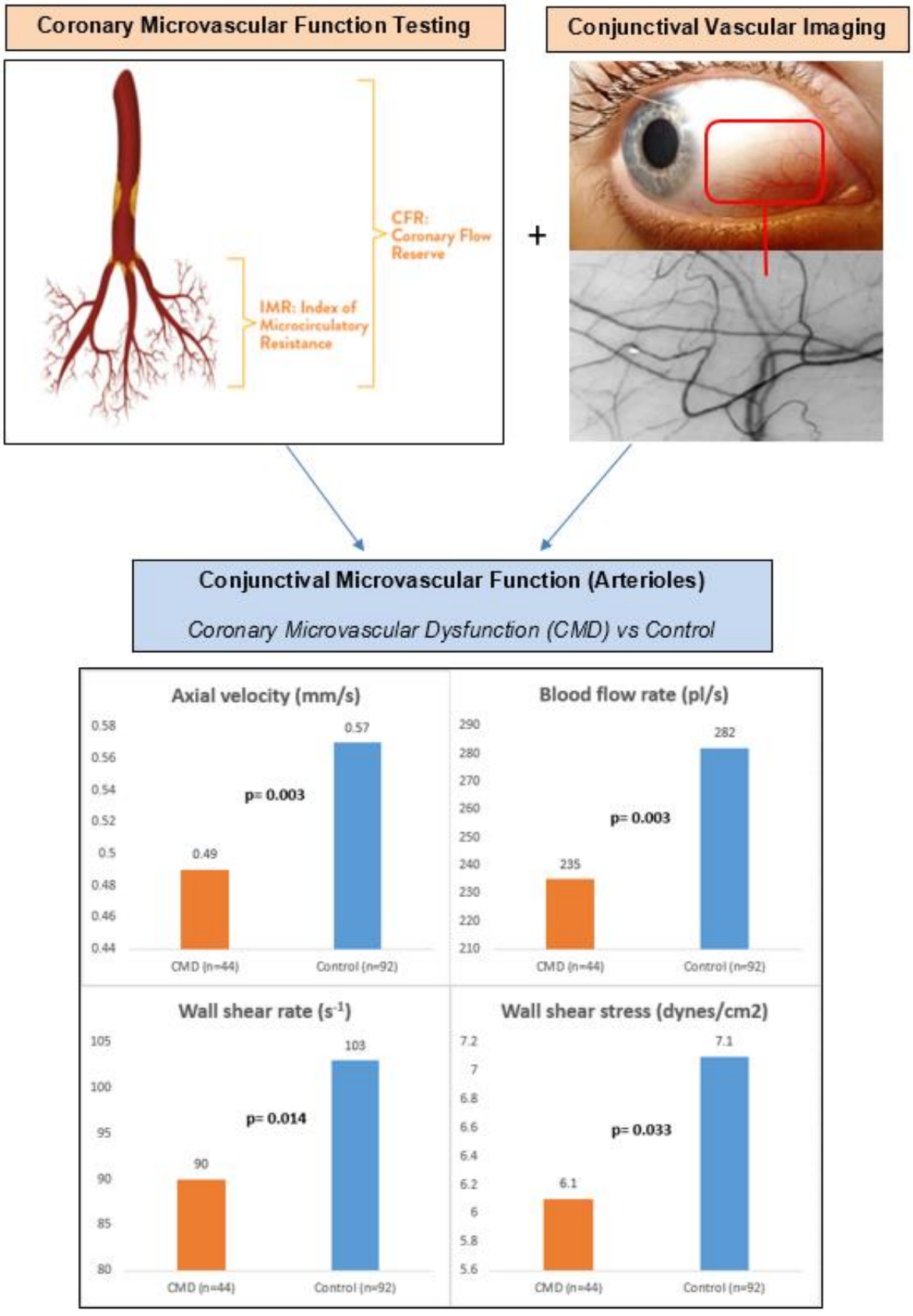
Conclusion

The changes in microvascular function linked to CMD can be observed non-invasively in the bulbar conjunctiva. Conjunctival vascular imaging may have utility as a non-invasive tool to both diagnose CMD and augment conventional cardiovascular risk assessment.

Keywords

INOCA; microvascular angina; microvascular dysfunction; conjunctiva; cardiovascular screening.

GRAPHICAL ABSTRACT



INTRODUCTION

It is estimated that approximately 112 million people globally experience angina pectoris (1). Between 40 and 50% of patients undergoing invasive coronary angiography for the investigation of angina have no obstructive epicardial disease (2, 3). In the setting of abnormal functional ischaemic testing these symptoms are commonly due to ischaemia with non-obstructive coronary arteries (INOCA). The most frequently encountered sub-types of INOCA are coronary microvascular dysfunction (CMD) and epicardial vasospastic angina (VSA) (4). These conditions are notoriously underdiagnosed leading to recurrent angina, impaired quality of life, unplanned hospitalizations, repeated coronary angiography and adverse long-term cardiovascular outcomes (5, 6, 7). The CorMicA trial highlighted the importance of invasive coronary function testing in INOCA with significant reductions in angina and improvement in quality of life with stratified medical therapy in the intervention arm of the study vs standard of care (3). The intervention in this study led to a mean improvement of 11.7 U in the Seattle Angina Questionnaire summary score at 6 months (95% confidence interval [CI]: 5.0 to 18.4; $p = 0.001$). In addition, the intervention led to improvements in the mean quality-of-life score (EQ-5D index 0.10 U; 95% CI: 0.01 to 0.18; $p = 0.024$) and visual analogue score (14.5 U; 95% CI: 7.8 to 21.3; $p < 0.001$) (3).

CMD can occur due to structural remodelling of the microvasculature (fixed reduction in microcirculatory conductance) and/or functional vasomotor disorders affecting the coronary arterioles (dynamic arteriolar obstruction) (8, 9). VSA is caused by

abnormal dynamic epicardial coronary obstruction. There can be overlap between VSA and CMD sub-types, particularly with functional CMD.

Significant epicardial coronary artery disease can be excluded non-invasively using CT coronary angiography (CTCA) and ischaemia demonstrated with a functional imaging test. However, the gold-standard for the diagnosis of CMD remains invasive coronary angiography to exclude obstructive epicardial CAD and perform a physiological evaluation of microvascular function including vasoreactivity testing. Current European Society of Cardiology (ESC) guidelines for the diagnosis and management of chronic coronary syndromes suggest that invasive coronary function testing should be considered in patients with suspected CMD (IIa recommendation) (10). The downside to invasive angiography is the exposure of the patient to infrequent, but potentially life-threatening iatrogenic complications (11).

Whilst a link between systemic microvascular dysfunction and INOCA has been suggested from previous studies (12), it remains to be definitively shown. We hypothesized that if microvascular dysfunction in an alternative vascular network could be demonstrated non-invasively in patients with CMD, this would have potential clinical utility in both the non-invasive diagnosis of CMD and the enhancement of conventional cardiovascular risk assessment tools such as SCORE, ASSIGN and Q-RISK III. A diagnostic algorithm for CMD that utilises a non-invasive assessment of systemic microvascular dysfunction has clear advantages. It would avoid the cost and time requirement for invasive coronary angiography and benefit the patient by avoiding discomfort, anxiety and potential procedural complications.

The conjunctival microcirculation is a readily assessable microvascular network in which physiological parameters can be non-invasively evaluated (13, 14, 15, 16, 17, 18). Microvascular dysfunction has previously been observed in the bulbar conjunctiva in a variety of cardiovascular disorders and levels of CV risk (14, 15, 19, 20, 21, 22). In this study we compare physiological parameters of conjunctival microvascular function in symptomatic subjects with and without invasive evidence of CMD.

