RETINAL AND SYSTEMIC VASCULAR FUNCTION IN HEALTH AND DISEASE: THE EFFECT OF SMOKING AND CORONARY ARTERY DISEASE

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Summary

The impact of coronary artery disease (CAD) and its systemic vascular implications have been extensively researched. However, the effect upon the ocular circulation and visual function remained largely unknown. The purpose of the following studies was to explore the effect of systemic vascular and endothelial dysfunction upon the ocular circulation and functionality of the retina. Furthermore, the relationship between different vascular beds and its markers were explored. There are 6 principal sections to the present work:

Retinal vessel reactivity in smokers and non-smokers:

The principal findings of this work were:
- Chronic smoking affects retinal vessel motion at baseline and during stimulation with flickering light
- Chronic smoking leads to a vaso-constrictory shift in retinal arteriolar reactivity to flicker
- Retinal arteriolar elasticity is decreased in chronic smokers

The effect of acute smoking on retinal vessel dynamics in smokers and non-smokers:

- The principal finding of this work was that retinal reactivity in chronic smokers is blunted when exposed to flicker light provocation immediatly after smoking one cigarette

Ocular blood flow in coronary artery disease:

The principal findings of this work were:
- Retrobulbar and retinal blood flow is preserved in CAD patients, despite a change pulse wave transmission
- Arterial retinal response to flickering light provocation is significantly delayed in CAD patients
- Retinal venular diameters are significantly dilated in CAD patients

Autonomic nervous system function and peripheral circulation in CAD:

The principal findings of this work were:
- CAD patients demonstrate a sympathetic overdrive during a 24hr period
- A delay in peripheral vascular reactivity (nail-fold capillaries) as observed in patients suffering from CAD could be caused by either arteriosclerotic changes of the vascular walls or due to systemic haemodynamic changes

Visual function in CAD:

The principal findings of this work were:
- Overall visual function in CAD patients is preserved, despite a decrease in contrast sensitivity
- Applying a filtering technique selecting those with greater coefficient of variance values which in turn represents a decrease in reliability, some patients appear to have an impaired visual function as assessed using FDT visual field evaluation

Multiple functional, structural and biochemical vascular endothelial dysfunctions in patients suffering from CAD:

relationships and possible implications:

The principal findings of this work were:
- BMI significantly correlated with vWF (a marker of endothelial function) in CAD patients
- Retinal vascular reactivity showed a significant correlation with peripheral reactivity parameters in controls which lacked in the CAD group and could reflect a loss in vascular endothelial integrity
- Visual field parameters as assessed by frequency doubling technology were strongly related with systemic vascular elasticity (ambulatory arterial stiffness index) in controls but not CAD patients, a finding that further supports the hypothesis of multilevel endothelial damage in CAD patients contributing to the developement of impaired visual function

Keywords: Ocular blood flow, coronary artery disease, retinal vessel reactivity, capillary microscopy, endothelial function

Contents

1 Introduction **20**

1.1 Background 20

1.2 Ocular anatomy and physiology 21

1.2.1 Retrobulbar circulation 21

1.2.2 Retinal circulation 23

1.2.3 Venous drainage 24

1.2.4 Ocular perfusion pressure 25

1.2.5 Ocular blood flow and haemodynamic regulation of the retina 25

1.2.5.1 Metabolic mechanisms 26

1.2.5.2 The vasospastic syndrome 28

1.3 Techniques for assessment of ocular blood flow parameters 28

1.3.1 Colour Doppler imaging 28

1.3.2 Scanning laser Doppler flowmetry 31

1.3.3 Static retinal vessel analysis 32

1.3.3.1 Assessment of the arterio venous ratio (AVR) with the software Visualis (Imedos GmbH, Germany) . 32

1.3.3.2 Dynamic retinal vessel analysis 32

1.3.3.3 Novel analysis of retinal vascular dynamic parameters 40

1.4 Peripheral circulation 40

1.4.1 Cutaneous blood flow 40

1.4.2 Anatomy and physiology 40

1.4.3 Current techniques for evaluating peripheral blood flow . . . 43

1.4.3.1 Measurement of micro vascular perfusion 43

1.4.3.2 Nail fold Capillaroscopy 43

1.4.3.3 Peripheral vasospasm and ocular blood flow . . . 46

1.5 Techniques to assess visual function 46

1.5.1 Colour vision assessment 46

1.5.2 Visual field assessment 47

1.5.2.1 Flicker Perimetry 47

1.5.2.2 Frequency Doubling Perimetry 49

1.5.3 Contrast sensitivity assessment 49

1.6 Coronary circulation 50
1.6.1 Heart anatomy 50
1.6.1.1 Coronary arteries 51
1.6.1.2 Coronary veins 51
1.6.1.3 Coronary Blood Flow 52
1.6.2 Cardiac physiology 53
1.6.2.1 The cardiac cycle 53
1.6.2.2 Regulation of cardiac rhythm 53
1.6.3 The electrocardiogram: 54
1.6.3.1 Sequence of cardiac activation 54
1.6.3.2 Electrocardiogram analysis 54
1.6.4 Heart rate variability 55
1.6.4.1 Heart rate variability in cardiovascular disease . . 56
1.6.4.2 Heart rate variability in ocular disease 56
1.6.5 Coronary disease - Atherosclerosis 56
1.6.5.1 Systemic implications of arteriosclerosis 56
1.6.5.2 Risk factors and progression of arteriosclerosis . 57
1.6.5.3 Ocular implications of arteriosclerosis 57
1.6.6 Cardiac ischaemia 59
1.6.6.1 Chronic 59
1.6.6.2 Acute ischaemic heart disease/ Unstable angina . 59
1.6.6.3 Angina pectoris 59
1.6.6.4 Myocardial Infarction 60
1.6.7 Stable angina 60
1.6.8 Cardiac Syndrome X (CSX) 60
1.7 Systemic circulation 61
1.7.1 Blood pressure and formulas 61
1.7.1.1 Regulation of systemic blood pressure 62
1.7.1.2 Baroreceptor reflex 63
1.7.1.3 Circardian variation in systemic blood pressure . . 64
1.7.2 Techniques for assessment of systemic circulatory parameters: Ambulatory blood pressure monitoring 66
1.7.2.1 Cardio Tens: Ambulatory Blood Pressure and ECG monitor 66
1.7.2.2 Ambulatory Arterial Stiffness Index (AASI) 66
1.7.3 Heart rate variability analysis 67
1.8 Biomedical studies 67
1.8.1 Background 67
1.8.2 Vascular endothelium 68
1.8.3 Normal endothelial function 68

1.8.4 Endothelial dysfunction 68
1.8.5 Circulating markers of endothelial function 69
1.8.5.1 Von Willebrandt factor 69
1.8.5.2 Endothelin-1 70
1.8.6 Techniques for assessing circulating markers of endothelial function 71
1.8.6.1 Measurement of plasma vWf levels 71
1.9 Statistical analysis 72
1.9.1 Sample size calculation 73

2 Chronic Smoking **75**
2.1 Abstract 75
2.2 Introduction 76
2.3 Hypothesis 76
2.4 Aims 77
2.5 Subjects and methods 77
2.5.1 Recruitment of healthy individuals 77
2.5.1.1 Inclusion criteria 77
2.5.1.2 Exclusion criteria 77
2.5.2 Ethical approval 78
2.5.3 Experimental protocol 78
2.5.3.1 Intraocular pressure measurement 78
2.5.3.2 Blood pressure and pulse assessment 78
2.5.3.3 Assessment of retinal vessel dynamics 78
2.5.4 Statistical Analysis 80
2.6 Results 80
2.6.1 Sample 80
2.6.2 Non-smoking subjects 81
2.6.2.1 The effect of age on measurement variability in non-smokers 81
2.6.2.2 The effect of baseline diameter before flicker stimulation on the amount of vascular dilation after flickering 81
2.6.3 Chronic smokers 83
2.6.3.1 The effect of age on measurement variability in smokers 83
2.6.3.2 The effect of baseline diameter before flicker stimulation on the amount of vascular dilation after flickering: 84
2.6.4 Smokers versus non-smokers 84
2.6.4.1 Retinal vascular stiffness 88

2.7 Discussion 88
2.7.1 Main findings 88
2.7.2 The effect of age on the retinal vascular reactivity 89
2.7.3 The effect of baseline diameter and baseline diameter fluctuation on retinal vascular reactivity to flickering light stimulation in chronic smoking 90
2.7.4 The effect of chronic smoking 90
2.7.5 Vascular effects: systemic and ocular 90
2.7.6 Conclusion 92

3 Acute Smoking **93**
3.1 Abstract 93
3.2 Introduction 94
3.3 Hypothesis 95
3.4 Aims 95
3.5 Subjects and methods 95
3.5.1 Recruitment of healthy subjects 95
3.5.1.1 Inclusion criteria 95
3.5.1.2 Exclusion criteria 95
3.5.2 Ethical approval 96
3.5.3 Experimental protocol 96
3.5.3.1 Intraocular Pressure 96
3.5.3.2 Systemic blood pressure 96
3.5.3.3 Retinal vessel analysis 97
3.5.3.4 Smoking 97
3.5.4 Statistical analysis 97
3.6 Results 97
3.6.1 Sample 97
3.6.2 The effect of acute smoking: chronic smokers vs non-smokers 98
3.6.2.1 The effect on systemic BP, IOP and vascular diameter 98
3.6.2.2 The effect of retinal vessels function 99
3.7 Discussion 100
3.7.1 Main findings 100
3.7.2 The effect of acute smoking 102
3.7.3 Conclusion 103
3.7.4 Limitations 103

4 Ocular blood flow parameters in coronary artery disease **104**
4.1 Abstract 104
4.2 Introduction 105

4.3 Hypothesis . . . 106
4.4 Aims . . . 106
4.5 Subjects and methods . . . 106
4.5.1 Patient recruitment . . . 106
4.5.2 Inclusion criteria . . . 106
4.5.3 Exclusion criteria . . . 107
4.5.4 Ethical approval . . . 107
4.5.5 Experimental protocol . . . 107
4.5.5.1 Intra-ocular Pressure (IOP) . . . 108
4.5.5.2 Systemic Blood Pressure . . . 108
4.5.5.3 Ocular blood flow assessments . . . 109
4.5.6 Statistical Analysis . . . 111
4.6 Results . . . 111
4.6.1 Sample . . . 111
4.6.1.1 General characteristics of the study groups . . . 111
4.6.2 Ocular blood flow assessments . . . 111
4.6.2.1 Retrobulbar blood flow velocities . . . 111
4.6.2.2 Peripapillary retinal perfusion . . . 114
4.6.3 Retinal vessel reactivity . . . 115
4.6.3.1 Diameter Response . . . 115
4.6.3.2 Time course to reach max dilation (RT) . . . 116
4.6.3.3 Slope . . . 116
4.6.3.4 Arterio-venous coupling . . . 119
4.6.4 Static retinal image analysis . . . 120
4.6.5 Relationship between static and dynamic vascular variables 120
4.7 Discussion . . . 123
4.7.1 Main findings . . . 123
4.7.2 The effect of coronary artery disease on ocular blood flow at all levels of its vascular tree . . . 123
4.7.2.1 Retrobular blood flow alterations . . . 123
4.7.2.2 Retinal blood flow . . . 124
4.7.2.3 Retinal vascular reactivity . . . 124
4.7.2.4 Structural changes . . . 125
4.7.2.5 The relationship between structural and functional parameters of retinal vessels . . . 125
4.7.3 Conclusion . . . 126
4.7.4 Limitations . . . 126

5 Autonomic Nervous Stystem and peripheral circulation in Coronary Artery Disease 127
5.1 Abstract . . . 127

5.2 Introduction 128
5.3 Hypothesis 129
5.4 Aims 129
5.5 Subjects and methods 130
5.5.1 Patient recruitment 130
5.5.1.1 Study's inclusion criteria 130
5.5.1.2 Study's exclusion criteria 130
5.5.2 Recruitment of healthy individuals 131
5.5.3 Ethical approval 131
5.5.4 Experimental protocol 131
5.5.4.1 Patient preparation 131
5.5.4.2 Ambulatory BP and ECG monitoring 131
5.5.4.3 Peripheral blood flow 132
5.5.5 Statistical analysis 134
5.6 Results 134
5.6.1 Sample 134
5.6.2 Systemic blood pressure 134
5.6.2.1 Ambulatory arterial stiffness index 135
5.6.3 Heart rate variability 136
5.6.4 Peripheral blood flow assessment 139
5.6.5 Correlations 139
5.7 Discussion 141
5.7.1 Main findings 141
5.7.2 Circadian blood pressure and arterial stiffness 141
5.7.3 Autonomic function 141
5.7.4 Peripheral blood flow 142
5.7.5 The effect of cardiac rehabilitation on endothelial and ANS function in CAD 142
5.7.6 Conclusion 143
5.7.7 Limitations 143

6 Visual function in CAD **145**
6.1 Abstract 145
6.2 Introduction 146
6.3 Hypothesis 146
6.4 Aim 146
6.5 Subjects and recruitment 147
6.5.1 Patient recruitment 147
6.5.1.1 Inclusion criteria 147
6.5.1.2 Exclusion criteria 147
6.5.2 Ethical approval 148

6.5.3 Experimental protocol 148
6.5.3.1 Colour vision assessment 148
6.5.3.2 Contrast sensitivity assessment 148
6.5.3.3 Visual Field assessment 148
6.5.4 Statistical Analysis 149
6.6 Results 149
6.6.1 Sample 149
6.6.2 Contrast sensitivity 150
6.6.3 Visual field assessment 150
6.6.3.1 Frequency doubling technology 150
6.6.3.2 Flicker perimetry 151
6.6.3.3 Comparison: Frequency doubling technology vs flicker perimetry 153
6.6.3.4 Visual field variability 153
6.6.4 Visual field - sub group analysis 154
6.6.4.1 Frequency doubling technology 154
6.6.4.2 Flicker perimetry 156
6.7 Discussion 157
6.7.1 Main findings 157
6.7.2 Contrast sensitivity 157
6.7.3 Visual fields 158
6.7.4 Conclusion 158
6.7.5 Limitations 158

7 Coronary Artery Disease: Multi level endothelial dysfunction 160
7.1 Abstract 160
7.2 Introduction 161
7.3 Hypothesis 162
7.4 Aims 162
7.5 Subjects and Methods 162
7.5.1 Patient recruitment 162
7.5.1.1 Inclusion criteria 162
7.5.1.2 Exclusion criteria 162
7.5.2 Ethical approval 163
7.5.3 Experimental protocol 163
7.5.3.1 Obtaining venous blood samples 164
7.5.3.2 Analysis for von Willebrandt factor 164
7.5.4 Statistical analysis 165
7.6 Results 165
7.6.1 Sample 165

7.6.2 Relationship between retinal and peripheral (nail-fold capillaries) vascular reactivity 165
7.6.3 Relationship between systemic blood pressure parameters and ocular circulation 168
7.6.4 Relationship between circulatory (ocular and systemic) parameters and visual function 168
7.6.5 Circulating marker for endothelial function 169
7.7 Discussion 170
7.7.1 Main findings 170
7.7.2 Relationship between structurally and functionally different vascular beds 170
7.7.3 Vascular impairment/ stiffness and visual function 171
7.7.4 Conclusion 172

8 Summary and Conclusions **173**
8.1 Summary 173
8.1.1 The effect of chronic smoking in healthy individuals 173
8.1.2 The acute and chronic effect of smoking on vascular reactivity 174
8.1.3 Ocular Blood Flow and Retinal vessel reactivity in patients suffering CAD compared to healthy controls 175
8.1.4 Visual function in CAD 176
8.1.5 Multiple functional, structural and biochemical vascular endothelial dysfunctions in patients suffering from CAD: relationships and possible implications 176
8.2 Conclusions 177
8.2.1 To investigate the novel analysis techniques for the assessment of retinal vascular dynamics and the impact of age upon the analysed parameters 177
8.2.2 To assess the effect of smoking (chronic and acute) upon retinal vascular reactivity in healthy individuals 177
8.2.3 To assess systemic and ocular vascular function in patients suffering from CAD 178
8.2.4 To assess visual function in CAD 178
8.2.5 To assess the relationship of functional, structural and biochemical vascular endothelial dysfunction in patients suffering from CAD 178
8.3 Implications for CAD patients arising from these results 179
8.4 Future areas of research arising from this work 179
8.4.1 The acute and chronic effect of fasting and dehydration upon retinal vessel reactivity and ocular blood flow 179
8.4.1.1 Publications 179

8.4.2 The effect of fundus pulsation and retinal reflectivity on the assessment of retinal vessel reactivity 180
8.4.3 Analysis of retinal vascular pulsation in health and cardiovascular disease 180
8.4.4 Cardiovascular Rehabilitation and its effect on ocular vascular parameters 180

Bibliography **182**

Appendix **214**

List of Figures

1.1 Retrobulbar blood vessels 22
1.2 Venous drainage of the eye, via vortex veins into the sinus cavernous. 24
1.3 Colour Doppler Imaging: spectral wave shapes 29
1.4 Arterio venous ratio 33
1.5 Rentinal Vessel Aanalyser: controlling and measurement components 34
1.6 Measurement protocol for dynamic retinal vessel assessment . . . 35
1.7 Retinal Vessel Analyser output display 36
1.8 Retinal Vessel Analyser recording sequence 38
1.9 Illustration of the retinal diameter response to flicker provocation . 39
1.10 Illustration of slope calculation of retinal blood vessels when stimulated with flickering light. 41
1.11 Skin section showing vascular plexi system of the outer skin 42
1.12 Nail-fold capillaries, aprox. 200 times magnified 44
1.13 Nail-fold capillary flow measurement 45
1.14 Heart section showing atria and ventricles 50
1.15 Vascular supply of the heart via coronary arteries 51
1.16 Mechanisms involved in the regulation of coronary blood flow . . . 52
1.17 Variation of cardiac and aortic pressure 54
1.18 Schemata showing atheroma development 57
1.19 Risk factors and progression of arteriosclerosis 58
1.20 Factors involved in the regulation of systemic blood pressure . . . 63
1.21 Normal nocturnal blood pressure dip (blood pressure drops up to 10% during sleep compared to wake period) 64
1.22 Abnormal nocturnal blood pressure dip: overdipper (blood pressure drops between 10% and 20% during sleep compared to wake period) 65
1.23 Absent nocturnal blood pressure dip: non-dipper (blood pressure does not change, or increase during sleep compared to wake period) 65

2.1 Measurement sequence of the retinal diameter assessment 79
2.2 Correlation of of arterial and venous baseline diameters 82

2.3 Correlation of the baseline diameter fluctiation and the dilation amplitude in retinal arteries 83
2.4 Correlations of arterial and venous baseline diameters 85
2.5 Correlation between baseline diameter fluctuation and dilation amplitude 85
2.6 Group mean differences of the arterial retinal reactivity parameters 87
2.7 Retinal arterial stiffness in smokers 89

3.1 Measurement sequence 96
3.2 Change in baseline diameter fluctuation after smoking 101
3.3 Comparison of arterial baseline diameter fluctuation and dilation amplitude: pre and post smoking 101

4.1 Spectral wave shapes of retrobulbar velocity measurements 109
4.2 Schematic of quadrant division of the peri-papillary retina; ONH: optic nerve head 110
4.3 Body mass index in CAD patients and controls 112
4.4 Retinal blood Flow: minimum and maximum volume in CAD patients and controls 114
4.5 Time course of retinal arterial reactivity to flicker stimulation 117
4.6 Graphical determination of retinal vascular slope 118
4.7 Arterial slopes in CAD patients compared to controls: a schematic outline 119
4.8 Comparison of retinal arterial and venous slopes 120
4.9 Comparison of static retinal vein assessment 121
4.10 Correlation between static and dynamic retinal parameters measured122

5.1 Measurement protocol of nail-fold capillary assessment 133
5.2 Arterial stiffness: Differences between non-dippers, dippers and over-dippers 137
5.3 Comparison of heart rate variability parameters 138
5.4 Difference in baseline capillary flow and recovery time between CAD patients and controls 139
5.5 Correlation between peripheral reactivity and large artery stiffness 140

6.1 Difference in contrast sensitivity between coronary artery disease patients and controls 150
6.2 Comparison of visual field parameters (inferior and superior mean sensitivity): frequency doupling technology 152
6.3 Visual field parameters (flicker perimetry): measurement reliability parameters 153

6.4 Between group comparison of measurement variability: flicker perimetry 155

7.1 Correlations between peripheral and retinal vascular parameters . 167
7.2 Correlaion between the systemic arterial stiffness and the retrobulbar blood flow velocity 168
7.3 Plasma vWF levels in CAD patients and controls 169
7.4 Correlation between body mass index (BMI) and von Willebrandt factor (vWF) in coronary artery disease patients 170

List of Tables

1.1 Overview of vaso-active substances involved in blood flow auto-regulation 27
1.2 Overview of the current methods to assess ocular blood flow . . . 29
1.3 Hypertension classifation 62
1.4 Blood pressure aquisition time 62

2.1 Group characteristics: non-smoker vs smoker 81
2.2 Effect of age on measurement variability 81
2.3 Arterial and venous baseline diameter (non-smokers) 82
2.4 Effect of age on the measurement variability 83
2.5 Arterial and venous baseline diameters (smokers) 84
2.6 Dynamic retinal parameter: arteries 86
2.7 Arterial and venous reaction time 87
2.8 Retinal venous diameter response 88

3.1 Group characteristics: pre and post-smoking 98
3.2 Pre- and post-smoking demographic data 99
3.3 Parameters of retinal vessel reactivity (arterial and venous) 100

4.1 Group characteristics: CAD patients and controls 112
4.2 Retrobulbar blood flow velocities in CAD patients and controls . . . 113
4.3 Retinal blood flow parameters in CAD patients and controls 114
4.4 Retinal arterial reactivity parameters 115
4.5 Retinal venous reactivity parameters 115
4.6 Time course of retinal arterial dilation 116
4.7 Time course of retinal venous dilation 117
4.8 Retinal arterial slope 118
4.9 Retinal venous slope 119
4.10 Correlation of arterial and venous slopes 120
4.11 Parameters of static retinal evaluation 121
4.12 Correlations between static and dynamic variables measured . . . 122

5.1 Group characteristics 134
5.2 Results for ambulatory blood pressure assessment 135

5.3 Subgroup analysis: Arterial stiffness 136
5.4 Heart rate variability parameters neasured in CAD patients and controls 137
5.5 Group differences in circardian changes of heart rate variability parameters 138
5.6 Results of the nail-fold capillary assessment 139
5.7 Correlations between nail-fold capillaroscopy parameter and large artery arterial stiffness 140

6.1 Contrast sensitivity results for coronary artery disease patients and controls 150
6.2 Visual field parameters: frequency doubling technology 151
6.3 Visual field parameters: flicker perimetry 152
6.4 Correlation between flicker perimetry and frequency doubling technology 153
6.5 Within group comparison of the measurement variability of the inferior ans superior visual field for flicker perimetry and frequency doubling technology 154
6.6 Comparison of the measurement variability of visual field parameters: flicker perimetry and frequency doubling technology 155
6.7 Results for the visual field sub-group analysis: frequency doubling technology 156
6.8 Results for the flicker perimetry sub-group analysis 156

7.1 Group characteristics 166
7.2 Results for correlations between nail-fold capillaroscopy parameters 166
7.3 Correlation between AASI and visual function parameters 169

Abbreviations

AASI	Ambulatory arterial stiffness index
AII	Angiotensin II
ANS	Autonomic nervous system
APR	Arterial peak ratio
ATS	Arteriosclerosis
AU	Arbitrary units
AVR	Arterio venous ratio
BCF	Baseline capillary flow
BF	Blood flow
BFR	Baseline corrected flicker response
BMI	Body mass index
BP	Blood pressure
BV	Blood vessel
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CDI	Colour doppler imaging
CHD	Coronary heart disease
CRA	Central retinal artery
CRAE	Central retinal artery equivalent
CRAO	Central retinal artery occlusion
CRV	Central retinal vein
CRVE	Central retinal vein equivalent
CS	Contrast sensitivity
CSX	Cardiac syndrom X
CO	Cardiac output
CPD	Cycle per degree

CV	Coefficient of variance
CVD	Cardio vascular disease
DA	Dilation amplitude
DBP	Diastolic blood pressure
DD	Disc diameter
DM	Diabetes mellitus
ECG	Electrocardiogram
EDV	End diastolic velocity
ET	Endothelium
ET-1	Endothelin-1
FDT	Frequency doubling technology
FP	Flicker perimetry
HF	High frequency
HR	Heart rate
HRF	Heidelberg Retinal Flowmeter
HRV	Heart rate variability
HT	Hypertension
IMT	Intima media thickness
ISH	International Society of Hypertension
IOP	Intra ocular pressure
LF	Low frequency
MABP	Mean arterial blood pressure
MC	Maximum constriction
MD	Maximum dilation (in retinal vessel reactivity)
MD	Mean deviation (in visual fields)
NFL	Nerve fibre layer
NO	Nitric oxide

NU	Normalised units
OA	Ophthalmic artery
ONH	Optic nerve head
OPP	Ocular perfusion pressure
PAD	Peripheral arterial disease
PSNS	Parasympathetic nervous system
PSD	Pattern standard deviation
PSV	Peak systolic velocity
PVD	Peripheral vascular disease
RI	Resistivity index
RVA	Retinal vessel analyser
SA	Sinoartial node
SBP	Systolic blood pressure
SD	Standard deviation
SNS	Sympathetic nervous system
SWAP	Short wavelenght automated perimetry
TD	Total deviation
TPR	Total peripheral resistance
vWF	von Willebrand Factor
WHO	World Health Organisation

Statement of Authenticity This thesis represents the work of Rebekka Heitmar during a 3 year postgraduate research at School of Life and Health Sciences (Ophthalmic Research Group), Aston University, Birmingham (UK).

The author has no commercial interest in any of the equipment or laboratory methods used in this work.

Chapter 1

Introduction

The purpose of this thesis was to explore retinal vascular function and its relationship with systemic circulation in health and disease with particular focus on coronary artery disease.

1.1 Background

Cardiovascular disease (CVD) is the main cause of premature death in the UK (2005: British Heart Foundation, UK). Of the 216,000 deaths in 2005 caused by CVD, about half of them were attributable to coronary heart disease (CHD) [Petersen et al., 2005]. These statistics are not unique for the UK and are similar throughout the western world (such as USA, Europe and Australia).

CVD has a serious impact on the country's economy in terms of health care costs, premature death of the workforce, time off work due to illness and informal care of people with the disease. Health care costs totalled £3.5 million in 2003, hospital care accounted for 79% of those costs and about 16% for drugs [Petersen et al., 2005]. Whereas mortality from CHD is falling, morbidity and other circulatory disease are rising, especially in older age groups.

The most common underlying causes of heart disease are diabetes mellitus (DM), hypertension (HT) and arteriosclerosis (ATS). ATS is the most common causative factor of heart disease; its development is triggered by a number of factors, most of them related to lifestyle, such as smoking, diet, lack of exercise, and alcohol abuse [Dasch et al., 2005]. In addition to ATS, vasospasm due to vascular endothelial cell dysfunction is also an important risk factor associated with heart attacks and coronary artery disease (CAD) [Wong et al., 2001a, Wong, 2004, Wong et al., 2003a, 2002a, Karseras, 2000, Witt et al., 2006]. Both vasospasm and ATS have important consequences on vascular beds throughout the human body. Since the eye is integrated in the general circulation, it could be affected by these disturbances and various eye pathologies may occur in patients suffering from CAD. Indeed, diseases such as artery and vein occlusions, ischaemic

neuropathies and glaucoma are just a few examples of ocular pathologies occurring in patients suffering from CAD or at risk of developing cardiovascular diseases [Ahuja et al., 1999, Wilson et al., 1979]. In addition, the autonomic nervous system (ANS) disturbances associated with CAD have disastrous hemodynamic consequences, which can potentially manifest at the ocular level.

The ocular vasculature is very sensitive to changes in blood flow and any minor change can have a serious effect on visual function and ocular perfusion. As many eye diseases have a systemic vascular disturbance as an underlying cause, commonly a compromised vascular supply, it is very important to detect abnormalities in the ocular circulation as early as possible as they precede structural changes and could be used as either diagnostic or monitoring tool for both the systemic disease and its ocular consequences.

Endothelial dysfunction and high cholesterol levels result in metabolic impairment and local toxicity, resulting in circulatory disturbances in vessels of varying calibre including the ocular vessels [Senn et al., 1999]. The resulting tissue ischaemia can cause preclinical changes that may be present before the destructive consequences of various ocular diseases. These subtle changes may, in theory, be detected using various visual function tests such as colour vision and contrast sensitivity measurements. Alterations of the ocular vessels' wall and structure (conjunctival and retinal) can also occur and may be detected through observation using evaluation of fundus photographs and conjunctival images [Stubiger et al., 1997]. These changes may be predictive of future systemic and ocular pathology and their detection may help with the diagnosis and treatment of some vision-disabling conditions. Therefore the aim of the present research is to study changes in the retinal vascular function in health and disease with particular attention to coronary artery disease (CAD).

In order to understand the mechanism and relationship of these vascular systems one has to know the function and physiology of systemic, cardiac and ocular circulation. Identifying subtle changes in the vascular function could predict future damage in a population at risk. New technologies assessing retinal vessel dynamics (see below), can be used to determine abnormalities in the function of a vascular bed that is known to reflect pathologies in major organs such as the heart and the brain [Patton et al., 2005, 2006].

1.2 Ocular anatomy and physiology

1.2.1 Retrobulbar circulation

Ophthalmic artery (OA) The ophthalmic artery is the first branch of the internal carotid artery, supplying the eye and other structures in the orbit. Where the

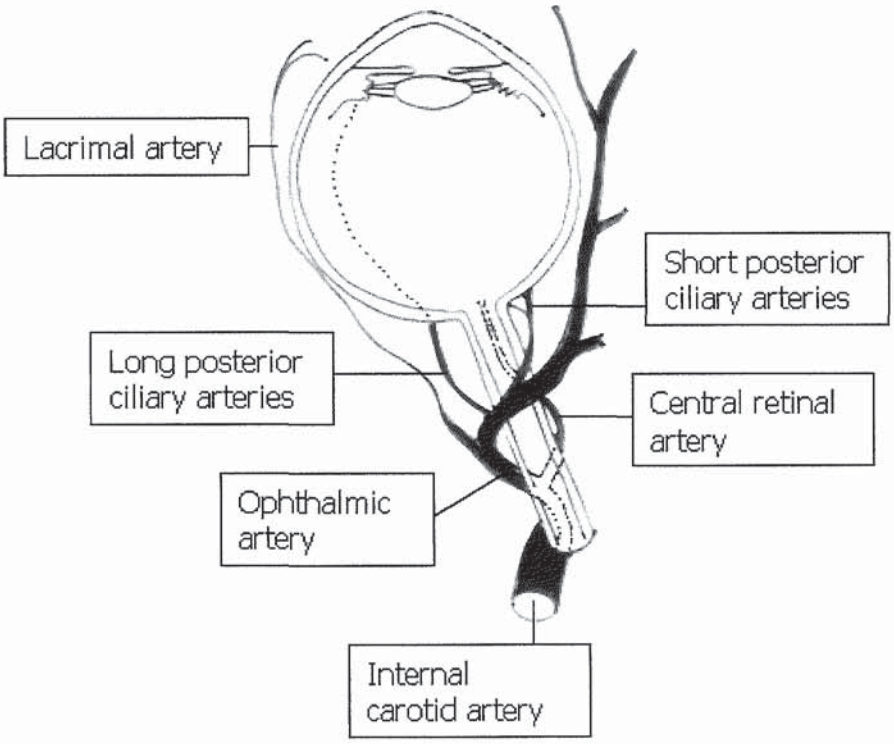

Figure 1.1: Retrobulbar blood vessels

ophthalmic artery crosses the ON, the central retinal artery and more distally the long and short posterior ciliary arteries branch off. Usually the OA passes over the ONH as it continues its way into the orbit, but in some individuals can pass below.

Central retinal artery (CRA) The inner retina is supplied from the branches of the central retinal artery, which derives from the ophthalmic artery about 4mm posterior to the ONH. The CRA enters the ONH centrally and divides to 2 main branches. These branches further divide to supply each quadrant of the eye, without overlap, with one artery. In about 20% of individuals there exists a cilioretinal artery which derives from the chroroidal circulation and supplies the macular area exclusively. This is of particular value in cases of central retinal artery occlusion (CRAO). If in such an event the patient has a cilioretinal artery his central vision might be preserved.

Posterior ciliary arteries (PCA) The posterior ciliary arteries, usually numbering between six and twelve, derive from the ophthalmic artery or its branches. They travel along the ONH towards the posterior part of the globe and pierce the sclera around the entrance of the ONH, supplying the nerve and the posterior choroidal vasculature.

1.2.2 Retinal circulation

The retina is the light sensitive layer lining the interior of the eye. The innermost part contains the photoreceptors (rods and cones) as well as their associated nerve fibres. Due to its high metabolic demands, the retina is nourished via two vascular beds: the outer retina receives its blood supply through the choriocapillaries (supplying 1/3 of the retina) and the inner retina through the retinal circulation (supplying the remaining 2/3 of the retina).

The major difference between the two vascular beds is the functionality of the vessels. The retinal vasculature is very similar to the cerebral vasculature; the blood vessel endothelium has tight junctions and no fenestrations and therefore maintains the blood-ocular barrier similar to the vessels in the cerebral circulation acting as the blood-brain barrier. The choroidal vasculature is characterised through its fenestrated blood vessels.

Choroidal circulation The choroidal vasculature is a thin capillary layer, characterized through its fenestrated vessels which are highly metabolic active. The choroid connects with the ciliary body toward the front of the eye and is attached to edges of the optic nerve at the back of the eye. Its main function is to nourish

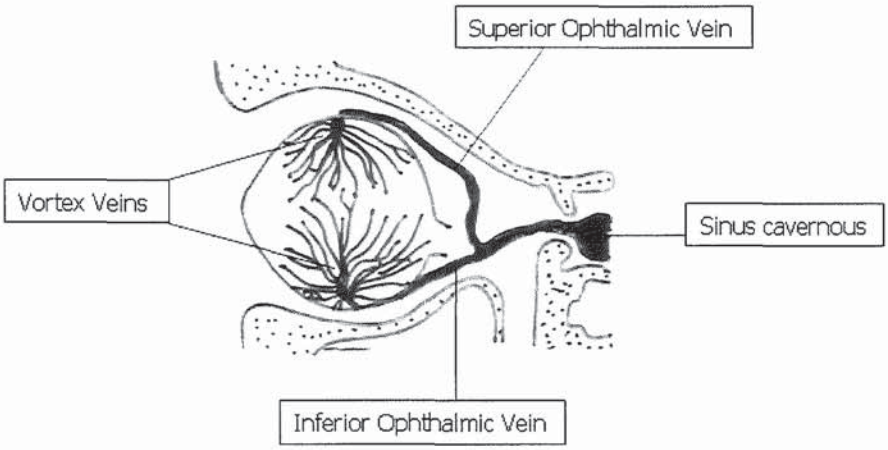

Figure 1.2: Venous drainage of the eye, via vortex veins into the sinus cavernous.

the outer layers of the retina as well as to absorb scattered light. The choroid is supplied by the ciliary arteries. The long posterior ciliary arteries and the anterior ciliary arteries nourish the iris and the sclera and conjunctiva respectively. The short posterior ciliary arteries are responsible for the blood supply to the posterior part of the choroid.

Structurally, the choroid is divided into four layers:

- Haller's layer: outermost layer of the choroid consisting of larger diameter blood vessels
- Sattler's layer: layer of medium diameter blood vessels
- Choriocapillaris: layer of capillaries
- Bruch's membrane: innermost layer of the choroid

1.2.3 Venous drainage

Venous drainage of the retina is maintained by the central retinal vein (CRV). Drainage of the nerve fibre layer (NFL) occurs through the retinal veins, later joining to form the CRV. In the pre-laminar, laminar and retro-laminar areas, drainage is provided either directly by the CRV or by axial tributaries of the CRV. The CRV then leaves the globe through the optic nerve head (ONH) parallel to the CRA, draining into the superior ophthalmic vein. In some individuals the CRV drains directly into the cavernous sinus.

Venous drainage of the choroidal vasculature is provided by the vortex veins. Most individuals have four to six vortex veins. Vortex veins can have anastomoses between each other but generally join to form the superior and inferior ophthalmic vein which drains into the cavernous sinus behind the globe.

1.2.4 Ocular perfusion pressure

Ocular perfusion pressure (OPP) is the term used to describe the force of ocular blood flow. OPP is defined as the difference of pressure between the arteries entering the eye and the veins leaving it [Alm, 1998]. As the pressure in the retinal vasculature cannot be measured directly the mean arterial pressure is used for calculation [Alm, 1998].

As blood pressure decreases along the way from the heart to the eye, the pressure in the arteries entering the eye is about 35-40 mmHg less than the MABP [Alm, 1998]. The pressure in the veins leaving the ocular circulation is approximately the same as the intra ocular pressure (IOP). The OPP can therefore be calculated according to following formula:

$$OPP = (2/3 \times MABP) - IOP, \tag{1.1}$$

OPP: Ocular perfusion pressure, MABP: Mean arterial blood pressure (MABP=2/3Diastolic BP + 1/3Systolic BP) and IOP: Intra ocular pressure

1.2.5 Ocular blood flow and haemodynamic regulation of the retina

The retina, classified as a mircrocirculatory vascular bed (definition: arteries <400µm and arterioles <100µm), has a very complex regulatory system similar to the brain [Riva et al., 1983, 1986]. Metabolic substrates and oxygen delivery to the retina is maintained through 2 pathways:

- Choroidal blood vessels (outer 1 third of the retina)
- Retinal blood vessels (inner 2 thirds of the retina)

As retinal blood vessels differ not only morphologically from carotid and ophthalmic arteries, they do not possess any anastomoses, arterio-venous shunts or sphincters [Ball and Henkind, 1967]. Furthermore, autonomic vaso-active nerves involved in the regulation of vascular tone on the level of the choroids are not present in the retinal vessels or the optic nerve head as they only reach up to the lamina cibrosa [Ehinger, 1966a,b,c, Laties, 1967]. Hormones involved in the regulatory process of many vascular beds are not effective at the retinal vessels' level [Delaey and de Voorde, 1998, Delaey and Voorde, 2000].

The rate of blood flow in the retina is therefore mainly dependant on 2 factors:

- Perfusion Pressure: the pressure driving the blood into the retinal vascular system

- Vascular Resistance: (generated by retinal vessels and their blood viscosity) Viscosity depends in turn on shear rate, hematocrit, rheological factors due to branching and the velocity profile of red blood cells

Changes in the length of the blood vessels themselves are not considered to play any major role in the OBF regulation. Due to those factors, retinal vascular tone is mainly regulated by auto-regulation. Auto-regulation is the intrinsic ability of a given tissue to maintain stable blood flow despite variations in perfusion pressure [Guyton et al., 1973]. Auto regulation involves a number of factors that can be categorised by two mechanisms; metabolic and myogenic.

1.2.5.1 Metabolic mechanisms

Metabolic auto-regulation is defined as the capability of an organ to regulate its blood supply in accordance to its needs. Changes in metabolism during muscle contraction and neuronal activity in the brain increase blood flow. This increase is thought to happen due to the release of vaso-active substances of the surrounding tissue and ensures adequate supply of oxygen and removal of metabolites such as CO2 and lactate.

Factors involved in the metabolic mechanism:

Hypoxia: Hypoxia is caused when increased oxygen demand or decreased oxygen supply results in vaso-dilation in order to meet the increased needs. This dilation might be a direct or indirect response to either inadequate oxygen supply to smooth muscle cells or production of vasodilator metabolites.

Tissue Metabolites and Ions

Adenosine: Formation of adenosine is increased during hypoxia, this plays an important role in the mechanisms involved in regulating coronary blood flow.

Potassium ions: These play an important role in in Na+/K+-ATPase pump that regulates muscle contraction.

Carbon dioxide (CO2): CO2 is produced by parenchymal cells and causes vasodilation in vascular smooth muscle cells. Carbon dioxide plays an important role in regulating cerebral blood flow.

Nitric Oxide (NO)	Stimulated by increased shear force, bradykinins, insulin-like growth factor-1, acetylcholine, thrombin and platelet products of various types	Binds to guanylate cyclise Causing cGMP accumulation and **vasodilation** **Vessel protection** due to inhibition of platelet aggregation and granule sectretion, leukocyte adhesion and smooth muscle cell proliferation.
Endothelin (ET)	Released by endothelial cells 21 amino acid peptide Divided in subtypes: ET-1, ET-2, ET-3)	Most potent **vasoconstrictor**: ET-1 Action occurs through receptor mediation: in humans: ETR-A
Superoxide anions	Generated by the vascular endothelium	Inactivation of NO **Indirect vasoconstriction**
Angiotensin-converting enzyme (ACE)	Renal origin	ACE converts angiotensin I to Angiotensin II ACE inactivates bradykinin Angiotensin II mediates **vasoconstriction** by stimulation of smooth muscle cells and pericytes

Table 1.1: Overview of vaso-active substances involved in blood flow auto-regulation

Hydrogen ion (H+): Their numbers increases if carbon dioxide levels rise or during anaerobic metabolism, potentially causing metabolic acidosis. H+ cause, like CO2, vasodilation and plays an important role in the cerebral vascular regulation.