METHODS

We conducted a study (Integrated Research Application System study number 166742) comparing conjunctival microcirculatory function in a group of patients with coronary microvascular dysfunction (CMD cohort) (n=43) as a cause of INOCA to a group of patients with non-obstructive coronary artery disease and normal indices of coronary microvascular function (Control cohort) (n=68).

All subjects provided written informed consent for participation in this study. The experimental protocol was approved by the Research Ethics committee in the Belfast Health and Social Care Trust (BHSCT) and Ulster University (UU). The study was carried out in accordance with the Declaration of Helsinki.

Baseline clinical data and characteristics were obtained using a recruitment questionnaire, clinical notes, hospital cardiology database (Cardiovascular

Information System Tomcat, Phillips, Eindhoven, Netherlands) and each patient's national electronic healthcare record.

Diagnosing coronary macro- and microvascular disease

Defining the presence or absence of hemodynamically significant coronary artery disease based on a visual assessment of coronary stenoses is limited by significant inter-observer variability, in addition to underdiagnosing the presence of microvascular dysfunction. Contemporary interventional cardiological practice thereby suggests the utilisation of coronary physiology for the investigation of symptoms suggestive of stable angina.

Fractional flow reserve (FFR) is a well validated tool (23, 24) that measures the hemodynamic significance of a coronary stenosis. FFR is performed by inserting a coronary guidewire with pressure transducing capabilities beyond the stenosis, inducing pharmacological stress (usually with intravenous adenosine) and comparing the distal to proximal coronary pressure during stress. In addition to FFR commercially available coronary pressure wires also allow microvascular assessment using thermodilution, whereby the injection of cold saline allows measurement of temperature change from proximal to distal within the coronary. This in turn allows the calculation of mean transit time of blood within the coronary and the calculation of coronary flow reserve (CFR) and the index of microcirculatory resistance (IMR). All pressure wire measurements are performed following adminstration of intraarterial unfractionated heparin and nitroglycerine.

A summary of the formulae used to derive the relevant coronary hemodynamics can be found below:

The derivation of IMR is based on Ohm's law, whereby:

$$\text{Resistance (R)} = \text{Voltage (V)} / \text{Current (I)}$$

In the coronary circulation V is analogous to the pressure difference (ΔP) across the coronary microvasculature. ΔP is calculated by subtracting the mean coronary wedge pressure (P_v) from the mean distal coronary arterial pressure (P_d):

$$\Delta P = P_d - P_v$$

Current (I) is equivalent to coronary blood flow (Q), whereby:

$$Q = 1 / \text{Mean transit time } (T_{mn})$$

IMR is thereafter calculated using this formula:

$$\text{IMR} = (P_d - P_v) / \text{Hyperaemic coronary blood flow } (Q_{(Hyp)})$$

P_v is however challenging to measure and usually of negligible value, so the formula can be simplified without creating significant inaccuracy to:

$$\text{IMR} = P_d / Q_{(Hyp)}$$

In its simplest form given the inverse relationship of Q and T_{mn}:

$$IMR = P_d \times T_{mn(hyp)} \quad (25)$$

CFR is simply a ratio of hyperaemic to resting coronary flow, thereby:

$$CFR = Q_{(Hyp)} / Q_{(rest)}$$

$$CFR = (1 / T_{mn(hyp)}) / (1 / T_{mn(rest)})$$

$$CFR = T_{mn(rest)} / T_{mn(hyp)} \quad (25)$$

Inclusion criteria

All subjects were recruited following invasive coronary angiography for the investigation of symptoms of chest pain (angina) and/or dyspnoea (angina equivalent). Only patients with both angiographically and physiologically non-obstructive epicardial coronary disease were eligible for recruitment. Non-obstructive coronary disease was defined angiographically if there were no epicardial stenoses >50% and physiologically in the context of any intermediate stenoses (50-70%) as a fractional flow reserve (FFR) ≥0.80. All subjects underwent an evaluation of coronary microvascular function with measures of coronary flow reserve (CFR) and index of microcirculatory resistance (IMR) calculated using standard thermodilution techniques and commercially available software. Subjects were only considered eligible if measurements of mean transit time during both rest and maximal hyperaemia were deemed to be repeatable (<20% variation in measurements).

Exclusion criteria

1. Inability to consent
2. Age less than 18 years of age
3. Pregnancy at time of recruitment
4. History of conjunctival inflammation or contact lens use in the 24 hours prior to recruitment
5. Presentation that fulfilled the ESC 4th universal definition of myocardial infarction (26)
6. Hemodynamically significant valvular heart disease
7. Left ventricular ejection fraction <40%
8. Heart failure with preserved ejection fraction
9. Previous coronary artery bypass grafting (CABG)

CMD cohort

All subjects fulfilled the COVADIS diagnostic criteria for CMD (8). Thus, all patients, in addition to symptoms suggestive of INOCA, had objective evidence of CMD with an elevated IMR (≥25), a reduced CFR (<2.0) or the combination of both of these abnormalities in microvascular function.

Control cohort

Subjects without evidence of CMD were recruited to the control arm of the study. Both indices of coronary microvascular function were normal in this cohort (IMR <25 and CFR ≥2.0).

All subjects underwent conjunctival imaging at least 4 hours after coronary angiography. Given the short half-lives of the administered intravenous and intra-arterial medications (unfractionated heparin, nitroglycerine and adenosine), this allowed time for these agents to wash out of the subjects' system and hence avoid any confounding impact on conjunctival microvascular function.