Myogenic mechanisms Myogenic vascular regulation is defined as non neural regulation of vascular tone mediated through endothelial cells releasing vaso-active molecules acting directly on vascular smooth muscle cells. Myogenic response of smooth muscle cells, is particularly important in small arteries and arterioles. If intravascular pressure is increased, smooth muscles contract in order to maintain constant flow. The exact mechanism behind this is still unknown. However, when referring to myogenic regulation, this is mainly achieved by a change in vascular resistance triggered through the variation in transmural pressure due to variations in BP [Alm and Bill, 1973]. In order to maintain constant flow, various vaso-active molecules are released by the vascular endothelial cells to act on the smooth muscle cells lining the BV wall. When assessing this function, one has to take into account the decrease in myogenic regulation with increasing age [Jeppesen et al., 2004].

Vaso-active molecules Please see Table 1.1 on page 27.

1.2.5.2 The vasospastic syndrome

Vasospastic syndrome can be divided in two groups:

- Primary: occurs without any underlying disease
- Secondary: occurs with an underlying disease (due to vascular endothelial dysfunction) or in raised Endothelin-1 levels [Flammer et al., 2001]

Patients classified as having primary vasospastic syndrome have been characterized in previous studies as having cold hands/feet, low BP, delayed sleep onset and being of slim build [Orgul et al., 1995]. It is also linked to migraine and ischaemic heart disease [Elliott, 2008, Hassan et al., 2008]. Diagnosis of this condition is very important, especially in CHD, as patients with vasospastic syndrome have a much better prognosis than those with arteriosclerosis [Fagan and Sunthareswaran, 2002].

1.3 Techniques for assessment of ocular blood flow parameters

There are various instruments and methods available to assess ocular blood flow as described in more detail in this section. All measurements have advantages and disadvantages; however, none of them provides all the necessary information in one reading. One has to choose the appropriate instrument based on the vascular bed that is being assessed.

1.3.1 Colour Doppler imaging

This is a method combining B-mode ultrasound imaging with Doppler sonography and more widely known as colour duplex sonography or colour Doppler imaging (CDI). The technical details of the method have been described in detail elsewhere [Haerten and Kim, 1993]. Doppler shift frequency is used to calculate blood flow velocity which is plotted against time. The resistivity index (RI) is then calculated from the values obtained for peak systolic velocity (PSV) and end diastolic velocity (EDV).

$$RI = \frac{PSV - EDV}{PSV} \tag{1.2}$$

Colour Doppler imaging has been a useful measurement in diagnosing and monitoring various ocular disease, because it is a flexible and non-invasive method of assessing orbital blood flow [Tranquart et al., 2003, Baxter and Williamson, 1995, Cianci et al., 2000]. The technique has been reported to have

Device/ Method	Vascular bed assessed	Measurement
Colour Doppler Ultrasound/ Colour Doppler Imaging [CDI]	Retrobulbar blood vessels	Blood flow velocities of retrobulbar vessels (OA, CRA, CRV, PCA)
Heidelberg Retina Flowmeter [HRF]	ONH microcirculation, Retinal microcirculation	ONH and retinal microcirculation
Pulsatile OBF [POBF]	Choroidal OBF	IOP, pulsatile choroidal blood flow
Retinal vessels analyser [RVA]	Retinal vessels >60µm diameter	Retinal vascular diameter and reactivity
Laser interferometry	Choroidal and ONH blood flow	Pulsatile OBF
Laser Doppler velocimetry	Retinal vessels	Blood velocities in the retinal vessels
Laser Doppler flowmetry	ONH Choroidal blood flow	Capillary blood flow at the ONH and in the choroid
Blue field entopic technique	Retinal circulation in the fovea	Capillary retinal blood flow
Fluorescein angiography	Retinal circulation	Retinal blood flow velocity, Perfusion mapping
Indocyanine green angiography	Choroidal circulation	Choroidal blood flow velocity

Table 1.2: Overview of the current methods to assess ocular blood flow

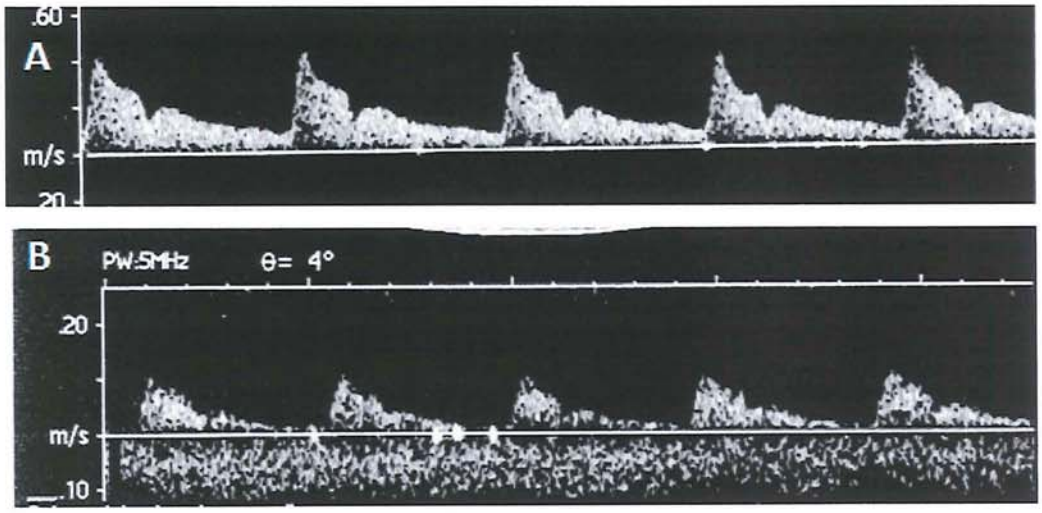

Figure 1.3: Graphical representation of the spectral waveshapes as recorded using Colour Doppler Imaging (A) Spectral wave shape of the ophthalmic artery; (B) Spectral wave shape of the central retinal artery

a high level of reproducibility, but inter-observer variability has been poor [Senn et al., 1996]. When performing repeated measurements it is therefore recommended to have the same examiner throughout the study. According to a number of other studies it has been shown that the most reliable results obtained are the ones from the ophthalmic artery (OA), the central retinal artery (CRA) and the central retinal vein (CRV). Whereas results obtained from the posterior ciliary arteries vary significantly more due to their size, location and tortuosity compared to the OA and CRA [Baxter and Williamson, 1995, Lieb et al., 1991, Senn et al., 1996, Tranquart et al., 2003, Németh et al., 2002]. CDI measurements have also been shown to have good correlation with other blood flow measurements such as laser Doppler flowmetry (LDF) and the method of Langham (LOBF) [Zeitz et al., 2002, Dimitrova et al., 2005]. Please see Figure 1.3 on page 29 which illustrates the spectral wave and its shape from the OA and the CRA. Where the PSV is taken from the peak of any vessels' wave shape and the trough represents the measurement basis for the EDV.

Factors influencing the measurement There are various factors known to potentially influence blood flow. It is therefore of great importance to address and minimise those in order to obtain reliable and reproducible results.

These factors include:

- Observer experience [Németh et al., 2002]
- Fasting and dehydration [Inan et al., 2002]
- Stimulants (caffeine, alcohol, chocolate)[Vlachopoulos et al., 2004, 2005]
- Age [Gillies et al., 1999]
- Vasospastic syndrome [Gherghel et al., 1999]
- Gender [Harris et al., 2000, Kaiser et al., 1996]
- Systemic Disease [Williamson and Baxter, 1994, Goebel et al., 1995]

Advantages of the method

- Non invasive
- No diagnostics (e.g. anasthetics, mydriatics,...) needed
- Good reproducibility
- Quick: results can be obtained within as little as 10 minutes

Limitations of the method

- Evaluation only of retrobulbar vasculature
- High variability of smaller vessels measured due to low resolution
- High inter observer variability
- Experience of the observer

1.3.2 Scanning laser Doppler flowmetry

Riva et al. (1972) first introduced this method to ophthalmic research as a non-invasive method of measuring perfusion of the retina and ONH [Riva et al., 1982, 1972]. Laser doppler flowmetry is based on the principle of frequency shift once a beam of light hits a moving object, in this case moving red blood cells (RBC). Scanning laser doppler flowmetry (SLDF) combines laser doppler flowmetry with a scanning laser system to visualize a perfusion map of a selected area of the retina. The device used in this thesis is more widely known as the Heidelberg Retinal Flowmeter (HRF). It uses a confocal scanning laser system (low intensity infrared laser, 670nm) to assess various depths of tissue. SLDF is able to analyse perfused vessels of the juxtapapillary retina and the ONH in order to obtain reliable and reproducible measurements of retinal capillary blood flow.

Advantages of the method

- No pupil dilation is necessary
- Non invasive

Limitations of the method

- Difficult to obtain a good quality measurement due to errors in the imaging process if subject does not have a stable fixation
- Results are highly dependent on selected measurement area [Bohdanecka et al., 1998]
- Results can be influenced by brightness of the illuminated fundus [Tsang et al., 1999]
- Results can vary due to ocular pulsation related to cardiac rhythm similar to the variation in intra ocular pressure depending on the cardiac cycle.

1.3.3 Static retinal vessel analysis

Static retinal vessel analysis can be carried out by manual or semi automatic measurement of the arterio-venous-ratio (AVR) according to a method described by Michaelson et al. and others [Michelson et al., 2005, Neubauer et al., 2008]. There is a large variety of devices available to obtain retinal images along with software to analyse them. The device used in the present thesis was a high resolution fundus camera (FF450, Carl Zeiss Meditech, Jena, Germany) to obtain retinal images. According to the protocol used in a large population study by Michaelson et al (2005), a black and white image (30 degree wide), with the ONH centred, of the chosen eye is obtained. Analysis of retinal vessels has long been shown to be a valuable technique in the diagnosis of hypertensive and diabetic retinopathy [Wong et al., 2003b, Patton et al., 2006, Kristinsson et al., 1997]. Moreover, recent studies have assessed the importance of AVR as a maker for cardiovascular disease [Wong et al., 2001b], stroke [Wong et al., 2001a] and cerebrovascular disease [Neubauer et al., 2008].

1.3.3.1 Assessment of the arterio venous ratio (AVR) with the software Visualis (Imedos GmbH, Germany)

To assess the arterio venous ratio (AVR) from retinal images the Visualis software was used. It semi-automatically measures retinal arterial and venous diameter in a predefined area around the ONH as shown in Figure 1.4 on page 33. First, the measurement area is defined as a concentric ring centerd around the ONH at 1/2 to 1 disc diameter (DD) from the optic disc margin. Arteries and veins were then manually chosen by automatic vessel tracking using the Visualis software.

From these measurements, the software generates the AVR, the central retinal artery equivalent (CRAE) and the central retinal vein equivalent (CRVE). The latter two are an estimate based on an algorithm described elsewhere [Hubbard et al., 1999, Parr and Spears, 1974a,b] for the diameter of the CRA and CRV and is given in micrometers. CRAE and CRVE give additional information about the contribution of arteries and veins to the AVR. This is important, as for example a subject exhibiting a stable AVR can still have a major change in vascular morphology. This is so, because if arteries and veins simultaneously change diameter the AVR can stay the same.

1.3.3.2 Dynamic retinal vessel analysis

Dynamic retinal vessel analysis is a technique to assess the behaviour of retinal arteries and veins with diameters larger than 60 micro meters. Measurements were obtained using a Retinal Vessel Analyzer (RVA, IMEDOS, Germany). The device consists of a high resolution fundus camera (FF450, Carl Zeiss Meditech,

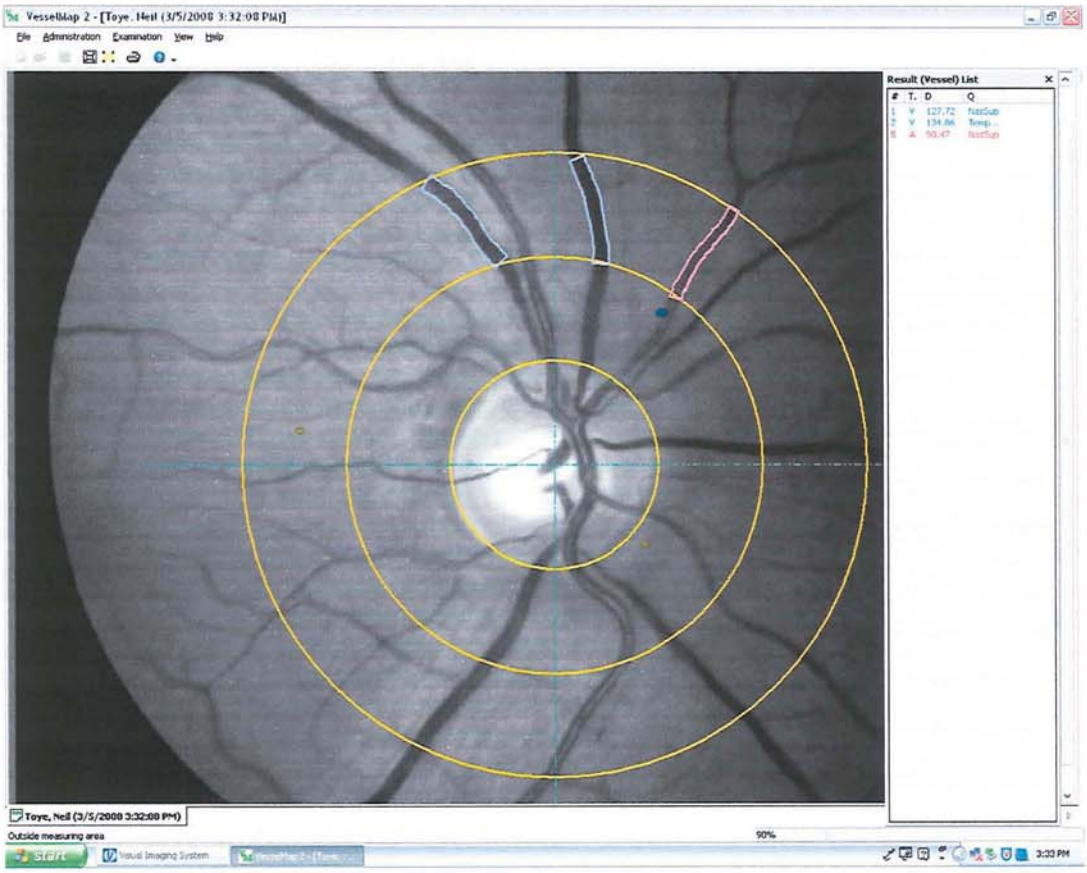

Figure 1.4: Example of an arterio venous ratio (AVR) measurement using the VesselMap soft ware

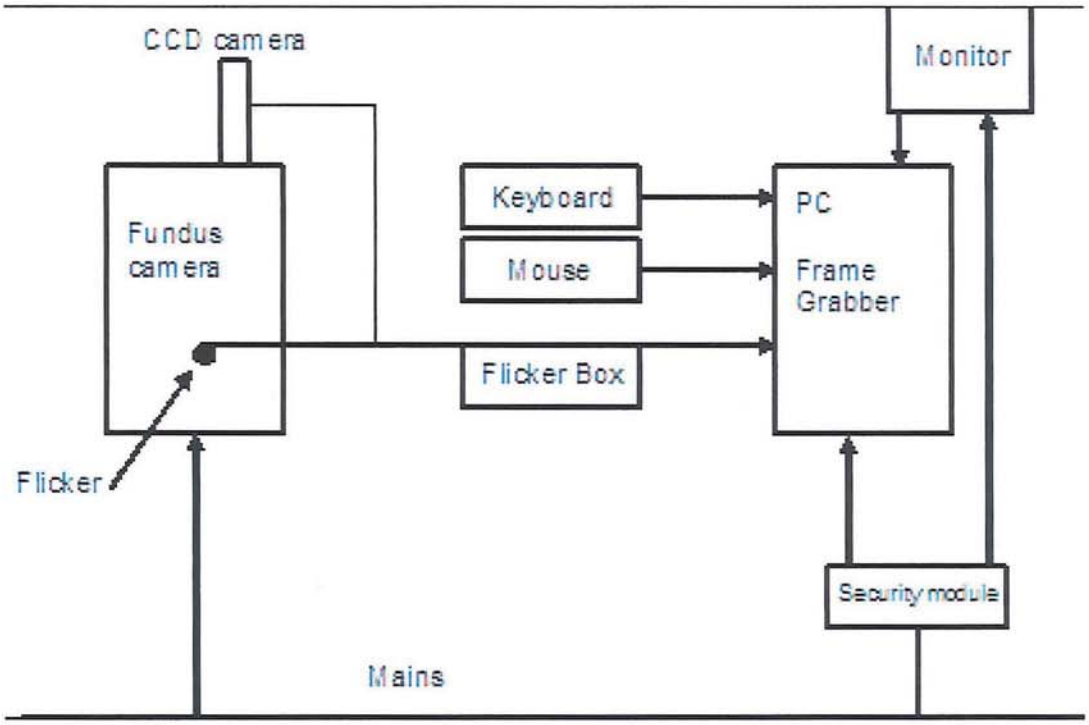

Figure 1.5: Rentinal Vessel Aanalyser: controlling and measurement components

Jena, Germany) and is capable of measuring retinal vessel diameter continuously along a selected vessel segment over a defined time. Recordings can be stored on tape by a video recorder coupled to the CCD camera that is imaging the retina. This enables subsequent additional offline measurements of other vessels and vessel segments within the recorded retinal area.

Retinal vessel analysis (RVA) measurement basics To obtain a good quality measurement the patient's pupil has to be fully dilated. It is necessary to obtain a clear fundus image with good contrast and illumination (without reflections) with the angle of the camera set at 30 degrees. The temporal resolution is 40 ms, such that 25 video frames are captured per second (i.e. sampling rate = 25 Hz). The RVA measures the width of the red blood cell column within the vessel of interest [Seifertl and Vilser, 2002]. Measuring points along the selected vessel segment are approximately 12.5 µm distant from each other. All size related measurements of the RVA are given in UM, whereas 1 UM relates to 1µm in an emmetropic individual. Frame to frame analysis is used to process the recordings as described elsewhere [Vilser et al., 2002].

There are two methods of assessing auto regulatory mechanisms of retinal vessels with the RVA.

- **Measurement of velocity of autoregulation by use of various substances to challenge autoregulation to its limits, e.g. inhaling carbon dioxide, increasing IOP by using a suction cup, and others.**

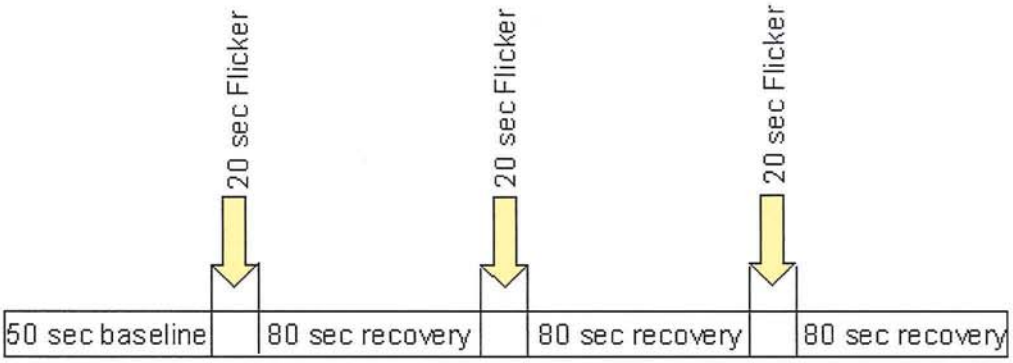

Figure 1.6: Measurement protocol for dynamic retinal vessel assessment

- **Measurement of vessel dilation due to visual stimulation by use of flickering light, as an indirect provocation of the retinal vessels via metabolic increase.**

For the purpose of this thesis, flickering light provocation was used to assess the reactivity of a chosen vessel segment. It has been shown to be capable of detecting changes in vessel diameter and time course to flickering light under various conditions and disorders, e.g. oxygen breathing, suprasystolic IOP provocation, hypertension, hyperglycaemic clamps and isomeric exercise [Frederiksen et al., 2006, Dorner et al., 2003a, Garhofer et al., 2004b, Nagel et al., 2004]. Flicker is a light modulation and due to the natural purpose of the retina to respond to light modulation this is the most natural stimuli possible to assess its function. Furthermore it offers the advantage of stimulating the retina exclusively as it does not affect any other vascular bed as opposed to other methods such as an increase in IOP with a suction cup.

RVA flicker measurement For the assessment of retinal diameter reaction to flickering light an optoelectronic shutter is inserted in the optical pathway of the camera illuminating the retina. Flicker is generated with the shutter by interrupting the observation illumination to the fundus, producing a bright to dark contrast ratio of at least 25:1. Rectangular light interruption of 12.5 Hz has been shown to be in the range of maximal exciting flicker frequency [Nagel and Vilser, 2004a]. As video frequency is set at 25 Hz the flicker frequency will give one dark image every second frame, translating into a sampling rate of 12.5 Hz during flicker provocation. The standard protocol introduced by Nagel et al. [Nagel et al., 2005] is described below:

Whole measurement duration is 350 seconds, consisting of a baseline measurement of 50 seconds and followed by 3 cycles of flickering and recovery. The flickering comprises 20 seconds light provocation followed by 80 seconds of recovery (see Figure 1.7 on page 36).