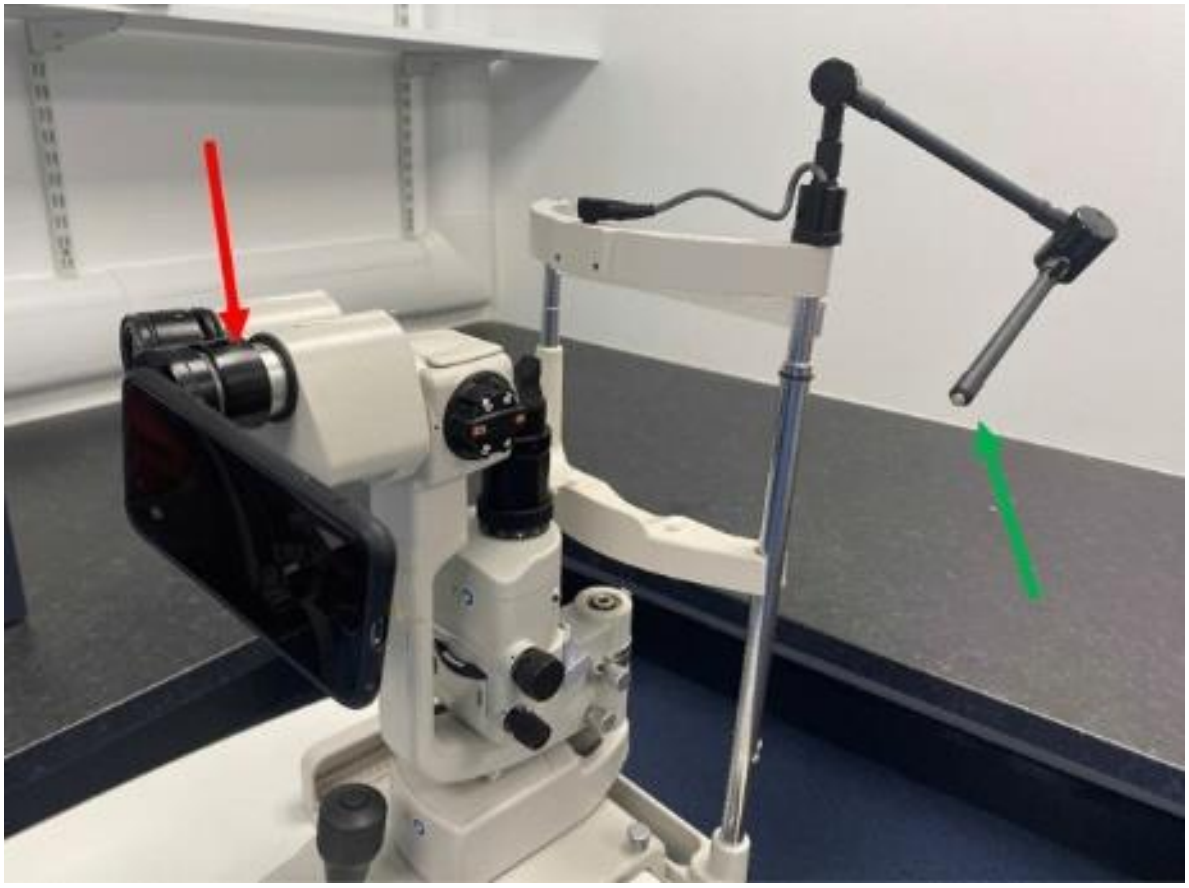
Conjunctival Microvascular Assessment

Imaging Equipment

In order to obtain video imaging of the conjunctival microvasculature of sufficient quality to allow quantification of hemodynamic parameters, a combination of hardware that provides sufficient illumination and magnification of the vessels is required. The equipment used for conjunctival vascular imaging (**Figure 1**) included:

1. Topcon SL-D4 Slit Lamp Biomicroscope (Topcon Medical Systems Inc., Oakland, NJ, USA)
2. Apple iPhone 11 Pro Smartphone (Apple Inc., Cupertino, CA, USA)
3. Digital Photomicrography Slit Lamps Lens Adapter (Zarf Enterprises Inc., Spokane, WA, USA)

Figure 1. Smartphone and slit-lamp biomicroscope imaging system with the adapter and external fixation target



The slit lamp biomicroscope was used for both illumination and magnification of the bulbar conjunctival microvasculature. This provided a 40x magnification of the micro-vessels being imaged. Images were then also magnified by the smartphone camera by a further 2x factor of magnification. This allowed sufficient image quality, whilst not compromising the size of the field of view and hence number of blood vessels visualised. The slit lamp and iPhone were coupled using a bespoke adapter.

A smartphone gives little control over relevant camera properties (focus, ISO, shutter speed, aperture and compression). In this study we overcame this by using a commercially available third-party application “ProMovie Recorder” (www.promovieapp.com). This enabled conjunctival imaging to be performed in line with a set imaging protocol (as described below), providing consistent imaging settings with respect to zoom, ISO, focus, shutter speed and exposure. This allowed calculation of a pixel to millimetre conversion factor for the video settings applied (454.8 ± 22.4 pixels/mm). This conversion factor was used for the downstream measurement of vessel diameter and blood flow velocity, in addition to the calculation of other hemodynamic parameters of microcirculatory function.

Protocol for Image Acquisition

1. All imaging was performed with surrounding external lighting dimmed and the primary source of ocular illumination provided by the slit-lamp biomicroscope.
2. The 12-megapixel rear facing wide lens on the iPhone 11 pro was used to acquire videos.
3. Magnification on the slit lamp biomicroscope was set at a factor of 40x.
4. An external fixation target was used to minimise blinking and eye movement.
5. Videos were captured using the camera application “Promovie Recorder”. The fixed camera settings used were as follows:
    a. Aspect ratio 16 : 9
    b. Resolution 3840 x 2160 pixels
    c. Frame rate 60 frames per second
    d. Maximal available compression bitrate (120Mbps)
    e. Camera zoom 2x magnification
    f. Focus 0.5
    g. ISO set to the minimum level (30)
    h. Shutter speed set to minimum level (61)
6. For each subject 5 to 10 second videos were obtained from both the nasal and temporal fields of each eye (a total of 4 videos per subject)
7. Videos were saved under a unique anonymised study number prior to being electronically transferred to a University laptop for image processing

Image Processing and Microvascular Hemodynamic Parameter Quantification

A summary of the image processing steps used to estimate hemodynamic parameters and analyse conjunctival microvascular function is provided in the supplementary appendix (**Figure S1)**.

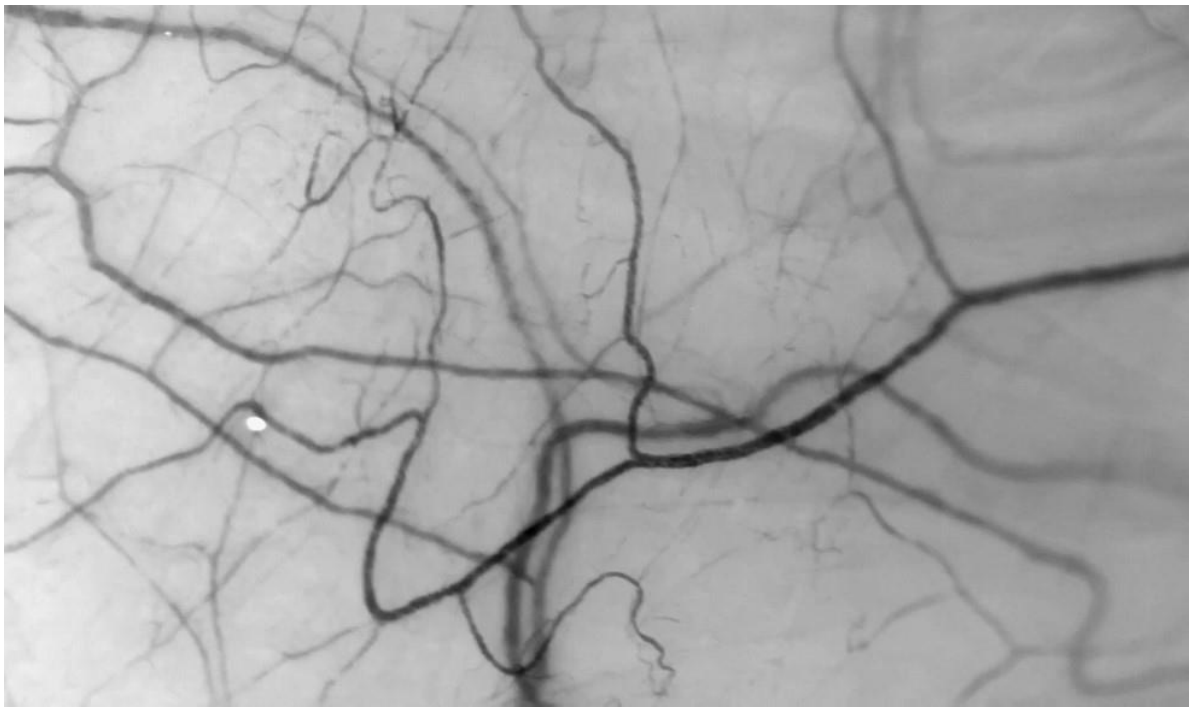
Following image acquisition, an initial manual visual inspection of the videos was performed (see **Figure S2** in the supplementary appendix for an example of initial conjunctival image). This allowed for consecutive frames of the highest quality to be selected for subsequent image processing and analysis by researchers. The criteria applied to select these frames included:

- Conjunctival microvasculature in focus
- No eye blinking
- Minimal eye movement
- Field of view did not drift by more than 25% of the width of the frame

Colour videos were converted into grey scale and any underexposed or out of focus regions were excluded. The sharpest frame in the selected sequence was then chosen as a reference frame and all other frames registered to it through an affine registration procedure (27). A vessel enhancement filter was then applied to the mean registered images (28) to enhance the performance of the Frangi filter (29). A binary map of the conjunctival vasculature and corresponding centrelines was extracted via standard skeletisation techniques. This allowed small spurious branches to be removed and for the detection of the end and branch points of the

vessels connected vessel network was broken into individual vessel segments by setting the branch points' neighbouring pixels to zero. Any vessel segments longer than 30 pixels were selected for further assessment. **Figure 2** demonstrates the final grey-scale conjunctival vascular network generated.

Figure 2. Stabilised conjunctival image obtained at 80 times magnification



Estimation of vessel diameter

The Euclidean Distance Transform (EDT) was used for vessel diameter estimation. This method can be applied to binary images to measure the straight-line distance in pixels between two points on the image. The value at each pixel of EDT map was calculated based on the Euclidean distance between the pixel and its' nearest nonzero pixel in the binary vessel image. The centreline of the vessel was used to obtain the central EDT values and thus the radius along the vessel axis. This measurement in pixels is then converted to millimetres using the previously calibrated pixel to mm conversion factor. Using this method, the vessel centreline is used to obtain the central EDT values and thus the radius along the vessel axis. The average of the diameters along the analysed vessel length was used to provide the final vessel diameter estimation.

Estimation of axial velocity

The axial velocity (V_a) of blood flow within the vessel was estimated via 1D+T continuous wavelet transform (1DTCWT) based on spatial-temporal image (STI) generated for each vessel segment (**Figure S3** in the supplementary appendix). In these STI graphs a change in signal intensity is reflective of erythrocyte movement through the blood vessel. The graphs provide a plot of signal intensity against vessel segment length on the y-axis and the frame number on the x-axis. All imaging was recorded at a consistent setting of 60 frames per second, meaning 1 frame= 0.01667 seconds. Since the change of intensity in STI represents the erythrocyte flowing through the vessel within the given time (video frames), V_a can also be obtained by finding the slope of the prominent intensity bands in STI. The process of generating STI graphs is automated using specially designed software. However, the flow analysis methods described in this study required human input to differentiate and select the graphs of sufficient quality (without artefact) to enable erythrocyte tracking and hence estimate axial velocity.

Additional conjunctival hemodynamics

Cross-sectional velocity, blood flow rate, wall shear rate and wall shear stress were estimated using the formulae described below. These calculations are performed using the mathematical formulae for diameter and axial velocity described in previous publications (30, 31).

Cross-sectional velocity (V_{cs})

V_{cs} is impacted by the diameter of the vessel in which blood is travelling. In this study it was estimated according to these formulae:

Diameter / human erythrocyte diameter (D_c) ≤ 0.6:

$$V_{cs} = V_a$$

Diameter / human erythrocyte diameter (D_c) > 0.6:*

$$V_{cs} = V_a / 1.58 \times (1 - e^{-\sqrt{2}D_c})$$

* In these equations D_c was taken to be a constant, equal to 7.65 μm.

Blood flow rate (Q)

Q has a linear relationship to V_{cs} and is exponentially related to diameter according to this formula:

$$Q = V_{cs} (\pi D^2 / 4)$$

Wall shear rate (WSR)

WSR has a linear relationship to V_{cs} and an inverse relationship to diameter according to this formula:

$$WSR = (8V_{cs}) / D$$

Wall shear stress

Wall shear stress (WSS) is calculated as the product of wall shear rate (WSR) and whole blood viscosity (η):

$$WSS = WSR \times \eta$$

Newton's law defines the relationship between shear stress and the shear rate of a fluid subjected to mechanical stress. The ratio of shear stress to shear rate is a constant for a given temperature and pressure, and hence in Newtonian fluids the viscosity is independent of the shear rate (32). Blood does not follow Newton's law of viscosity, and hence is described as a non-Newtonian fluid. The primary determinants of η are plasma viscosity (η_p) (in turn primarily influenced by total protein concentration), haematocrit (HCT) and the mechanical properties of red blood cells (33).

In this study it was not possible to measure η directly on participants due to the lack of the specialised equipment required. The Quemada model for estimation of η was therefore chosen to obtain results and in turn estimate WSS (34). This model takes into consideration HCT, η_p and WSR as defined in the equation below:

$$\eta = \eta_\rho \left(1 - \frac{1}{2} \frac{\kappa_0 + \kappa_\infty \sqrt{\frac{\dot{\gamma}}{\gamma_c}}}{1 + \sqrt{\frac{\dot{\gamma}}{\gamma_c}}} H_t \right)^{-2}$$

In this equation k_0, k_∞ and γ_c are constants (4.33, 2.07 and 1.88 respectively)(34). HCT and η_p were obtained from blood sampling during the recruitment process and WSR estimated for the individual vessels as described previously.

In addition to quantification of microvascular hemodynamics, vessels were manually differentiated into arterioles and venules using the principle of blood flow direction in relation to bifurcations. This allows a more accurate comparison of microvascular function to be formed. This method of vessel differentiation has been described previously (35, 36). Vessels were defined as arterioles if blood flow was towards a diverging bifurcation; venules if blood flow was towards a converging bifurcation; and undifferentiated if no bifurcation was present in the imaging field to allow vessel differentiation. Undifferentiated vessels were excluded from subsequent sub-group analyses.

Given the significant impact of diameter on Q, WSR and WSS; hemodynamic parameters were further analysed in two distinct diameter groupings (10 – 25 μm and 25 – 40 μm). These diameter groups were chosen by including only conjunctival vessels with a diameter that fell within 2 standard deviations of the total mean of all conjunctival vessels analysed. The range of these vessels was 10 to 40 μm, which was then divided evenly into the two groups.

Statistical Analysis

The results of a pilot study published by our research group (14) were used for a formal power calculation. We estimated that a sample size of 100 patients (3600 conjunctival vessels) would provide the study with a power of at least 80% to reject the null hypothesis of no between-group differences in conjunctival hemodynamics.

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) for Apple iOS version 27 (property of IBM). Continuous variables were described using the mean and standard deviation of the mean. Kolmogorov–Smirnov testing was used to assess normality of the continuous variables. Categorical variables were expressed as a number and percentage of the total category number to which the variable belonged.

Normally distributed variables were compared between the two populations using the independent-samples t-test. Non-normally distributed continuous variables were compared using non-parametric tests e.g. Mann–Whitney U test. Categorical comparisons were made between the two groups using Pearson Chi-Square or Fisher's exact test as appropriate. Repeatability was assessed using Intraclass Correlation Coefficient for continuous variables and Fleiss Kappa for categorical variables.

RESULTS

Baseline Characteristics

Between November 2020 and February 2022, a total of 119 patients were recruited to this study. There were two patients excluded due to symptoms that did not fulfil the above specified inclusion criteria and six patients due to non-reproducibility of the measured coronary microvascular indices (>20% variation in the measurements of coronary mean transit time obtained during microvascular function testing). The remaining 111 patients had a mean age of 64.2 ± 9.5 years (range 38 – 81 years). A small majority of patients were male (56.8%).