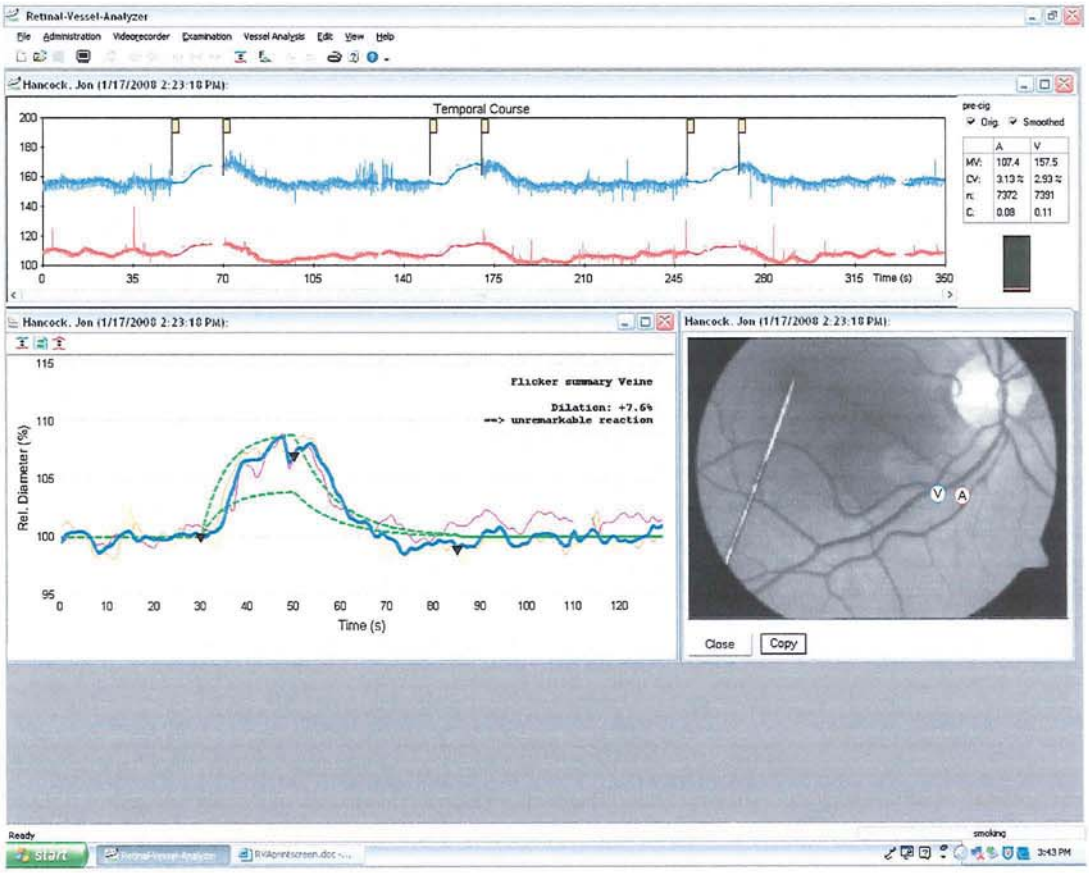

Figure 1.7: Retinal Vessel Analyser (RVA) display showing measurement location, dynamic analysis (next to fundus image) and the diameter time course on top of the screen (arterial diameter shown in red, veonous diameter shown in blue)

This measurement method has been of particular value in a number of studies in recent years as it allows direct vascular observation by a non-invasive technique. In particular it has proved useful in the examination and analysis of:

- Administration of vaso-active substances [Dorner et al., 2003b, Huemer et al., 2003]
- Administration of intra venous glucose [Dorner et al., 2003a]
- Glaucoma [Garhofer et al., 2004a]

Advantages of the method

- Non invasive measurement
- Highly reproducible [Pache et al., 2002]
- Low variability [Nagel et al., 2006]
- High potential for monitoring drug effectiveness [Vilser et al., 2002]

Limitations of the method

- The patient needs to have reasonably clear media in order to obtain a fundus image of sufficient quality
- The patient needs to have good fixation and compliance as the measurement takes approximately 5 minutes
- Pupil dilation is required, but can only be obtained by use of tropicamide as other substances would have an adverse effect on the vascular dynamics.

Reaction mechanism Flickering light provocation indirectly stimulates the vascular system via the metabolic pathway. An increase in the metabolic rate of the photoreceptor layer triggers the release of nitric oxide (NO) in retinal vascular endothelial cells to meet increased metabolic demand (in order to meet the increased oxygen demand of the photoreceptors due to their stimulation with flickering light). After stimulus removal, the vessel diameter relaxes to baseline values. This is particularly true for the retinal venous response; however, the artery demonstrates a sub-baseline constriction immediately after the end of the flicker. In a normal subject, maximum dilation is reached after approximately 20 seconds of stimulation and needs an average of 80 seconds to relax to baseline diameter afterwards. The reactive constriction after stimulation is thought to be triggered by an overshooting regulatory mechanism of the retinal vascular endothelium.

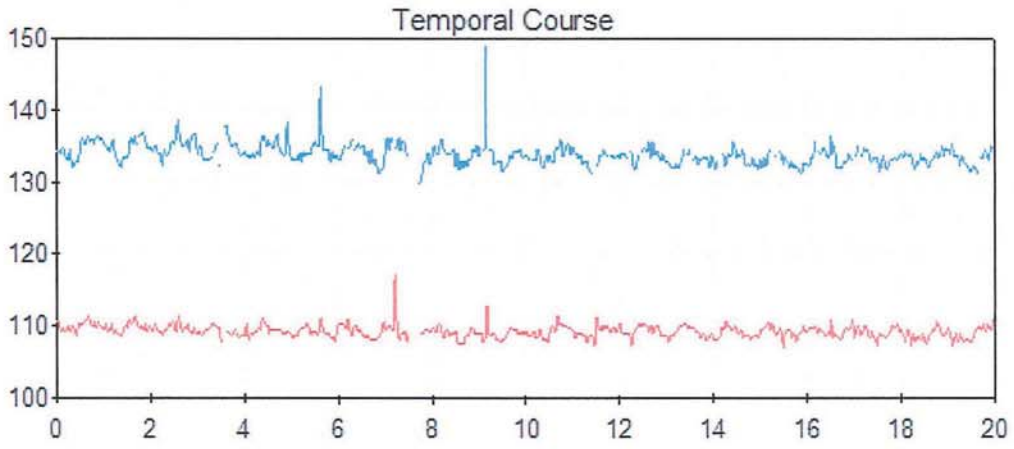

Figure 1.8: Retinal Vessel Analyser (RVA) recording of retinal arteriole (red line) and retinal venule (blue line) diameter change over time

Analysis of diameter changes The device provides a variety of predefined analysis profiles. Figure 1.8 on page 38 shows a 20 second extract of a continuous baseline diameter measurement of both, the artery (red trace) and vein (blue trace).

In recent years a number of different analysis of diameter responses have been published. Some authors used the analysis of the supplied RVA software, in which the maximum response is calculated by first averaging all three stimulation cycles and then calculating the average of the last +/-3 seconds after light stimulation as the maximum diameter response to flickering light provocation [Mandecka et al., 2007, Dawczynski et al., 2007]. This analysis has a major shortfall as it incorporates both time and diameter response. Subjects reaching maximum dilation before 17 seconds or later than 23 seconds after flicker start will have their maximum dilatory response underestimated. Furthermore, when averaging all stimulation cycles, differences in reaction pattern or time course of each cycle cannot be assessed. In order to assess differences between cycles others have incorporated an analysis of each individual flicker cycle [Gugleta et al., 2006b].

Another problem arising when discussing maximum dilatory response to flickering light is the baseline diameter fluctuation (BDF) due to vascular tone and venous pulsation. To take this fluctuation into account, Nagel et. al introduced a value termed baseline corrected flicker response (BFR) [Nagel et al., 2004]. In this approach, BDF is accounted for by subtracting it from the dilation amplitude (DA) after flicker stimulation (see Figure 1.9 on page 39 and Equation (1.3)). However, they did not examine each individual flicker cycle separately.

$$BFR = DA - BDF \tag{1.3}$$

The second problem is that of defining a reaction pattern, to do so, one does

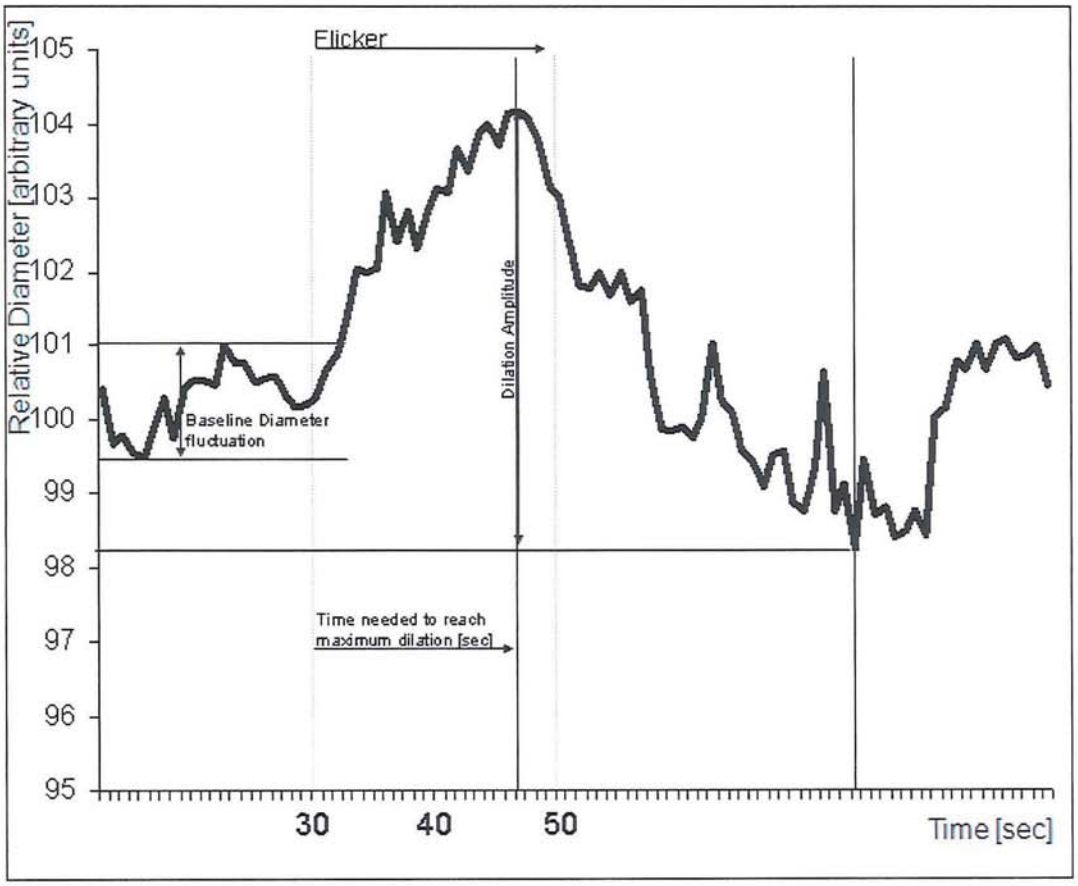

Figure 1.9: Illustration of the retinal diameter response to flicker provocation

not only need to know the point of maximum dilation (MD) but also needs to know the point of maximum constriction (MC), DA and time course of the reaction.

1.3.3.3 Novel analysis of retinal vascular dynamic parameters

For the present thesis we approached these problems by analysing each individual flicker cycle separately. In addition to the calculation of MD, MC and DA we investigated the time course of retinal vascular dilation. This was done by calculating the time needed to reach MD for each flicker cycle separately for the arterial and venous response respectively.

Retinal Vessel Ananalyser: slope analysis As atherosclerotic changes have an impact on vessel stiffness, we introduced a novel approach to assess changes in vascular reactivity at the retinal level. Depending on a vessels' elasticity, reaction can be fast or slow. Therefore the rate at which the vessel diameter increased seemed an ideal approach to assess retinal vessel reactivity. The reaction to flickering light stimulation is very quick and plateaus after about 15 seconds of stimulation. We determined the slope by fitting a regression line for the diameters measured against the time by using the original unedited measurements (for the vein and artery).

1.4 Peripheral circulation

1.4.1 Cutaneous blood flow

Parallel changes in peripheral and ocular blood flow have been studied previously [Gherghel et al., 1999, Flammer et al., 1996, Gasser and Flammer, 1991, Gasser et al., 1999, Senn et al., 1999]. Therefore assessing peripheral blood flow in patients suffering CAD could help to understand how changes in the ocular vascular system relate to systemic vascular dysfunction.

1.4.2 Anatomy and physiology

The vascular system of the skin comprises two horizontally arranged plexi, each of them containing arterioles and venules. The superficial plexus is located in the deep dermis and is characterized by its nutritive capillary loops. These capillary loops are arranged perpendicular to the skin surface. The deep plexus is located in the subcutis, nourishing sweat glands, hair follicles and nerve endings [Page et al., 1997]. The skin protects the body against the environment and prevents excessive loss of protein, electrolytes, water and heat. The latter is regulated mainly by the superficial plexus by acting like a large thermal radiator. Extreme

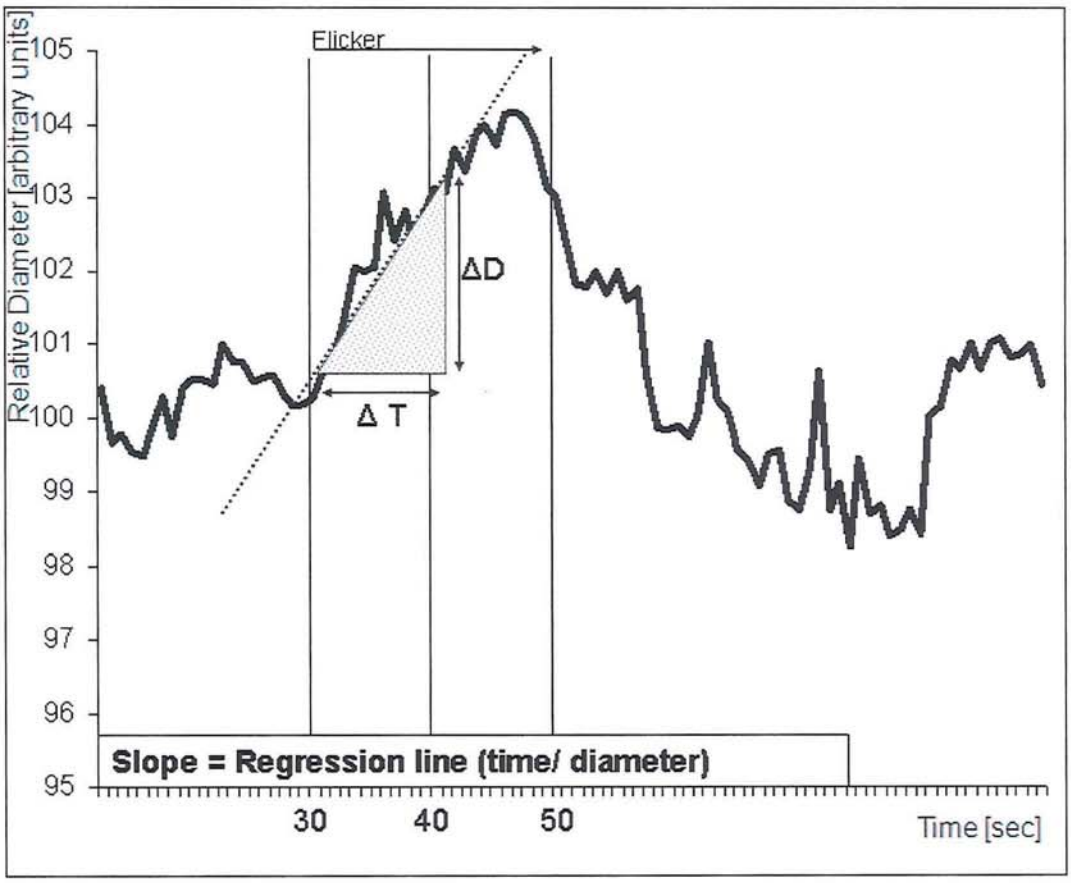

Figure 1.10: Illustration of slope calculation of retinal blood vessels when stimulated with flickering light.

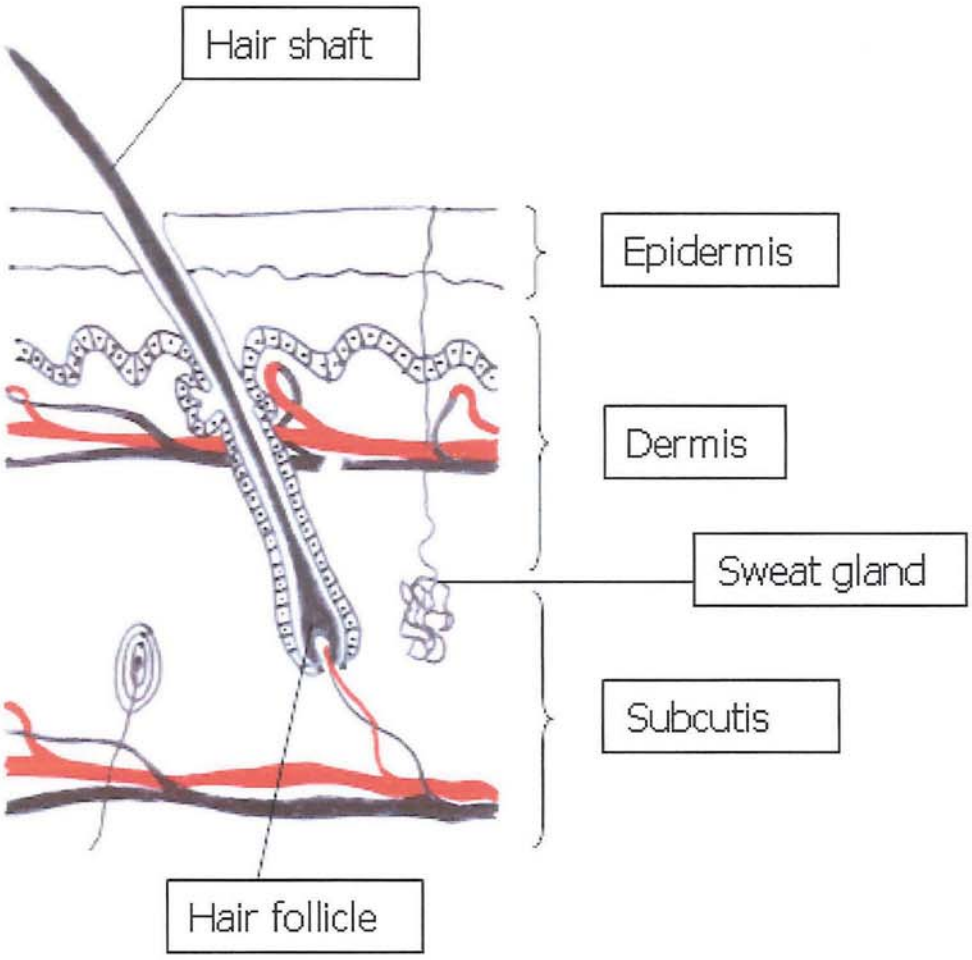

Figure 1.11: Skin section showing vascular plexi system of the outer skin

differences in blood flow are necessary in order to cope with large temperature differences.

1.4.3 Current techniques for evaluating peripheral blood flow

Depending on the instrumentation used, there are two different possibilities for evaluating peripheral blood flow. These are described below.

1.4.3.1 Measurement of micro vascular perfusion

This method assesses micro-vascular perfusion using an infrared laser beam. The laser is directed at the tissue of interest with a fibre optic probe. Perfusion is measured by calculation of the frequency shift produced by the movement of cells within the tissue (Doppler principle). Moving objects, such as red blood cells, hit by a laser beam reflect the incident light. Due to their movement the backscattered light interferes with the incident beam which causes a frequency shift detected by the measuring unit. Tissue flow is determined from the measured frequency shift.

1.4.3.2 Nail fold Capillaroscopy

This technique is performed either in the nail fold area of the foot (toe) or hand (finger) of a patient. In this particular area capillary loops are arranged parallel to the skin surface which in turn makes it possible to assess them by laser Doppler flowmetry. Capillaroscopy is a test more commonly known to be used to assess and monitor the progress of peripheral vascular disease, e.g. Raynaud's syndrome [Mahler et al., 1983, Gasser and A, 1991]. It has also been used to evaluate peripheral vasospasm in patients with angina and other systemic vascular disease [Saner et al., 1987b,a]. In addition to the classic application for nail-fold capillaroscopy as diagnostic tool for Raynaud syndrome, scleroderma and reumathoid arthritis, it has more recently been suggested as an additional examination for cardiovascular disorders [Portig and Maisch, 2004, Pries et al., 2008].