A total of 43 patients were included in the CMD cohort and 68 patients in the control cohort. There were no significant differences in baseline characteristics between the groups (**Table 1**).

Table 1. Baseline Characteristics

Characteristic	CMD (n=43)	Control (n=68)	p-value
Age- *yrs ± SD*	66.0 ± 9.8	63.1 ± 9.2	0.08
Male sex- *n (%)*	21 (48.8)	42 (61.8)	0.18
Body mass index- *kg/m² ± SD*	29.4 ± 5.7	30.9 ± 6.8	0.13
Systolic BP- *mmHg ± SD*	124.6 ± 17.0	125.2 ± 15.8	0.58
Diastolic BP- *mmHg ± SD*	70.5 ± 9.6	72.4 ± 10.7	0.64
Smoking history- *n (%)*	23 (53.5)	35 (51.5)	0.84
Hypertension- *n (%)*	22 (51.2)	36 (52.9)	0.86
Diabetes mellitus- *n (%)*	13 (30.2)	21 (30.9)	0.94
Hypercholesterolaemia- *n (%)*	37 (86.0)	51 (75.0)	0.16
Ischaemic heart disease- *n (%)* • Previous myocardial infarction • Previous percutaneous coronary intervention	13 (30.2) 10 (23.3) 13 (30.2)	26 (38.2) 16 (23.5) 25 (36.8)	0.39 0.97 0.48
Stroke- *n (%)*	4 (9.3)	6 (8.8)	1.0
Peripheral vascular disease- *n (%)*	3 (7.0)	1 (1.5)	0.30
Chronic kidney disease- *n (%)* • eGFR >60 • eGFR 45-59 • eGFR 30-44	7 (16.3) 36 (83.7) 6 (14.0) 1 (2.3)	9 (13.2) 59 (86.8) 8 (11.8) 1 (1.5)	0.66
Chronic lung disease- *n (%)*	8 (18.6)	4 (5.9)	0.06

Biomarkers/Blood tests			
HbA1c *(mmol/mol)*	43.7 ± 15.8	44.2 ± 12.8	0.38
Creatinine *(µmol/L)*	79.9 ± 23.7	84.3 ± 15.5	0.057
Creatinine Clearance *(ml/min)*	99.1 ± 30.6	104.6 ± 39.7	0.73
Haemoglobin *(g/L)*	137.1 ± 12.6	138.9 ± 13.6	0.47
Haematocrit *(l/l)*	0.41 ± 0.03	0.41 ± 0.04	0.57
Platelets *(10^9/L)*	258.9 ± 65.5	244.9 ± 59.4	0.36
NT-proBNP *(ng/L)*	910.0 ± 3000.5	199.4 ± 290.6	**0.01**
Cholesterol *(mmol/L)*	3.7 ± 0.9	3.8 ± 1.1	0.75
Triglycerides *(mmol/L)*	1.65 ± 1.51	1.79 ± 0.88	**0.046**
High Density Lipoprotein *(mmol/L)*	1.32 ± 0.34	1.19 ± 0.31	**0.042**
Low Density Lipoprotein *(mmol/L)*	1.71 ± 0.76	1.86 ± 0.96	0.95
Urate *(mmol/L)*	0.33 ± 0.08	0.33 ± 0.07	0.78
C-reactive protein *(mg/L)*	3.6 ± 5.0	2.8 ± 3.3	0.60

The majority of patients had the physiological assessment of microvascular function performed in the left anterior descending artery (LAD) (91.0%). In the remainder of cases, this was performed in the left circumflex artery (LCX) (5.4%) and right coronary artery (RCA) (3.6%).

The mean qualitative % coronary stenosis (defined by the interventional cardiologist performing the procedure) did not differ between the CMD and control cohorts (left main stem (LMS) 3.7 ± 8.7% vs 4.7 ± 12.4%, p= 0.93; LAD 37.7 ± 22.9% vs 33.7 ±

18.5%, p= 0.17; LCX 13.0 ± 15.8% vs 13.8 ± 15.8%, p=0.89; RCA 17.4 ± 22.2% vs 13.1 ± 15.2%, p=0.57). The measurements of resting full-cycle ratio (RFR) and FFR did not differ between the CMD and control cohorts (0.92 ± 0.03 vs 0.93 ± 0.03, p=0.08 and 0.88 ± 0.05 vs 0.89 ± 0.05, p=0.83 respectively). Indices of microvascular coronary function were significantly different between the groups, as expected given the nature of the study design. The CMD cohort had mean reductions in CFR (2.5 ± 1.3 vs 5.2 ± 2.5, p<0.001) and elevations in IMR (28.4 ± 11.8 vs 13.7 ± 5.0, p<0.001).

Baseline blood results demonstrated significant differences between the CMD and control cohorts in NT-proBNP (910 ± 3001 ng/L vs 199 ± 291 ng/L, respectively; p=0.01), triglycerides (1.65 ± 1.51 mmol/L vs 1.79 ± 0.88 mmol/L, respectively; p=0.046) and high density lipoprotein (1.32 ± 0.34 mmol/L vs 1.19 ± 0.31 mmol/L, respectively; 0.04).

Conjunctival microvascular hemodynamics

Hemodynamic parameters were obtained from a total of 2295 conjunctival vessels across all 111 subjects. A mean of 22.6 ± 13.2 vessels (3.1 ± 2.7 arterioles, 16.5 ± 10.9 venules and 3.1 ± 3.6 undifferentiated vessels) were analysed in the CMD cohort and 20.0 ± 12.5 (3.2 ± 3.9 arterioles, 13.8 ± 10.7 venules and 3.0 ± 3.2 venules) in the control cohort (p=0.18).

Table 2 demonstrates a comparison of measured conjunctival microcirculatory parameters in CMD and control cohorts across all analysed vessels. Mean diameter did not differ between the groups. V_a, V_{cs}, WSR and WSS were all significantly lower in the CMD cohort. Q was numerically lower in the CMD cohort and the difference approached statistical significance (p=0.06).

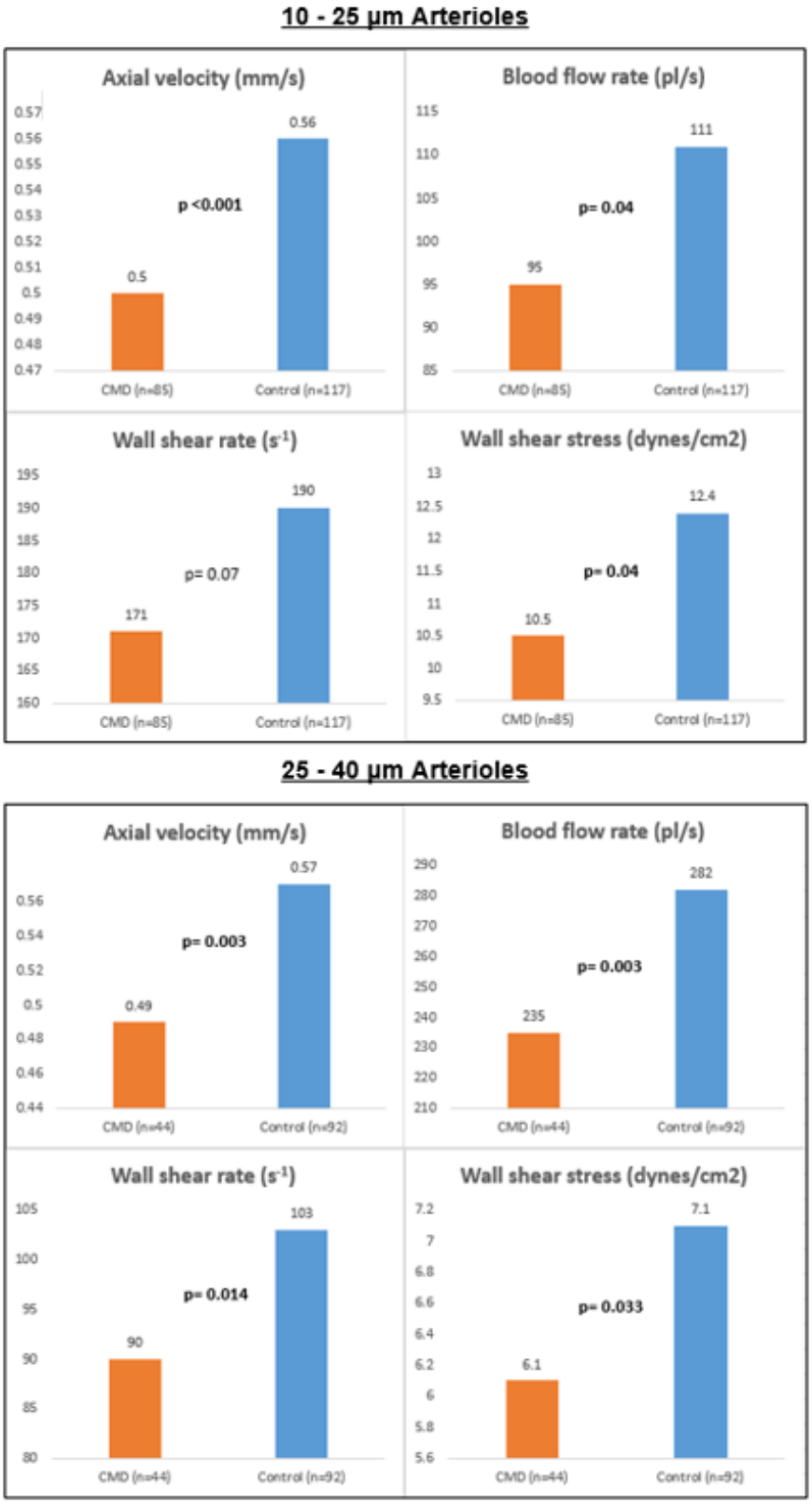
Table 2. Comparison of conjunctival microcirculatory parameters in all vessels

Parameter	CMD (n=975)	Control (n=1320)	p-value
Diameter- *µm* ± *SD*	24.9 ± 8.4	24.8 ± 7.9	0.88
Axial velocity- *mm/s* ± *SD*	0.52 ± 0.15	0.55 ± 0.14	**<0.001**
Cross-sectional velocity- *mm/s* ± *SD*	0.36 ± 0.10	0.38 ± 0.10	**<0.001**
Blood flow rate- *pl/s* ± *SD*	193.6 ± 132.8	200.5 ± 131.4	0.06
Wall shear rate- s^{-1} ± *SD*	136.4 ± 75.5	142.3 ± 74.6	**0.03**
Wall shear stress- *dynes/cm*^{2} ± *SD*	8.8 ± 4.5	9.6 ± 5.0	**<0.001**

Significant reductions in V_a (0.53 ± 0.15 mm/s vs 0.55 ± 0.14 mm/s, p= 0.01), V_{cs} (0.37 ± 0.10 vs 0.38 ± 0.10 mm/s, p= 0.009) and WSS (8.6 ± 4.4 dynes/cm^2 vs 9.2 ± 4.9 dynes/cm^2, p= 0.01), but not Q or WSR were observed in venules in the CMD cohort. A full list of results can be found in **Table S2** in the supplementary appendix.

The number of arterioles per patient in which results were obtained was lower than venules (354 vs 1605), but the most marked numerical hemodynamic differences were observed in this vessel type. In the CMD cohort reductions were observed in V_a (0.50 ± 0.14 mm/s vs 0.56 ± 0.13 mm/s, p <0.001), V_{cs} (0.36 ± 0.10 mm/s vs 0.39 ± 0.09 mm/s, p <0.001) and Q (137.7 ± 96.9 pl/s vs 180.3 ± 116.9 pl/s, p <0.001). WSR (155.4 ± 89.8 s^{-1} vs 160.4 ± 85.5 s^{-1}, p= 0.40) and WSS (9.8 ± 5.2 dynes/cm^2 vs 10.5 ± 5.8 dynes/cm^2, p= 0.30) did not differ, however WSR and WSS are inversely related to vessel diameter. Vessel diameter in isolation is not a marker of microvascular function; instead, being predominantly influenced by the field of imaging, vessel selection and the height and weight of the subject. To overcome this difference and measure differences in comparable vessels, arterioles were further analysed in two distinct diameter groups. These groups were selected as described in the methods. In both 10 - 25 µm and 25 – 40 µm arterioles, reductions were observed in all measured microcirculatory parameters in the CMD cohort (**Figure 3**).

Figure 3. Comparison of conjunctival arteriolar microcirculatory parameters divided by diameter