CAM1 Laser Doppler Capillary Anemometer Measurements were performed using the CAM1 Laser Doppler Capillary Anemometer (KK Technology, Devon; England). The velocity measurement is aquired by use of the laser Doppler effect as described by [Stuecker et al., 2006].

There are two possibilities to measure capillary velocity with this particular device:

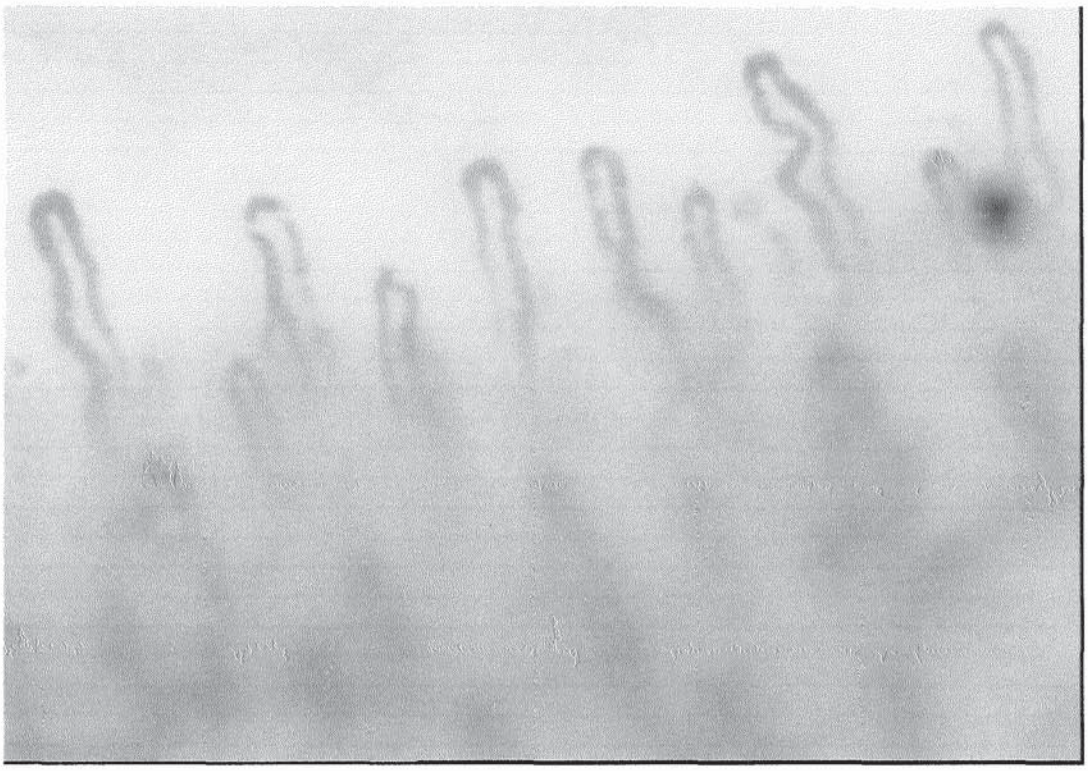

Figure 1.12: Nail-fold capillaries, aprox. 200 times magnified

1. Online measurement: Capillary velocity can be measured online, by means of laser Doppler flowmetry. Therefore the inbuilt infrared laser is used while examining the patient.

2. Offline measurement: Capillary velocity is measured after obtaining a video recording. Analysis of velocity is carried out by means of the frame to frame analysis. This allows reassessment of the measurement. Furthermore, hand movements can be corrected by using a movement correction filter and measurements from different locations can be obtained.

Factors influencing the measurement

- Room temperature
- Seasonal temperature differences
- Movement/Tremor

Assessment of peripheral vasospasm: In order to trigger a vasospastic behaviour/ reaction, two methods have been described in the literature. Both of them use cold exposure to provoke vasospasm in a subject.

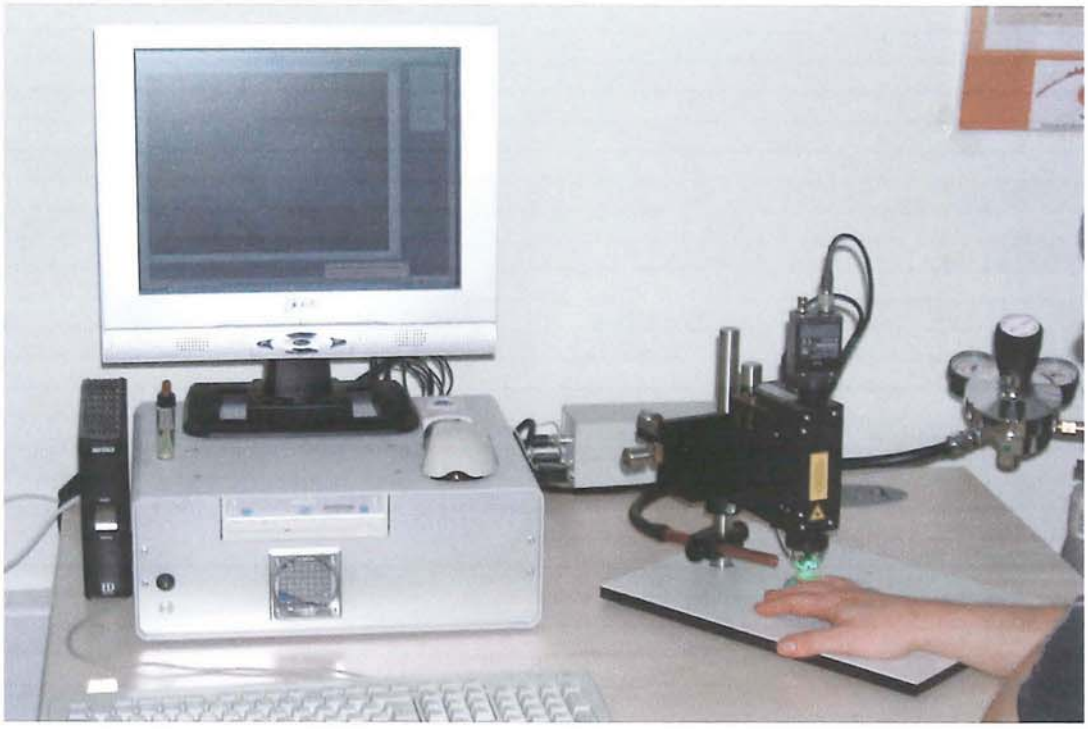

Figure 1.13: Measurement set up for nail-fold capillaroscopy and cold provocation with carbon dioxide via custom tube

Cold provocation with rapidly decompressing carbon dioxide In this procedure first described by Saner et al (1987) and applied here, the patient is acclimatised in a room with a stable temperature of approximately 23 to 25 degrees Celsius. The hands are then immersed in 40 degree Celsius warm water for 3 minutes in order to achieve maximum blood flow through the capillary system and to minimize any differences between subjects arising through seasonal changes in hand temperature [Gasser and Flammer, 1991]. The measurement is then performed while the patient is in a sitting position with the hand positioned at heart level. After a baseline recording of maximum capillary flow, cold provocation is applied by blowing rapidly decompressing CO2 on the patient's nail fold area for 60 seconds. The stream temperature of CO2 is calibrated at -15 degree Celsius which has the effect to cool down the nail-fold tissue to +15 degree Celsius. Recordings are taken until flow has been restored to baseline values. The recordings are then analysed not only for mean capillary flow and mean recovery time, but also to assess if any capillary flow stopped for a period longer than 12 seconds. In this case the patient is classified as having peripheral vasospasm. This has been shown to be a very valuable method in examining peripheral vasospasm. Futhermore, it gives a better understanding of a number of ocular diseases related to systemic vascular disorders. It led to the term ocular vasospastic syndrome, which describes an entity of ocular diseases linked to vasospastic vascular behaviour [Flammer et al., 2001].

Cold provocation using ice water Similar to the method described previously, the patient is acclimatized in a room providing constant temperature. After a period of acclimatisation of about 15-20 minutes in a sitting position an initial recording is taken in order to select the appropriate limb for the measurement. Then, both hands are immersed in 40 degree Celsius warm water for 2 min and a measurement of maximized flow in the chosen limb is recorded. After this, cold provocation is performed using ice water. For this both hands are immersed in 4 degree cold water for 10 sec to obtain minimized flow and the recording is taken [Hafez et al., 2005].

In order to measure velocity in a single capillary various analyses can be carried out, depending on the system used. The measurements differ by the way the recordings are analysed; by flying spot, frame-to-frame or photometric analysis. In this thesis all recorded measurements have been analysed online using the laser Doppler technique.

1.4.3.3 Peripheral vasospasm and ocular blood flow

Individuals with peripheral vasospasm have been shown to have altered retrobulbar blood flow [Gherghel et al., 1999] and their reaction to flickering light stimulation at the retinal level is blunted [Gugleta et al., 2006b]. As described previously, individuals suffering primary vasospasm tend to have lower BP [Orgul et al., 1995] and therefore have an increased risk of suffering from transient periods of low ocular perfusion pressure. This in turn puts them at higher risk of developing ocular vascular disease due to reduced autoregulatory capability [Hasler et al., 2002, Haufroid and Collignon-Robe, 2004, Broadway and Drance, 1998]. Furthermore, ocular disease, in particular glaucoma has been shown to be linked to both ocular blood flow alterations and vasospasm .

1.5 Techniques to assess visual function

To assess visual function, a variety of tests can be applied. For the purpose of this thesis visual function was assessed using colour vision, visual field evaluation and a contrast sensitivity test. Previous studies have shown abnormal findings in visual field, colour vision and contrast sensitivity assessments in patients suffering systemic vascular diseases [Erb et al., 2000, Ghirlanda et al., 1997, Di Leo et al., 1992].

1.5.1 Colour vision assessment

Currently there are various colour vision tests available, ranging from simple random dot versions, in the form of booklets, to computerised screen versions. In

order to chose the appropriate test it is therefore necessary to know more about the diagnostic capabilities of the test. In our study the aim was to obtain a reliable, reproducible result with a test that is able to detect and classify deficiencies/ defects. Another important fact was that the test could be done within only a few minutes and with minimal patient compliance. We therefore chose the Waggoner HRR 4th Edition. Testing was conducted in a quiet room with an average illumination of 450 lux. It was performed binocularly under a light box with a D6500 natural daylight fluorescent tube, providing an illumination of 1000 lux. The subjects were asked to wear their best correction (i.e. spectacles with which the patient reaches their best corrected visual acuity), while doing the tests at a distance of 35 to 40 cm in a sitting position.

1.5.2 Visual field assessment

In order to assess visual fields (VF) in an individual a variety of tests and procedures are available. The examiner can choose to assess various parts of the retina by different types and sizes of stimuli and background illumination. Because the most commonly used form of VF testing, white-on-white perimetry, has been shown to have low test-retest reliability [Artes et al., 2002, Wild et al., 1999a,b], especially in areas of VF loss and is furthermore unable to assess retinal ganglion cell layer sufficiently [Harwerth et al., 1999, 2004], new tests have been developed [Arend and Plange, 2006]. Those new tests developed include frequency doubling technique (FDT), short wavelength automated perimetry (SWAP), flicker perimetry (FP), high-pass resolution perimetry and rarebit perimetry. Many of them have been developed with the aim to identify damage of a special subset of retinal ganglion cells and are often referred to as visual function specific or selective perimetry tests. These new tests are meant to assess selectively the function of one of the three major visual pathways; parvocellular pathway (transmits colour and form), magnocellular pathway (transmits flicker/ motion) and koniocellular pathway (transmits short-wavelength/ blue information) [McKendrick, 2005]. In order to assess early neural damage FDT and FP have been shown to be highly reliable and efficient [McKendrick, 2005, Sample et al., 2006, Arend and Plange, 2006]. For the purpose of this thesis flicker perimetry and frequency doubling perimetry were chosen and will be described in more detail below.

1.5.2.1 Flicker Perimetry

Background FP is a more recently introduced method to assess visual field defects. FP has been shown to be capable to show early changes in visual field compared standard procedures such as white-on-white perimetry [Phipps et al.,

2004, McKendrick, 2005]. It is an alternative to FDT for assessing the magnocellular visual pathway and thought to represent function of the retinal y-ganglion cell layer [Lachenmayr et al., 1989]. Various ocular diseases are based on an underlying vascular disturbance altering blood constituents and flow parameters. This in turn can cause insufficient blood supply to the retina and therefore cause neural damage manifesting in visual field defects. Erb et al have reported VF defects in patients suffering from cardiovascular disease, despite having normal findings in a standard eye examination [Erb et al., 2000]. This suggests that subtle changes, not visible by routine eye examinations, could be picked up by FP testing. Further more FP findings have been shown to be directly related to poor metabolic control of blood glucose levels despite the signs of any retinopathy [Lobefalo et al., 1997].

Measurement principle There are two ways of performing FP: temporal modulation perimetry (TMP), measuring contrast thresholds for fixed temporal frequency and the other being critical flicker frequency (CFF), measuring the highest frequency for which flicker is detected at a fixed contrast level. Both methods have been shown to have similar test-retest reliability in normals [Yoshiyama and Johnson, 1997]. In the present thesis CFF was performed with the Octopus 311 (Interzeag AC, Schlieren, Switzerland).

Flicker perimetry with the Octopus 311 Octopus FP tests the critical flicker fusion frequency, the frequency at which a flickering/flimmering test stimuli appears to be still. This effect can be compared to the frame rate of a computer screen; At low frame rates of about 50-60 Hz the screen appears to flicker whereas at about 75 Hz frame rate the screen is perceived to be still. For a given test stimuli of Goldmann size III the critical flicker fusion frequency is approximately 40 Hz. The Octopus FP uses the same stimuli size, test locations and examination strategies as in its white-on-white perimetry tests. This in turn enables direct comparison of results with regard to regional pattern deviations. In the present study we use the 32 pattern with Goldmann size III stimuli of 1000 ms duration to examine the central 30 degree of the retina. The threshold algorithm used is that of tendency-oriented perimetry (TOP) which has been developed to speed up the thresholding procedure compared to standard staircase techniques [McKendrick, 2005, Morales et al., 2000]. Unlike other procedures that test the same location many times, the TOP thresholding procedure is based on testing each point only once. However, the sensitivity estimate at each location is based on the response at that point and the response of three neighbouring points. This in turn speeds up test time, which is very valuable, since patients suffering cardiovascular disease are often of older age and have a limited attention span.

1.5.2.2 Frequency Doubling Perimetry

Background and measurement principle Frequency doubling perimetry (FDT) is a more recently developed technique to assess visual fields. The term frequency doubling does not refer to a true frequency doubling. It describes the perceptual effect occurring when viewing sinusoidal gratings that are rapidly counterphase flickered at frequencies higher than 15 Hz [McKendrick, 2005]. An individual will therefore perceive twice as many light and dark bars as are physically present, similar to the spatial frequency of a grating being doubled. This effect was thought to be a response of the magnocellular retinal ganglion cells [Johnson and Samuels, 1997] but more recent literature refers to a cortical mechanism being responsible for the doubled perception or at least a combined response of both [White et al., 2002].

In this procedure the subject does not have to distinguish if doubling is present or not. When the stimuli is seen, the patient had to press a button for the machine to register detection, meaning FDT measures grating contrast detection thresholds for sinusoidal stimuli, not CFF.

1.5.3 Contrast sensitivity assessment

This measurement was carried out by using the CSV-100E Contrast Testing Unit (Vector Vision Inc., Drayton; OH). Central contrast sensitivity (CS) was tested at a distance of 8 feet, at four spatial frequencies, 3, 6, 12 and 18 cycles per degree using a standardized testing luminance level of 85 cd/m^2 (achieved through retro illumination by the device itself). This in turn minimizes the error caused by illumination variability of the examination room. In this test each spatial frequency is presented on a separate row of the test panel, and each double row presents 17 circular patches 1.5 inches in diameter. The first patch in the row presents a very high contrast grating, which can be seen easily; this is the sample patch. The remaining 16 patches appear in pairs, vertically offset in eight columns across the row. For each pair of patches, either the top or the bottom presents a grating, and the other patch is blank. The patches containing gratings decrease in contrast moving from left to right across the row. The patient is directed to observe the first sample patch and is told to look for a similar line pattern in either of the remaining pairs. While reading across the row, the patients indicate whether the grating appears in the top or bottom patch for each column. If the grating is not visible in either patch, patients are asked to respond that both patches appear blank. The contrast level of the last correct response is considered the contrast threshold. Log contrast sensitivity levels in each row range from 0.70 to 2.08, 0.91 to 2.29, 0.61 to 1.99 and 0.17 to 1.55 log units for 3, 6, 12 and 18 cycles/ degree, respectively. The contrast sensitivity difference between the sample patch

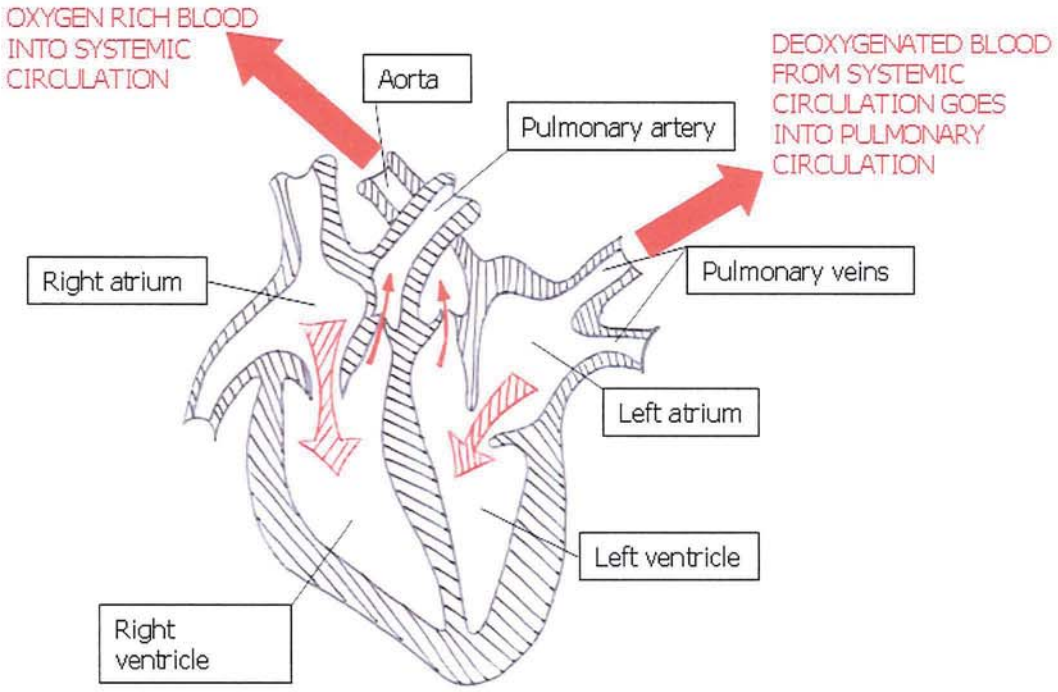

Figure 1.14: Heart section showing atria and ventricles

and level 1 is 0.3 log units. For the remaining test patches, contrast sensitivity levels diminish in a uniform logarithmic fashion in steps of 0.15 log units at each step. CS is evaluated using the patient's best refractive correction (spectacles with which the patients reaches his best corrected visual acuity) monocular. To avoid test learning or bias, two practice trials were conducted with each patient before the first baseline measurement.

1.6 Coronary circulation

1.6.1 Heart anatomy

The human heart resembles a double pump (see Figure 1.14 on page 50). It comprises of two muscular pumps, the right and left ventricle, each of which has its own reservoir, the right and left atrium. Each of them belongs to a specific circulation. The right ventricle serves as a pump for the pulmonary system and receives deoxygenated blood from the systemic circulation and transfers it into the lung. After losing carbon dioxide and acquiring oxygen the blood enters the left atrium through the pulmonary veins and leaves the heart via the left ventricle and aorta into systemic circulation [Fagan and Sunthareswaran, 2002].

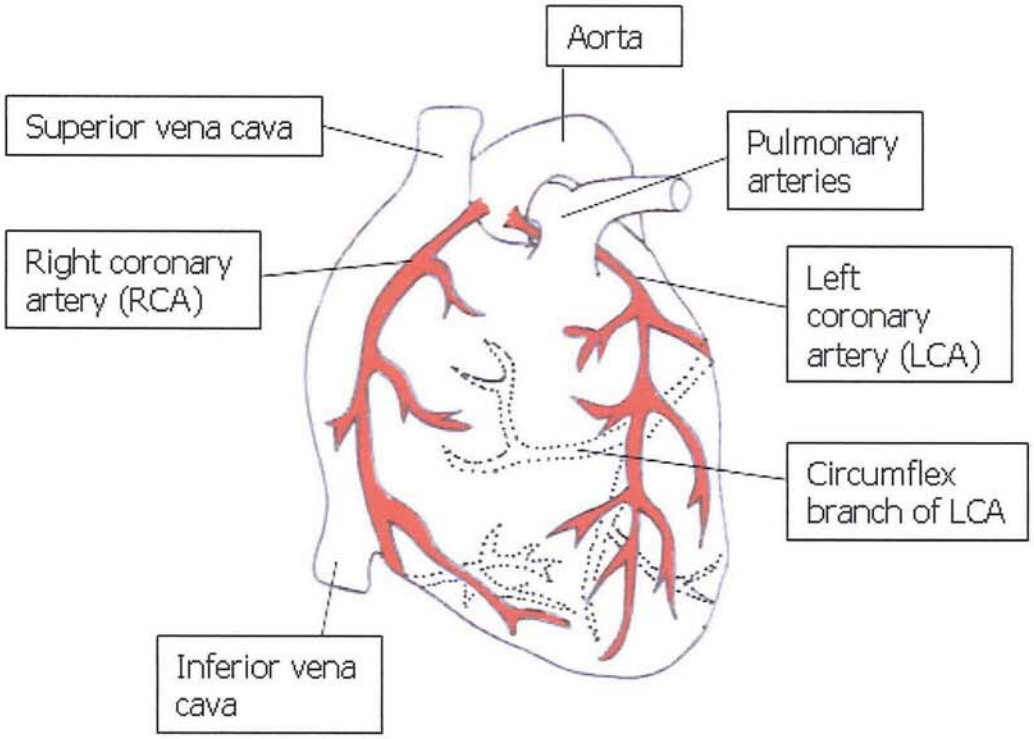

Figure 1.15: Vascular supply of the heart via coronary arteries

1.6.1.1 Coronary arteries

The heart's blood supply is maintained by the coronary arteries (Figure 1.15 on page 51). The right coronary artery originates from the right anterior aortic sinus and the left coronary artery arises from the left anterior cusp of the aortic valve. Whereas the right coronary artery mainly supplies the right atrium, ventricle, arterioventricular node and the interventricular septum, in some cases it may also supply parts of the left atrium and left ventricle.

The left coronary artery is divided in two major branches, one called the anterior interventricular branch or left anterior descending artery and the circumflex branch. The left anterior descending artery nourishes both ventricles, and the circumflex branch nourishes the inferior part of the left ventricle and the left atrium.

1.6.1.2 Coronary veins

Coronary veins are similarly arranged to the major coronary arteries. Venous drainage is mainly provided by the coronary sinus, draining directly into the right atrium. There are also some smaller veins draining directly into the right heart chambers.

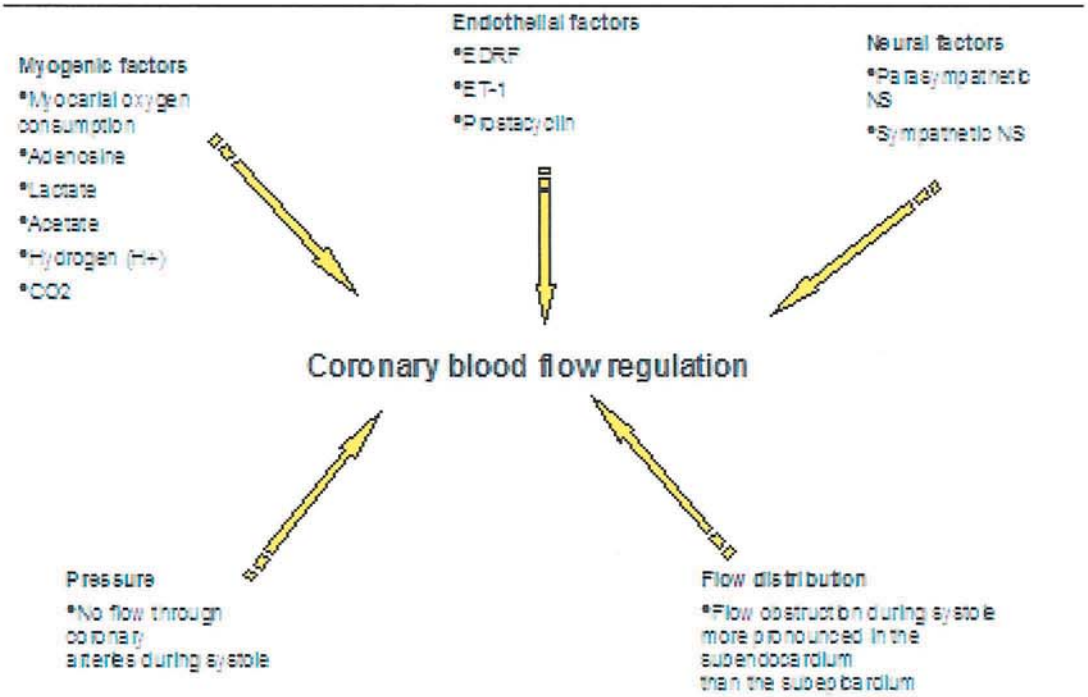

Figure 1.16: Mechanisms involved in the regulation of coronary blood flow

1.6.1.3 Coronary Blood Flow

The blood flow through coronary arteries can be calculated by following formula:

$$Q = P/R, \tag{1.4}$$

where Q=blood flow, P=vessel's perfusion pressure and R=vascular resistance.

In contrast to most arterial beds in the body, coronary blood flow occurs during cardiac diastole not systole [Lilly, 2003]. Blood flow of coronary arteries is regulated by many factors, as detailed in Figure 1.16 on page 52.

However, there are a number of factors interfering with this regulatory system that can potentially lead to myocardial infarction (MI). A brief overview of the most important factors involved:

- Family history of coronary heart disease (CHD);
- Diabetes Mellitus (DM);
- Hypertension (HT);
- Dyslipidemia;
- Cigarette smoking;
- Ageing and

- Menopause.

1.6.2 Cardiac physiology

1.6.2.1 The cardiac cycle

The cardiac cycle is the term used to describe the process of pressure and volume change in both atria and ventricles as they pump blood into the pulmonary and systemic circulation. During rest, the time of one cardiac cycle is approximately 0.9 seconds. The pressure and volume change occurring during cardiac activity arises through electrical and mechanical events (ventricular contraction).

Electrical properties of the heart The cardiac muscle's (myocardium) ability to contract rhythmically is maintained by nerve cells covering the outer structures of the heart, comprising the sinoatrial node (SA). The SA node is located in the posterior wall of the right atrium and is responsible for initiating the depolarisation by contracting the entire heart. Innervated by the autonomic nervous system (ANS), sympathetic activation causes more frequent action, parasympathetic stimulation having the opposite effect. Depolarisation then spreads to the arterioventricular (AV) node causing aterial systole (contraction) in both atria. Conduction continues from the AV node through the bundle of His and its branches and Purkinje fibres initiating ventricular contraction.

Mechanical properties of the heart Ventricular contraction, also known as systole, is the beginning of the cardiac cycle. As the ventricles contract, pressure inside them rises rapidly and is soon greater than that in the aorta and pulmonary artery. This in turn allows the blood to be ejected into both the systemic and pulmonary circulation. Following ejection, pressure decreases and the ventricles relax and refill, a process referred to as diastole.

1.6.2.2 Regulation of cardiac rhythm

Cardiac rhythm is regulated by the autonomic nervous system (ANS) via the sympathetic and parasympathetic pathways. Heart rate variability (HRV) is a reflection of cardiac pacemaker cell modulation. During active periods and daytime, sympathetic compared to parasympathetic (vagal) tone is increased but vagal activity is highest at rest [Goldberger, 1999]. Activation of the sympathetic pathway increases heart rate (HR), while parasympathetic activation decreases HR by acetylcholine release [Pumprla et al., 2002].

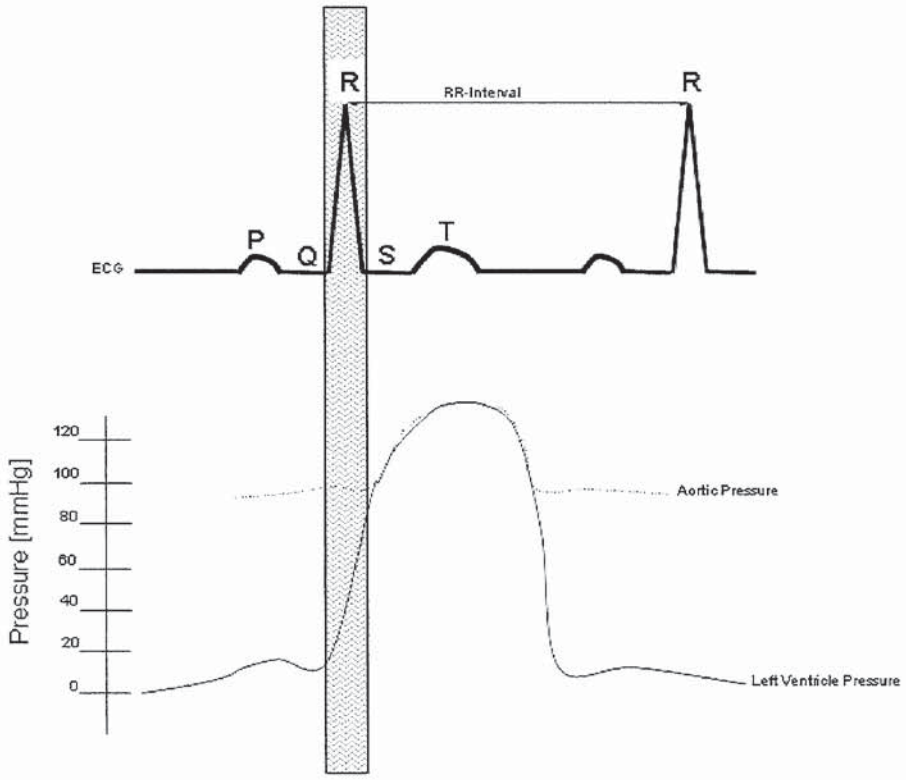

Figure 1.17: Variation of cardiac and aortic pressure during the cardiac cycle (bottom) and its resultatnt ECG recording (top); ECG: Electrocardiogram

1.6.3 The electrocardiogram:

The electrocardiogram (ECG) measures cardiac activity. It provides information on cardiac structure and cardiac function.

1.6.3.1 Sequence of cardiac activation

Each heart beat, as shown by ECG, can be divided in four major parts [Fagan and Sunthareswaran, 2002]

- P-wave: Atrial depolarisation and contraction
- QRS-complex: ventricular depolarisation
- QT-interval : contiunuing ventricular muscle depolarization
- T-wave: ventricular repolarisation

1.6.3.2 Electrocardiogram analysis

The following parameters can be assessed by ECG:

- Heart rate (HR): the number of ventricular contractions in one minute

$$HR = \frac{25mm}{t \times B}, \tag{1.5}$$

where t is the time in minutes and B refers to the number of mms between beats

Or

$$HR = \frac{1500}{C}, \tag{1.6}$$

where C refers to the number of small boxes between 2 consecutive beats

- Heart rhythm: normal heart rhythm is present at a HR between 60 – 100 beats per minute and referred to as normal sinus rhythm.
- Atrial and ventricular rhythm disturbances
- Length and size of intervals (e.g. PR, QRS, QT); Normal length: PR=120ms, QRS=80ms, QT=300ms
- ST-segment and T-wave abnormalities. These occur in myocardial ischaemia (MI) when there is a difference in oxygen demand and supply and present through:
  - ST-segment depression (e.g. metabolic abnormalities)
  - ST-segment elevation (e.g. cardiac infarct and pericarditis)

1.6.4 Heart rate variability

When resting, the sinus rhythm of the heart is irregular, referred to as heart rate variability (HRV). This variability is due to a number of physiological activities throughout the body, such as BP, respiration, body temperature, hormone levels, metabolic rate and sleep pattern. HRV has been extensively researched in normal physiology as well as pathological conditions.

Useful applications of HRV evaluations include:

- HRV as an independent risk factor to predict death in cardiovascular disease [Bjorkander et al., 2008]
- Evaluation of HRV during a variety of stress test, such as the cold pressor test [Moses et al., 2007]
- Evaluation before, during and after MI [Kochiadakis et al., 2000]
- HRV analyses to asses CAD risk [Kupari et al., 1993, Preckel and von Kaenel, 2004]
- Spectral analyses of HRV to asses the outcome of surgical cardiac intervention [Bellwon et al., 1996]

1.6.4.1 Heart rate variability in cardiovascular disease

The analysis of HRV has shown to be a useful tool in a number of ways, such as the assessment of severity of coronary atherosclerosis [Hayano et al., 1991], risk of CAD [Kupari et al., 1993, Preckel and von Kaenel, 2004] and the changes arising with increasing age [Jokinen et al., 2001]. Furthermore it has been used as a monitoring tool for outcome of coronary artery bypass grafting (CABG) [Bellwon et al., 1996] showing an improvement in cardiac vagal activity. In addition to monitor short and long term ANS function, HRV is an ideal tool for the assessment of ANS activity before, during and after episodes of MI [Kochiadakis et al., 2000].

1.6.4.2 Heart rate variability in ocular disease

Any change of the regulation of blood flow at the heart, cerebral or peripheral vascular level can have a detrimental effect on the ocular vascular supply. This is so because of the close proximity of the ocular circulation to the heart and brain via the carotid artery [Schilder, 1994]. Another factor contributing to this interaction is the homology between cerebral and retinal microvascular structure [Patton et al., 2005]. In fact, patients suffering from ocular vascular disease have shown a change in HRV compared to healthy controls [Gherghel et al., 2004].

1.6.5 Coronary disease - Atherosclerosis

Definition Arteriosclerosis (ATS) is the term used to describe the thickening or hardening of the arterial walls. There are two types of ATS:

Arterio-sclerosis: This is a slow but progressive inflammatory process of the intima of large and medium sized arteries. It is a result of lipid and collagen accumulation in the arterial wall.

Arterio-losclerosis: This describes the hyalinization of the walls of small arteries and arterioles.

1.6.5.1 Systemic implications of arteriosclerosis

ATS generally affects large and medium sized arteries. It is most commonly observed in coronary arteries, carotid arteries as well as in arteries of the lower extremities. As the disease progresses, plaques encroach on the lumen of the vessel wall and weaken it, subsequently triggering the development of aneurysm, calcification, thrombus formation, haemorrhage, ulceration and embolism [Lilly, 2003]. As a result it can damage various organs and tissues throughout the whole body, by causing ischaemia due to a compromised oxygen supply.

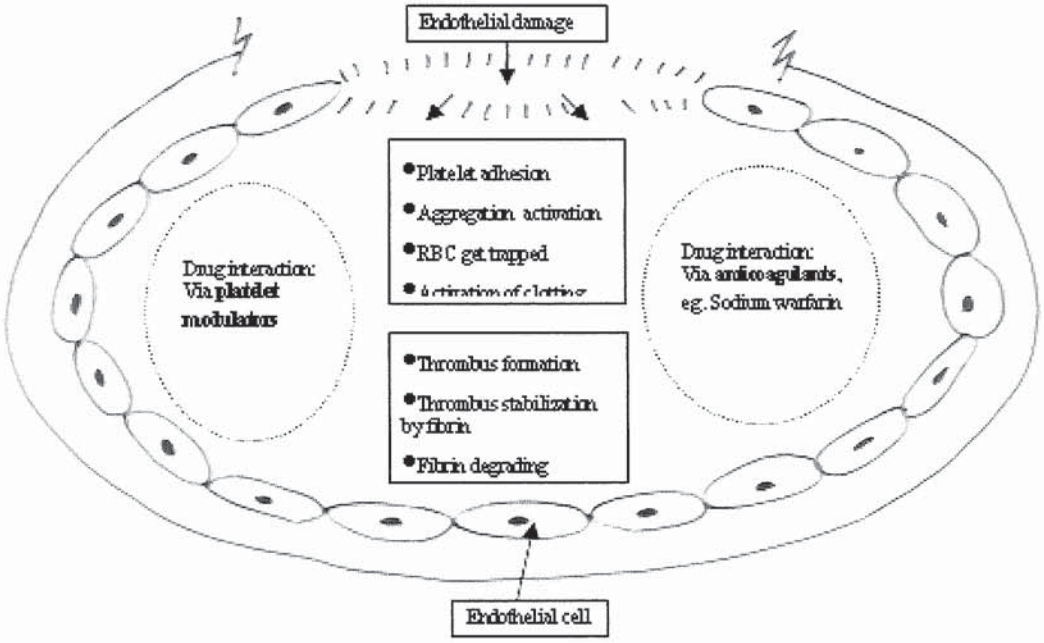

Figure 1.18: Schemata showing atheroma development

1.6.5.2 Risk factors and progression of arteriosclerosis

Besides hereditary predisposition and endothelial dysfunction [Lilly, 2003]; lifestyle and other factors involved in the disease process is a very powerful factor on progression of the disease [Hong et al., 2006, Dasch et al., 2005, Kawasaki et al., 2006]. It has been shown in previous studies that changes in lifestyle have a significant impact on the progression of the disease; e.g. 1% reduction in serum cholesterol levels may lower the rate of CAD by 3% [Fagan and Sunthareswaran, 2002].

Since the disease progression is often silent, patients may be asymptomatic and unaware of the disease for a very long time. The symptoms are usually picked up when the oxygen demand of a particular tissue/ organ is no longer met.

1.6.5.3 Ocular implications of arteriosclerosis

Assessment of ocular involvement is difficult as the clinical signs, such as widening of the arterial reflex also occur as normal age-related changes within the retinal vasculature. Fortunately, however other manifestations may be present such as [Karseras, 2000]:

- Copper wire/ silver wire appearance of retinal arteries
- Arterio-venous nicking
- Loss of auto-regulation in retinal arteries

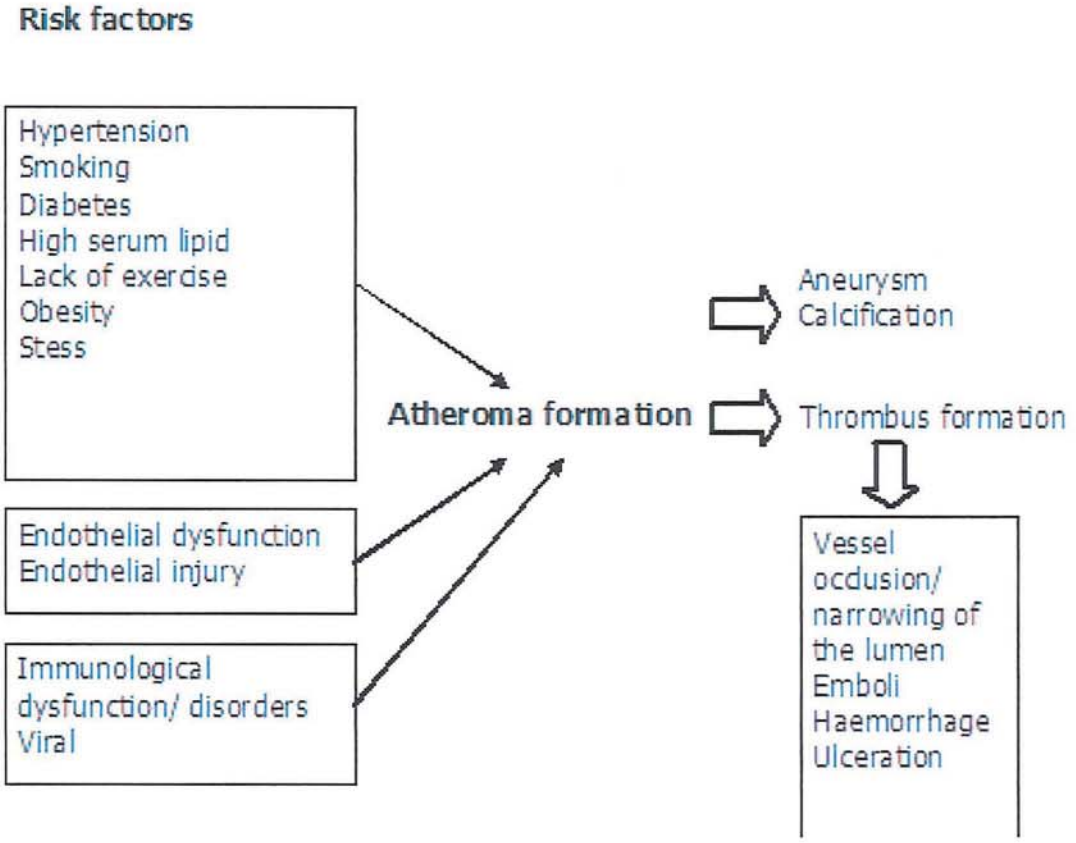

Figure 1.19: Risk factors and progression of arteriosclerosis

- Retinal ischaemic changes
- Retinal artery and/ or vein occlusions (central or branches)
- Atheromatous emboli seen in retinal vasculature
- Lipid emboli seen in retinal vasculature (Hollenhurst plaque)

Arteriolar narrowing has long been thought to be a major feature of ATS vascular changes in the ocular circulation, however, latest studies proving this wrong, showing more evidence for arterio-venous nicking as a clinical feature of ATS related vascular changes in the eye [Wong et al., 2003b]. These changes can lead to severe partial of complete vision loss, depending on the extent and site of the damage. As the disease progresses, the vascular system slowly loses its auto-regulation, due to the manifestations described above. While auto-regulatory mechanisms are no longer working reliably, permanent ocular damage can manifest:

- Central/ Branch Retinal artery and/ or vein occlusion [Ahuja et al., 1999, Wilson et al., 1979]
- Non-specific visual field defects [Erb et al., 2000]

- Ophthalmic artery aneurysm
- Diffuse colour vision disturbances [Erb and Fahle, 2006]
- Age-related maculopathy [Dasch et al., 2005]
- Glaucoma [Flammer et al., 1996, Geijssen and Greve, 1987, 1995]

1.6.6 Cardiac ischaemia

Cardiac ischaemia (also known as ischaemic heart disease) is a condition in which myocardial oxygen demand is insufficient. This in turn leads to hypoxia and accumulation of waste metabolites. The condition is most commonly caused by atherosclerotic disease of the coronary arteries, more widely known as coronary artery (CAD) or coronary heart disease (CHD). If the oxygen demand is not met, prolonged or the condition is undetected, this can result in myocardial ischaemia or, worse, myocardial infarction (MI). The latter often causing sudden cardiac death (SCD) [Fagan and Sunthareswaran, 2002].