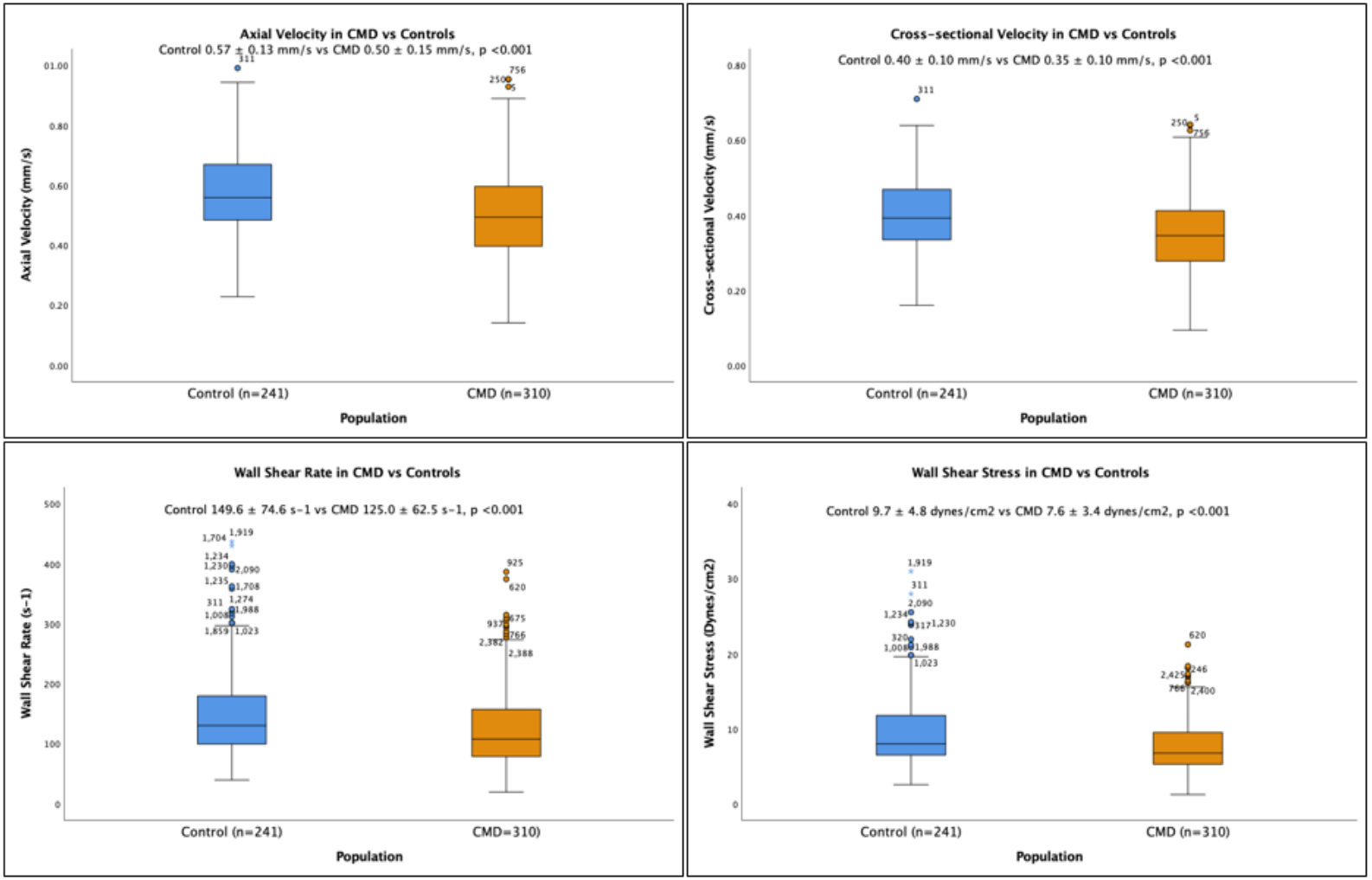


Baseline co-morbidities and pharmacological therapies

To evaluate the impact of potentially confounding medical conditions on microvascular hemodynamics, we performed a comparative analysis of the CMD and control cohorts, excluding subjects with a past medical history of percutaneous coronary intervention, myocardial infarction, diabetes mellitus or systemic hypertension. This enabled subjects with isolated CMD to be compared to healthy controls with no significant co-morbidities associated with conventional cardiovascular (CV) risk and the development of atherosclerosis. A total of 13/43 subjects in the CMD cohort and 16/68 subjects in the control cohort fulfilled this inclusion criteria for analysis. In the CMD cohort there were 310 analysable conjunctival vessels (50 arterioles, 221 venules and 39 undifferentiated vessels). In the Control cohort there were 241 analysable conjunctival vessels (37 arterioles, 163 venules and 41 undifferentiated vessels).

A comparison of all vessels demonstrated significant reductions in the CMD cohort in V_a (0.50 ± 0.15 mm/s vs 0.57 ± 0.13 mm/s, p <0.001), V_{cs} (0.35 ± 0.10 mm/s vs 0.40 ± 0.10 mm/s, p <0.001), WSR (125.0 ± 62.5 s^{-1} vs 149.6 ± 74.6 s^{-1}, p <0.001) or WSS (7.6 ± 3.4 dynes/cm^2 vs 9.7 ± 4.8 dynes/cm^2, p <0.001). Q was numerically lower in the CMD cohort, but this did not reach statistical significance (184.7 ± 117.9 pl/s vs 197.0 ± 121.2 pl/s, p=0.19) (**Figure 4**).

Figure 4. Boxplots comparing conjunctival hemodynamics in all vessels in subjects without established coronary artery disease, diabetes mellitus, systemic hypertension and hypercholesterolaemia



In this sub-population of patients with no confounding co-morbidities, similar hemodynamic differences were observed in both arteriole and venule sub-groups. In the CMD cohort reductions in arteriole V_a, V_{cs}, and Q; and venule V_a, V_{cs}, WSR and WSS were demonstrated (**Table 3**).

Table 3. Comparison of conjunctival hemodynamics in arterioles and venules (excluding subjects with a previous history of PCI, MI, diabetes mellitus or systemic hypertension)

Arterioles			
Parameter	**CMD (n=50)**	**Control (n=37)**	**p-value**
Diameter- *μm ± SD*	21.4 ± 6.8	22.8 ± 7.4	0.36
Axial velocity- *mm/s ± SD*	0.48 ± 0.12	0.56 ± 0.14	**0.002**
Cross-sectional velocity- *mm/s ± SD*	0.34 ± 0.09	0.40 ± 0.10	**0.004**
Blood flow rate- *pl/s ± SD*	129.9 ± 94.8	169.5 ± 100.9	**0.03**
Wall shear rate- s^{-1} ± *SD*	144.6 ± 78.4	161.7 ± 88.5	0.25
Wall shear stress- *dynes/cm²* ± *SD*	8.2 ± 3.8	10.4 ± 5.5	0.06
Venules			
Parameter	**CMD (n=221)**	**Control (n=163)**	**p-value**
Diameter- *μm ± SD*	26.2 ± 7.6	24.4 ± 7.4	**0.02**
Axial velocity- *mm/s ± SD*	0.51 ± 0.15	0.57 ± 0.13	**<0.001**
Cross-sectional velocity- *mm/s ± SD*	0.35 ± 0.11	0.40 ± 0.10	**<0.001**
Blood flow rate- *pl/s ± SD*	201.7 ± 121.1	200.2 ± 124.6	0.88
Wall shear rate- s^{-1} ± *SD*	120.8 ± 59.8	148.4 ± 74.3	**<0.001**
Wall shear stress- *dynes/cm²* ± *SD*	7.4 ± 3.3	9.6 ± 4.6	**<0.001**

These findings coupled with the lack of significant differences in baseline co-morbidities between CMD and control groups suggests that the differences observed in conjunctival hemodynamics in the CMD cohort are independent of these conventional CV risk factors for the development of atherosclerosis.

A comparison of baseline pharmacological therapies at the time of conjunctival vascular imaging is shown in **Table 4**. The only difference observed was a more prevalent use of angiotensin-2 receptor blockers (ARBs) in the CMD cohort. A comparison of conjunctival hemodynamics in patients taking ARBs vs ARB naïve participants revealed no differences in the conjunctival parameters of diameter (23.7 ± 2.4 vs 24.6 ± 3.3, p=0.22), V_a (0.54 ± 0.05 mm/s vs 0.54 ± 0.06 mm/s, p=0.93), V_{cs} (0.38 ± 0.03 mm/s vs 0.38 ± 0.04 mm/s, p=0.96), Q (181.3 ± 37.9 pl/s vs 193.6 ± 49.2 pl/s, p=0.36), WSR (141.9 ± 16.6 s^{-1} vs 141.9 ± 30.2 s^{-1}, p =0.78) or WSS (8.6 ± 1.8 dynes/cm^2 vs 9.3 ± 2.5 dynes/cm^2, p=0.28). Only 13.5% of the total number of patients in this study were on regular ARBs. This difference is therefore unlikely to impact or confound the results or conclusions that can be drawn from this study.

Hemodynamics were not influenced by the field of imaging (nasal vs temporal) or the eye that was imaged (right vs left). This was evaluated by comparing mean V_{cs} in the control cohort separated by the field of conjunctiva that was imaged (Left nasal 0.40 ± 0.10 mm/s; left temporal 0.38 ± 0.10 mm/s; right nasal 0.38 ± 0.09 mm/s; right temporal 0.38 ± 0.09 mm/s; p= 0.10). The hand dominance of the subject did not significantly impact mean V_{cs} in either the right (right dominant 0.38 ± 0.09 mm/s vs left dominant 0.38 ± 0.09 mm/s; p= 0.98) or left (right dominant 0.39 ± 0.10 mm/s vs left dominant 0.40 ± 0.12 mm/s; p=0.47) eyes.