Ischaemic heart disease can be sub-classified:

1.6.6.1 Chronic

This type of cardiac ischaemia is more prevalent amongst the elderly, especially those suffering advanced ATS and in such cases many coronary arteries are affected. If untreated, atrophy of the myocytes occurs followed by diffuse fibrosis causing acute MI and severe arrhythmia which can lead to SCD.

1.6.6.2 Acute ischaemic heart disease/ Unstable angina

Unstable angina and acute MI, sometimes described as acute coronary syndromes, both commonly result from atherosclerotic plaque rupture, platelet aggregation, thrombus formation or unopposed vasoconstriction. Its clinical presentation is a sudden onset of angina where the episodes are more frequent and prolonged in duration. It is often a precursor of MI and if untreated potentially leads to SCD.

1.6.6.3 Angina pectoris

Angina is a condition in which myocardial oxygen demand is not met sufficiently. Due to decreased perfusion, waste metabolites accumulate at the site of the obstruction, causing pain. It is often induced by exercise and the patient experiences transient episodes of chest pain. The pain commonly radiates towards the neck and left arm.

Classic angina This is the term used for angina caused by emotion or excitation. Usually the pain disappears with rest. This condition can be stable or unstable depending upon its clinical presentation. In a stable condition the pain can be due to an insufficient oxygen supply caused by a gradual narrowing of the artery, termed stenosis. In an unstable condition, the cause is more likely to be a thrombus blocking the blood supply.

Vasospastic Angina/ Prinzmetals angina This is the term used to describe angina which is caused by vasospasm in coronary arteries. Due to the spasm in the coronary arteries, oxygen supply is no longer met and potentially causes MI if untreated.

1.6.6.4 Myocardial Infarction

Myocardial infarction results as a consequence of prolonged interruption of blood supply to the heart muscle. During these prolonged episodes of lack of oxygen supply, myocyte atrophy occurs which progress into local necrosis of the heart muscle at the site of blood supply obstruction.

1.6.7 Stable angina

Stable angina is the term used to describe a pattern of recurrent, predictable but transient angina during exertion. In stable angina, flow through coronary arteries is obstructed by atheromatous plaques. This narrows the vessel lumen and can cause inappropriate vasoconstriction and therefore incapable to provide enough oxygen to the heart muscle. The symptoms experienced by the affected individual are usually pain triggered by exercise, excitation or emotional/mental stress causing sympathetic activation, increased heart rate (HR) and blood pressure (BP) and contractility. One can be without symptoms even if the affected coronary artery is stenosed up to 70% [Lilly, 2003]. This is because oxygen demand at rest is low and still maintained sufficiently. ATS-derived vascular endothelial dysfunction is thought to partially contribute to the inadequate oxygen supply. In healthy individuals increased oxygen demand during exercise is maintained by coronary blood flow auto-regulation; increased local accumulation of metabolites induces release of vaso-active molecules in order to dilate the artery, increasing the lumen to meet the increased oxygen demand.

1.6.8 Cardiac Syndrome X (CSX)

This term is used to describe a condition where patients experience typical symptoms of angina and yield a positive exercise stress test but have normal coronary

angiograms and no evidence of significant atherosclerosis. Inappropriate vasodilation of resistance vessels, microvascular dysfunction, vasospasm, abnormal pain perceptions are all reasons thought to contribute to the pathogenesis of ischaemia in this condition. In some cases it has also been associated with migraine and Raynaud's syndrome, supporting the hypothesis of a general vascular disorder. About 2% of hospital admissions with symptoms of unstable angina turn out to be Syndrome X after diagnosis [Kaski et al., 2004, Kaski, 2004]. The condition is more common in men than in women with a ratio of 5:1, appearing most frequently in the adult age (50yrs). Whereas in CAD of atherosclerotic origin many risk factors contribute to progression of the disease, in Syndrome X smoking and endothelial dysfunction are the major risk factors [Kaski, 2004, Kaski and Russo, 2000, Ashikaga et al., 2007].

Diagnosis is very difficult as there is no test which definitively proves that a patient is suffering CSX. Most of the time diagnosis is made upon excluding other causes of chest pain. Exercise testing, cardiac catheterisation and MRI scanning have shown to be the most helpful in diagnosing CSX. The latter has been employed in research centres, showing abnormal blood flow in the heart muscle in patients suffering CSX after using provocative tests such as adenosine or dobutamine intravenously. MRI scanning is still not used in routine examination, but has shown the strongest evidence that CSX patients exhibit a physiological abnormality associated with their angina.

1.7 Systemic circulation

1.7.1 Blood pressure and formulas

BP is defined by the two extreme ends of systemic BP, namely systolic and diastolic pressure. Systolic blood pressure (SBP) is the highest measured pressure, occurring during ventricular contraction. Diastolic blood pressure (DBP) is the lowest measured pressure, occurring during ventricular relaxation and refilling. Blood pressure can be measured in millimetres of mercury using a sphygmomanometer at the brachial artery of the arm. Both, systolic and diastolic BP rises with increasing age. Risk of complications due to elevated BP increases progressively with increasing BP values, therefore the cut-off point for the definition of hypertension has been constantly discussed and reassessed. The current guidelines for classification of hypertension are based on the initial publication from the World Health Organization (WHO) and the International Society of Hypertension (ISH) of 1999. However, the latest classification implies categories for optimal, normal and high-end normal pressure grouped in 3 grades of HT (see Table 1.3 on page 62). In the case of SBP and DBP falling in different categories, the

BP-Category	SBP [mmHg]	DBP [mmHg]
Optimal	<120	<80
Normal	120-129	80-84
High-end Normal	130-139	85-89
Hypertension		
Grade 1 - MILD	140-159	90-99
Grade 2 - MODERATE	150-179	100-109
Grade 3 - SEVERE	>180	>110
Isolated systolic hypertension		
(graded also as 1, 2 or 3)	>140	<90

Table 1.3: Classification table for hypertension according to the World Health Organisation and the International Society of Hypertension

BP Acquisition	SBP [mmHg]	DBP [mmHg]
Office/ Clinic	140	90
24-hour	125-130	90
Day	130-135	85
Night	120	70
Home	130-135	85

Table 1.4: Average arterial blood pressure according to different acquisition times

highest category is used for classification and assessment of cardiovascular risk [Brookes, 2007].

However, when based on multiple BP measurements other authors suggest a cut of at 137 SBP and 84 DBP [Verberk et al., 2007].

Mean arterial blood pressure (MABP) is another term used to describe BP; it describes the function of cardiac output in relation to arteriolar resistance.

$$MABP = (2/3 \times DBP) + (1/3 \times SBP) \tag{1.7}$$

Individual variations in blood pressure are common. Emotional stress such as fear and excitement and muscular exertion raise SBP. In systemic disease BP may be altered in many ways, depending on the underlying cause [Sunthareswaran, 2002].

1.7.1.1 Regulation of systemic blood pressure

Systemic BP is the product of cardiac output (CO) and total peripheral resistance (TPR) and can thus be calculated as:

$$BP = CO \times TPR \tag{1.8}$$

Due to these relationships at least three systems are directly involved in blood

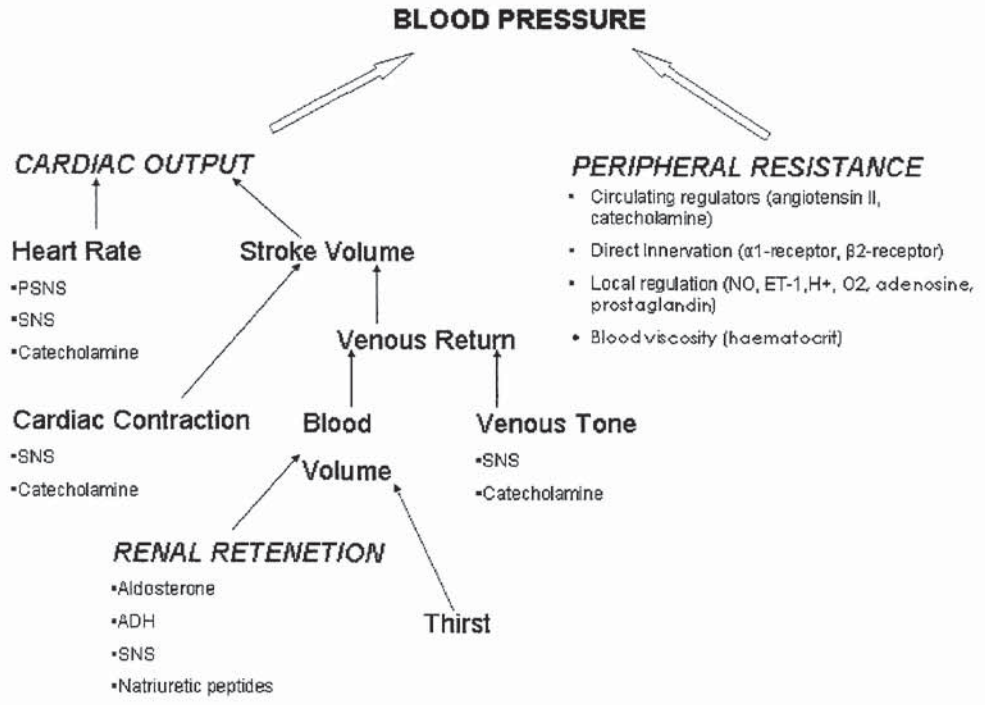

Figure 1.20: Factors involved in the regulation of systemic blood pressure

pressure regulation, namely

- The heart; acting as the pump responsible for pressure
- Blood vessel tone; vascular resistance
- The kidney; regulator for intravascular volume

1.7.1.2 Baroreceptor reflex

Baroreceptors adjust their response to an increase and decrease in BP. Dependent upon the cardiovascular system with its feedback mechanisms constantly monitoring arterial pressure, systemic blood pressure is included in, and influenced by this mechanism too. In order to maintain constant systemic BP, receptors within the aortic arch and carotid sinuses, namely baroreceptors monitor changes in pressure by detecting stretch and deformation within the arteries. Dependent on increase or decrease in pressure they increase (for increased pressure) or decrease (for decreased pressure) their impulses to the central nervous system (medulla), which in turn activates the ANS to restore BP to its baseline levels. This activation is conducted by impulse transmission through the glossopharyngeal (carotid sinus receptor stimulation) and vagus (aortic arch receptor

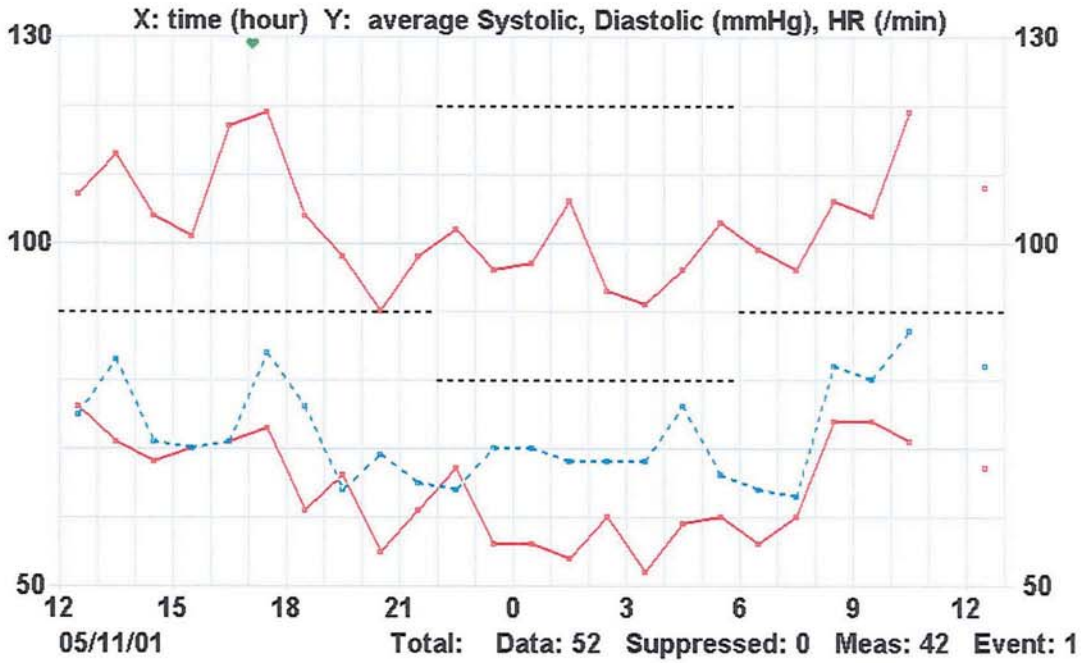

Figure 1.21: Normal nocturnal blood pressure dip (blood pressure drops up to 10% during sleep compared to wake period)

stimulation) nerve. The main role of baroreceptors is to monitor and adjust moment to moment pressure differences. It is therefore only responsible for short term BP modulation.

1.7.1.3 Circardian variation in systemic blood pressure

Circardian BP is defined as active minus passive BP, for each, SBP and DBP. In order to obtain reliable values, true daytime and true night time BP analysis is necessary. In order to achieve this, the subjects were asked to fill in a diary with information about their daily routine (active work, rest, exercise) and the time they went to sleep and got up [O'Brien et al., 2005, 2003]. Systemic BP, both SBP and DBP, varies throughout the day. It reaches a peak in the late afternoon and is lowest during sleep [Baumgart, 1991]. This variation arises due to physical and mental activity, stress and change in posture during sleep [Prill and Fahrenberg, 2007]. Typically, nocturnal BP is about 10% to 20% lower than diurnal BP; referred to as the nocturnal BP dip. This dip is present in approximately two thirds of the healthy population, referred to as dippers. Individuals exhibiting a nocturnal BP fall of higher than 20% are referred to as extreme-dippers or over-dippers. This can be partly due to intake of antihypertensive medication [Izzedine et al., 2006]. Non-dippers, which show either no dip in nocturnal BP or a reduction of less than 10% compared to diurnal values are at higher risk of target organ damage associated with CVD [Roman et al., 1997]. This is partly due to an altered baroreceptor function.

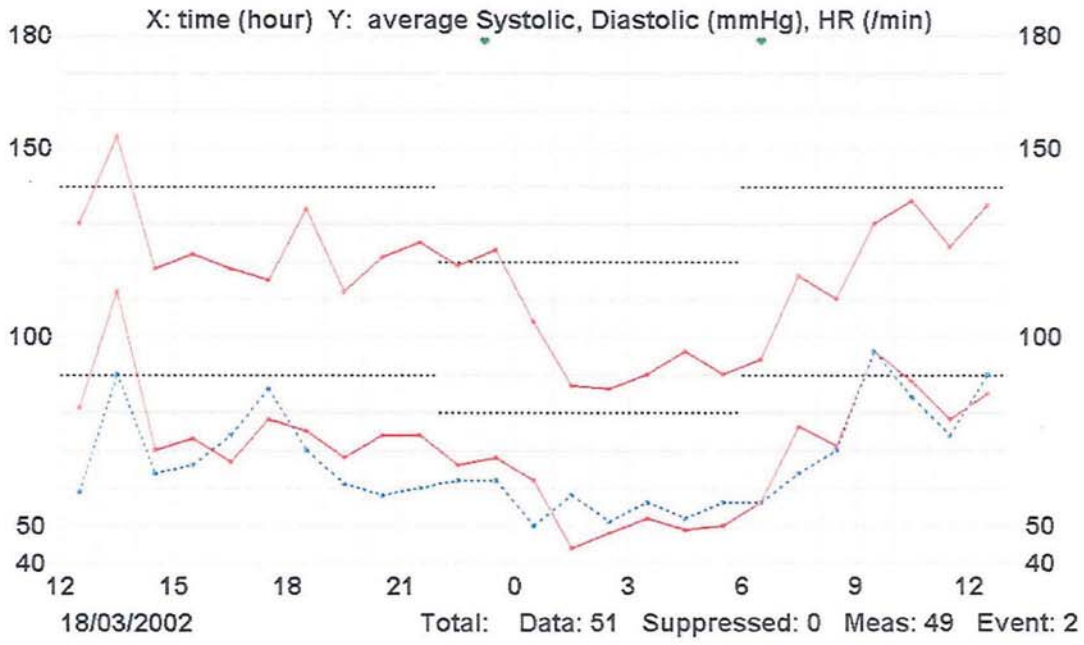

Figure 1.22: Abnormal nocturnal blood pressure dip: overdipper (blood pressure drops between 10% and 20% during sleep compared to wake period)

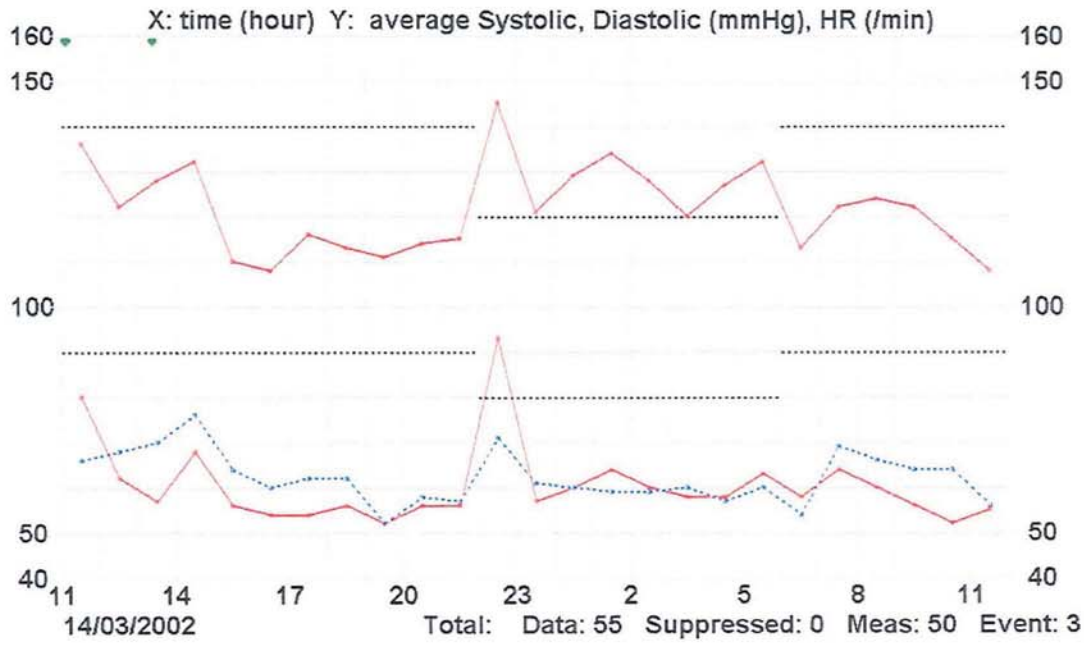

Figure 1.23: Absent nocturnal blood pressure dip: non-dipper (blood pressure does not change, or increase during sleep compared to wake period)

1.7.2 Techniques for assessment of systemic circulatory parameters: Ambulatory blood pressure monitoring

Ambulatory blood pressure monitoring Ambulatory blood pressure monitoring (ABPM) has proven to be a valuable addition to standard routine BP measurement in the physician consulting room [Dolan et al., 2005]. This method not only provides 24 hour BP values, but provides a better insight into the patients BP profile (eg. dipping status). Furthermore it has been shown to be of particular use in assessing those individuals termed "white-coat hypertensives", those who exhibit high BP values only in clinical settings [White, 2008]. Another useful parameter, readily accessible from ABPM, is the ambulatory arterial stiffness index (AASI). This parameter (described below) is receiving increased attention in recent studies examining its potential as independent predictor for CVD and cerebrovascular disease and its implications [Dolan et al., 2006b, Hansen et al., 2006, Ben Dov et al., 2008].