Table 4. Comparison of baseline pharmacological therapies between groups

Medication	CMD (n=43)	Control (n=68)	p-value
Antiplatelet- *n (%)*			
• Aspirin	29 (67.4)	41 (60.3)	0.45
• P2Y12 inhibitor	11 (25.6)	20 (29.4)	0.66
Anti-hypertensive- *n (%)*			
• ACE inhibitor	20 (46.5)	29 (42.6)	0.69
• Angiotensin-2 receptor blocker	10 (23.3)	5 (7.4)	**0.02**
• Mineralocorticoid receptor antagonist	1 (2.3)	1 (1.5)	1.0
• Calcium channel blocker	14 (32.6)	15 (22.1)	0.22
• Thiazide diuretic	5 (11.6)	5 (7.4)	0.51
SGLT-2 inhibitor- *n (%)*	7 (16.3)	4 (5.9)	0.10
Anti-anginal- *n (%)*			
• Beta blocker	31 (72.1)	41 (60.3)	0.21
• Ranolazine	8 (18.6)	5 (7.4)	0.07
• Nicorandil	4 (9.3)	3 (4.4)	0.43
• Long-acting nitrate	18 (41.9)	25 (36.8)	0.59
Statin- *n (%)*	37 (86.0)	55 (80.9)	0.48

DISCUSSION

This study demonstrates significant differences in parameters of conjunctival microcirculatory function in patients with CMD in comparison to an age and sex-matched group of controls. The findings suggest that the physiological changes involved in this sub-type of INOCA are associated with systemic microvascular dysfunction. To the best of our knowledge this is the first study to demonstrate a correlation with CMD and systemic microvascular dysfunction detected non-invasively in an alternative vascular network.

The elevations in IMR and reductions in CFR that are observed in CMD occur due to reductions in coronary blood flow velocity and rate. This is the result of structural and/or functional obstruction at a microvascular level. The findings of this study highlight that similar reductions in V_a, V_{cs} and Q can be observed in the conjunctival microcirculation in patients with CMD. The physiological differences were most pronounced in conjunctival arterioles, mirroring the site of pathophysiological changes observed in CMD.

Previous studies suggest that both low and high WSS are associated with atherosclerotic coronary artery disease. High WSS is associated with apoptosis of smooth muscle cells that might develop necrotic core progression and enhance plaque vulnerability (37). Endothelial cells exposed to low WSS are activated, displaying a pro-inflammatory state (38). Low WSS has therefore been associated with atherosclerotic plaque development and hence both early and advanced

coronary atherosclerosis (39). This study found reductions in conjunctival vessel WSS in a CMD cohort. These changes were demonstrated in all conjunctival vessels, but similar to V_a, V_{cs} and Q were most evident in arterioles.

The potential clinical utility for non-invasive vascular imaging to diagnose microvascular disease is two-fold. Firstly, the gold standard for the diagnosis of CMD involves invasive coronary angiography, thereby exposing the patient to a variety of potentially serious procedural risks. A diagnostic algorithm for CMD that incorporates the non-invasive demonstration of systemic microvascular dysfunction could, theoretically in combination with typical symptoms and non-obstructive epicardial CAD detected on computed tomographic coronary angiography (CTCA), replace the need for invasive angiography and coronary function testing. This hypothesis would need to be validated in future prospective studies evaluating the technique as a diagnostic tool for CMD.

Secondly, the demonstration of microvascular dysfunction may have a role in CV risk stratification and primary prevention. The underlying mechanisms involved in the development of atherosclerotic vascular disease can be observed earliest in the microcirculation of affected vascular beds (40). The presence of CMD has been shown to confer an adverse long-term CV prognosis (41, 42, 43, 44). This was highlighted in a recent large meta-analysis of 79 studies involving 59,740 patients. This study demonstrated that the presence of CMD, as evidenced by a reduction in CFR (multiple modalities of measurement across the included studies) was strongly associated with an increased risk of all-cause mortality (HR: 3.78, 95% CI: 2.39 –

5.97) and major adverse cardiovascular event (MACE) (HR 3.42, 95% CI: 2.92 – 3.99) (41). In this meta-analysis each 0.1 unit reduction in CFR was associated with a proportional increase in both mortality and MACE. The adverse prognosis was observed in patients with isolated CMD in addition to those with co-existent and potentially contributory pathologies such as acute coronary syndrome, previous cardiac transplant and diabetes mellitus. These findings highlight the potential value in utilising microvascular hemodynamics to identify individuals at an elevated CV risk and hence target vascular risk factor modification more aggressively.

Conventional CV risk stratification tools typically identify the majority of individuals as low-intermediate risk. The ability to detect systemic microvascular dysfunction therefore has potential clinical utility in enhancing CV risk assessment. Similar to CT coronary calcium scoring, this may allow appropriate CV risk re-categorisation and hence targeted primary preventative lifestyle and pharmacological recommendations. Conjunctival vascular imaging is advantageous as it is easy to perform, with limited expertise required for image acquisition and does not involve exposure of the patient to ionising radiation. Future research would be required to establish the prognostic benefit of conjunctival vascular screening and the ability to correlate to intermediate and long-term CV risk. Importantly the between group differences observed in this study are numerically small, and if conjunctival vascular imaging was to be clinically utilised a normal reference range would need to be established and overall sensitivity and specificity of the test validated.

LIMITATIONS

In this study coronary microvascular function testing did not incorporate coronary vasoreactivity testing to diagnose vasospastic angina. Therefore, a small number of patients in both the CMD and control cohorts may in fact have had this INOCA sub-type. A small minority of subjects had physiological evaluation of either the RCA or LCX, vessels in which, evaluation of microvascular function is less well validated. Whilst coronary physiology was heavily utilised to define coronary disease in this study, the definition of intermediate to severe coronary stenoses was still based on the subjective assessment of stenoses severity, which is less accurate than quantitative coronary angiography (QCA).

The utilised method of blood flow velocity measurement presumes constant velocity, and therefore does not account for the pulsatile nature of blood flow in arterioles.

Patients in the CMD cohort had evidence of coronary microvascular dysfunction during pharmacological stress, however conjunctival microvascular measurements were made at rest. Therefore, whilst differences between groups were observed the coronary and conjunctival microvasculature were assessed during different physiological conditions. However, one would hypothesis that similar to the coronary circulation, patients with systemic microvascular dysfunction will have a more pronounced reduction in blood flow velocity and rate during stress than at rest.

Given the nature of this study, a proportion of subjects had potentially confounding medical co-morbidities in addition to regular pharmacological therapies known to impact systemic microvascular function. Whilst we acknowledge this as a limitation, analysis of the impact of both co-morbidities (established coronary artery disease, diabetes mellitus, hypertension and hypercholesterolaemia) and medication use revealed no significant association with conjunctival microvascular parameters. There was also no difference in the prevalence of baseline co-morbidities between CMD and control cohorts.

CONCLUSION

This study demonstrates the presence of hemodynamic changes in the conjunctival microcirculation of patients with CMD that are consistent with systemic microvascular dysfunction in this population. The findings support the hypothesis that the microvascular changes in CMD are not limited to the coronary circulation. The potential clinical utilities of conjunctival vascular imaging lie both in the diagnosis of CMD and in the augmentation of conventional CV risk assessment. Future research is required to both validate this observation and importantly establish a threshold of abnormality for the various measured conjunctival hemodynamic parameters.

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