1.7.2.1 Cardio Tens: Ambulatory Blood Pressure and ECG monitor

The device used for this thesis was a computer operated ambulatory BP and ECG monitor (Cardiotens-01, PMS Instruments, Maidenhead, UK). BP measurements are indirectly obtained, based on an oscillometric technique, which measures BP by comparing the magnitude of the pressure oscillation to the pressure of the cuff. ECG can be measured by using a number of different lead setups, depending on the purpose. The monitor was connected via fiber-optic cable to a personal computer and programmed by using the BP monitoring software Cardiovisions 1.7.2 (PMS Instruments, Maidenhead, UK). The software provides a detailed protocol which can be customised for each patient measured, such as measurement intervals, trigger settings for additional readings and ECG compression. Furthermore, the software has a graphical and statistical package to analyse BP and ECG recordings. The device used has been validated and used in a number of clinical studies before, this is of great importance as variability and measurement errors kept a to minimum [O'Brien et al., 2002, Sims et al., 2005].

1.7.2.2 Ambulatory Arterial Stiffness Index (AASI)

Arterial stiffness depends on distending pressure, meaning a rise in arterial pressure leads to an exponentially increasing stiffness [Dolan et al., 2006a]. However, MAP varies throughout the day and depends on physical and mental activity and declines with sleep. In healthy individuals SBP and DBP changes occur in a parallel manner throughout the daily BP range. These changes may not occur in those suffering from vascular disease where arteries are less elastic. In such patients, increases in distending pressure are associated with higher increases in

SBP relative to DBP. The ambulatory arterial stiffness index reflects the dynamic relationship between SBP and DBP. AASI is defined as 1 minus the regression slope calculated from all 24 hour BP recordings by plotting DBP against SBP, for each subject separately [Dolan et al., 2006a, Li et al., 2006a]. Therefore the closer the value for AASI is to 1, the stiffer the arterial tree of a given subject.

$$AASI = 1 - R(DBP : SBP) \tag{1.9}$$

Recent studies showed a similar AASI between men and women without vascular disease. Women, however, exhibit higher AASI than men when suffering from diabetes mellitus or CHD [Dolan et al., 2006b]. The predictable value of such markers for cardio and cerebrovascular mortality has been evaluated recently in a number of studies [Dolan et al., 2006b, Hansen et al., 2006]. Normal values as determined by large multi-ethnic population studies, suggest that the upper boundary of AASI is 0.53 at 20 years through to 0.72 at 80 years [Li et al., 2006b]. ECG and ABPM were measured using the Cardiotens-01 (Meditech Ltd, Hungary for PMS Instruments, UK), an ambulatory BP and ECG monitor.

1.7.3 Heart rate variability analysis

HRV can be analysed in either the time domain or the frequency domain analysis of ECG recordings. Here we used frequency domain analysis which is therefore explained in more detail. The frequency domain analysis was chosen as it makes the analysis of global variations much simpler [Bracewell, 2003]. In order to analyse the data, ECG recordings had to be free of noise and needed pre-selection to ensure accurate analysis. After that recordings were Fourier transformed to obtain power spectra of the transform. From this power spectrum two frequency ranges were assessed: low frequency (LF: 0.04-0.15 Hz) and high frequency (HF: 0.15-0.40 Hz). LF is mainly a measure of sympathetic activity with minor influence of parasympathetic activity [Sleight et al., 1995] whereas HF represents solely parasympathetic activity [Pagani et al., 1984, 1986]. Furthermore LF:HF ratio was calculated as a measure of sympathovagal balance, an increase of this ratio indicating a predominance of the sympathetic versus parasympathetic nervous system activity.

1.8 Biomedical studies

1.8.1 Background

In recent years a number of circulating substances involved in blood flow (BF) regulation and those released by endothelial cells (cells lining the inner surface

of all blood vessels) have found to be strong independent markers of systemic vascular disease [Vischer, 2006, Ishihata and Katano, 2006, Lerman et al., 1991, Noll et al., 1996, Tsui and Dashwood, 2005, Wu and Thiagarajan, 1996]. Patients suffering from cardiovascular, peripheral vascular and ocular vascular disease have been shown to have above normal levels of these markers.

1.8.2 Vascular endothelium

The vascular endothelium is a single cell layer consisting of the so called endothelial cells (ET cells). Function of the vascular endothelium is essential for the maintenance of BF auto-regulation. ET cells provide the inner lining of every blood vessel. The two most important functions are, to

- provide a smooth inner surface in order to minimize sheer stress and prevent flow disturbances
- regulate blood flow by direct release of vaso-active molecules into the blood stream

Any impairment of those cells may result in an impairment of vascular function due to an imbalance of vaso-active molecules.

1.8.3 Normal endothelial function

In a healthy individual, both vaso-constrictive and vaso-dilatory molecules are in equilibrium. Furthermore, permeability of ET cells and secretion and expression of vaso-active substances is regulated according to local and global demand.

1.8.4 Endothelial dysfunction

In disease state such as e.g. CVD, ET cells and their function is compromised by multi-factorial mechanisms leading to an imbalance of vaso-constrictive and vaso-dilatory substances and hereby causing inappropriate regulatory responses of the vasculature changes in flow, pressure and volume. To date it is unclear if damaged ET cells are the cause of endothelial dysfunction or if they have been damaged due to alteration of other structural and regulatory factors such as e.g. ANS impairment. A number of circulating markers have been evaluated and found to be useful measures to quantify and describe endothelial function/ dysfunction in health and disease. For the purpose of the presented thesis we analysed venous blood samples for the markers described in the following section.

1.8.5 Circulating markers of endothelial function

1.8.5.1 Von Willebrandt factor

Von Willebrandt factor (vWf) is a glycoprotein involved in the regulation of haemostasis. It is mainly released by ET cells but also from platelets [Nichols et al., 1995], where it is stored in Weibel Palade bodies until activated. Primary function of vWf is to bind to other proteins such as factor VIII (as inactive form) to be activated by thrombin, eg when coagulation is stimulated. It binds to collagen when exposed to blood vessel damage and can bind to platelets in all circumstances but most efficient during high shear stress. During the latter, vWf which possesses a coiled structure, uncoils, decelerating the passing platelets and herby playing a major role in blood coagulation [Sadler, 1998]. Plasma levels of vWf are dependent upon genetic and non-genetic factors, whereas the genetic factors are: blood group and vWf type mutations and non-genetic factors such as ageing via impaired NO production, inflammation, free radical production and diabetes. A severe deficiency of vWf is known as von Willebrand Disease and characterised by its bleeding disorder [Sadler, 1998, Mannucci, 1998].

vWf in systemic vascular disease To date it is still unclear if plasma levels of vWf are a useful measure for arterial thrombus formation or merely reflecting the endothelial change in function. Plasma levels are thought to be an indicator of the state of endothelial function which is in turn a major contributor for the pathogenesis of CVD beyond the direct effect of vWf levels. Numerous studies have investigated the plasma levels and its association to cardiovascular risk factors. Investigators of the ARIC study [Folsom et al., 1997] assessed the relative risk for CV death using vWf plasma levels but found this to become negligible after adjusting for conventional CV risk factors, more so in the presence of diabetes. Other studies showing only a weak relationship with CHD risk [Rumley et al., 1999, Meade et al., 1994, Smith et al., 1997], supporting the current opinion that vWf levels are not a major predictor for CVD risk. However, those results do not exclude the possible potentiating effects of vWf upon conventional risk factors, possibly amplifying their detrimental effect in the pathogenesis of CVD and endothelial dysfunction.

vWf as a marker of endothelial dysfunction As discussed in more detail above, vWf plasma levels are a weak predictor for CV death in the general population, but a strong predictor within the high risk population especially those with previous CV events, diabetes or old age. More so, plasma levels of vWf correlate well with the finger pulse wave velocity (PWV), which is a marker of endothelial function as assessed by Troseid and co workers in CVD patients [Troseid et al.,

2006]. Authors of the "Aterhogene"-study assessing angiographically proven CAD patients also confirmed the week association of CV death and vWf levels. Plasma levels of vWf should not be underestimated but interpretation has to be taken with great caution as it does not show any correlation with structural measures of endothelial dysfunction such as intima media thickness (IMT) [Troseid et al., 2006]. It is playing a pivotal role in platelet adhesion and aggregation at sites of high shear stress, for example in coronary arteries with stenosis or ruptured atherosclerotic plaque lesions. Therefore, the evidence of it being not only a marker but a strong effector in the pathogenesis of endothelial dysfunction is growing.

1.8.5.2 Endothelin-1

Endothelin is a vasoactive, neural 21 amino acid peptide produced mainly by vascular endothelial cells. Three types of endothelin have been identified: ET-1, ET-2 and ET-3; whereas ET-1 has the strongest effect on the vascular system.

ET-1 and systemic disease It has been demonstrated in previous studies that ET-1 plasma levels are significantly raised in individuals with symptomatic ATS, independently from age [Lerman et al., 1991](Lerman et al. 997-1001) and patients suffering from CAD [Videm et al., 2006]. But it remains still unclear if the raised levels are causal for the development of ATS and endothelial dysfunction or if damaged endothelial cells release higher levels of ET-1. It has also been suggested to influence the inflammatory response to ATS [Videm et al., 2006]. Other studies have elucidated a relationship between low social status, future risk of vascular disease and increased levels of soluble intercellular adhesion molecule-1 (sIAM-1) and ET-1 [Hong et al., 2006]. Further more, Maeda et al were able to show a linear relationship between blood pressure, ET-1 levels and weight loss in obese individuals [Maeda et al., 2006]. This suggests a direct influence of obesity on ET-1 plasma levels as well as on vascular endothelial dysfunction. This is of importance as obesity associated with vascular endothelial dysfunction contributes to the development of DM, HT and ATS which are all known risk factors in the development of cardiovascular disease [Maeda et al., 2006]. Ishihata and Katano have used a rat model to investigate the role of angiotensin II (A II) and ET-1 in aging related functional changes of the cardiovascular system [Ishihata and Katano, 2006]. Their results show that both, angiotensin II and ET-1 are playing a key role in age related changes in the function of the cardiovascular system, being involved in the pathogenesis of cardiac fibrosis and coronary vascular ATS. Angiotiensin II (AII) and ET-1 caused greater vasoconstriction in aged rats than in young, whereas receptor affinity was not changed but receptor density was increased significantly in aged rats compared to younger ones. The precise mechanism of this receptor up-regulation remains unknown [Ishihata and

Katano, 2006].

ET-1 and peripheral vascular disease As mentioned above, ET-1 is a very potent vasoconstrictor and has therefore been of high interest in studies investigating the peripheral vascular system. Lipa et al have shown a significant vasoconstriction mediated mainly by ETA receptors of ET-1 on skin arteries [Lipa et al., 1999]. Furthermore Tsui and Dashwood suggest its involvement in peripheral vascular disease due to its proinflammatory, proliferative and vasoconsticting properties to the vascular system [Tsui and Dashwood, 2005].

ET-1 and ocular disease The ocular circulation is very vulnerable to perfusion changes. As described before, ET-1 one has been shown to be involved in the disease mechanisms altering the systemic vascular system, this in turn can affect the ocular perfusion being altered as well. More recent studies support the involvement of ET-1 in the development and progress of ocular diseases due to the fact that ET-1 plasma levels are raised in all diseases related to vasospasm [Zimmermann, 1997, Haufroid and Collignon-Robe, 2004]. The cellular distribution of ET-1 in the human eye as described by Wollensak et al. suggests that raised levels are of particular importance in a variety of ocular diseases, such as DM and HT [Wollensak et al., 1998]. DM and HT are known risk factors for the development and progression of CAD, and as mentioned before, showing raised ET-1 levels, demonstrate the importance of differential diagnosis in order to rule out any ocular involvement. Furthermore, ET-1 has shown to be involved in the pathogenesis of glaucoma due to its impact on the regulation of IOP and modulation of ocular blood flow [Haefliger et al., 1999].

1.8.6 Techniques for assessing circulating markers of endothelial function

1.8.6.1 Measurement of plasma vWf levels

Fasted venous blood samples were collected in citrated tubes. After spinning them in a centrifuge at 3000 rpm for 20 minutes the supernatant was collected and stored in a freezer at -80ºC. After all samples were collected, citrated plasma was thawed and analysed for vWf using standard ELISA with commercial antisera (Dako, Denmark). The analysis procedure as described elsewhere in more detail was in brief:

1. Coat microtitre plate with 100µl of a dilution of the primary antiserum (30µl in 20.5ml coating buffer pH 9.6) at room temperature for >60 minutes or overnight in a fridge

2. Wash 3x, add 100µl 1/40 serum or plasma in pbs/tween, neet tissure culture fluid, and standards, incubate for >60 minutes at room temperature

3. Wash 3x, add 100µl secondary antiserum – the peroxidase-labelled conjugate (30µl in 20.5ml PBS), incubate at room temperature for >45 minutes

4. Wash 3x, add 100µl substrate (orthophenylene diamine, hydrogen peroxide, ph 5 citrate buffer). The colour develops almost immediately.

5. Stop with 50µl acid

6. Read at 492nm

1.9 Statistical analysis

Statistical analysis for all studies presented in this thesis were performed using a comercially available program called STATISTICA version 6.0 (Statsoft Ltd., Tusla, USA). However, before ethical approval was granted, a power calculation to determine the number of patients/ subjects needed for each study had to be perfomed.

This is of great importance to any experimental design, because without these calculations, sample size may be too high or too low. If sample size is too low, the experiment will lack the precision to provide reliable answers/ results to the research questions it is investigating. If sample size is too large, time and resources are wasted, usually for minimal gain. Before explaining the procedure of how to determine sample size for a particular study one should know about the factors involved in it as well as what outline/ design the study will have.

Sample size: The number of patients/ subjects required for the study

Power: The power determines if a study/ experiment will have a significant (statistically significant, NOT necessarily clinically significant) result, at a p-value of less than the specified significance level (usually 5% or p=0.05). This probability is computed under the assumption that the difference or strength of association equals the minimum detectable difference.

Minimal detectable difference: The minimum difference between the treatments/ patient groups that one wants to detect. In a study of association it is the smallest change in the dependent (outcome variable, response), per unit change in the independent(input variable, covariate) that is plausible.

Parallel design: A parallel designed clinical trial compares the results of a treatment/ parameter on two separate groups of patients/ subjects (e.g. patients and healthy controls). The sample size calculated for a parallel design can be used for any study where two groups are being compared.

Crossover study: In a crossover design the results of two treatments on the same group of patients/ subjects is compared. Furthermore the sample size calculated for a crossover design can be used for a study which compares the value of a variable before and after treatment. The standard deviation (SD) of the outcome variable is expressed as either the within patient standard deviation or the standard deviation of the difference.

Study to find an association: In a study to find an association however, a variable, here the dependent variable, is affected by another, the so-called independent variable.

Success/Failure: The outcome of this design, usually with two values, can be treatment success or treatment failure.

Measurement: The outcome of this study design is a continuous measurement.

Time to an Event: The outcome of this study design is a time, e.g. the time to death, relapse, a.s.o....

In the presented thesis we applied a parallel design to study the difference between subject groups (smokers vs non-smokers, CAD patients vs healthy controls) as well as between treatments (non smoking vs acute smoking).

1.9.1 Sample size calculation

In order to calculate the sample size for a prospective study, it is important to know a minimum of a few parameters about the sample to be investigated, e.g. previous studies' data of a similar patient/ subject cohort (number of patients/subjects, parameters assessed, confidence limits, standart deviation, data distribution). Based on these values, normative data (if available) as well as data distribution (if available) and errors involved in data sampling power calculation for sample size can be applied. A more detailed view on errors involved in power calculations and formula derivation is given in Statistics: Methods and Applications [Hill and Lewicki, 2005]. However, when investigating a new patient group or using new equipment, there is hardly any data to compare available or at the most data of

only one of the parameters investigated in the new study. Furthermore, different parameters/ measurements have different sensitivities, variablility and distributions, therefore the power calculation to determine the necessary sample size can vary a lot as calculated for each parameter. In this case, the necessary number of patients/ subjects has to be determined within a balance of how powerful (=how predictive) each of the parameters choosen is in relation to address the research question.

In the presented theses new measurement techniques and novel analysis has been used in a sample of patients/ subjects which have so far not been examined with these techniques, therefore power calulation was only performed on the basis of a few parameters available, namely retrobulbar blood flow, retinal blood flow, retinal vascular diameter and visual fields [Baxter and Williamson, 1995, Williamson et al., 1995a,b][Roff Hilton et al., 2003][Nagel and Vilser, 2004a,b, Nagel et al., 2004].

Power calculation based on these studies has lead to the sample sizes described in the following chapter. However, having calculated the sample size, this does not guaranty to reach statistical significance. Furthermore, having reached a statistically significant result does not mean that this result has automatically clinical significance.

Chapter 2

The effect of chronic smoking on retinal vessel dynamics in otherwise healthy individuals

2.1 Abstract

Purpose: To assess the effect of chronic smoking on retinal vessels' reactivity to flickering light provocation in otherwise healthy smoking subjects compared to age-matched non-smoking controls.

Methods: 71 eyes (29 women and 42 men, mean age: 36 (14) yrs) of non-smokers and 21 eyes (13 women and 8 men, mean age: 31(10) yrs) of smokers subjects were examined using retinal vessel assessment by means of the Retinal Vessel Analyser (RVA). Systemic BP was measured using a manual sphygmomanometer (Digital BP Monitor: UA-767EX-C, PMS Instruments Ltd., Maidenhead, UK) in all subjects. In addition, IOP was also assessed by means of contact tonometry using a hand held device (TonopenXL, Medtronic Solan, PMS Instruments Ltd., Maidenhead, UK).

Results: There was no measurable effect of age upon retinal vascular reactivity in both smokers and non-smokers. In addition, the measured parameters of retinal vessels reactivity were not influenced by the level of systemic BP or IOP. In non-smokers, the one second mean baseline diameter immediately before flicker initiation was significantly inversely correlated with the proceeding amount of arterial dilation to flicker in all three stimulation cycles (cycle1:$p=0.001$, cycle 2: $p=0.009$, cycle3: $p=0.029$). Chronic smokers showed both an increased level of arterial BDF prior to flicker stimulation and an increased stiffness of retinal arterioles when stimulated repeatedly with flickering light as compared to age matched non-smoking subjects (ANOVA: $p=0.005$ and $p=0.035$, respectively).

Conclusions: Smoking individuals exhibited an abnormal vascular endothelial function at the level of retinal arterioles manifesting through a vasoconstrictory shift in reactivity to flicker light stimulation.

2.2 Introduction

Cigarette smoking represents the most important avoidable cause of mortality and morbidity in the western world and generally produces diffuse vascular injury in humans at many organ systems [Mayhan and Patel, 1997, Hanna, 2006, Barua et al., 2002b,a]; in addition, it represents a well established risk factor for atherosclerotic complications in both the coronary and cerebral circulation [Klein et al., 2000, Wang et al., 2006, Rahman and Laher, 2007b, Fang et al., 2004, Shinohara et al., 2006].

Morphological and functional effects of cigarette smoke and its extracts have been examined extensively [Hanna, 2006, Mayhan and Patel, 1997, Asmussen and Kjeldsen, 1975], however, the pathogenesis of cigarette induced vascular damage and functional impairment is still under investigation. The most common morphological change attributed to smoking at the retinal vessel level is chronic venous dilation [Wang et al., 2006, Wong et al., 2006a,b]. It has already been demonstrated that smoking results in abnormal increase in retrobulbar blood flow velocities [Kaiser et al., 1997], increased choroidal blood flow after isometric exercise [Wimpissinger et al., 2003] and abnormal constriction after inhalation of 100% oxygen [Wimpissinger et al., 2005]. In addition, carbogen breathing has been shown to cause significantly increased vascular reactivity as measured by fundus pulsation amplidude [Wimpissinger et al., 2004]. Functional vascular measures exploring the effect of chronic smoking on the eye are few; the methods used so far include the assessment of OBF in response to hyperoxia [Wimpissinger et al., 2005], carbogen breathing [Wimpissinger et al., 2004], retinal diameter assessment in response to arginine infusion [Garhofer et al., 2005] as well as the effect of chronic smoking on the retrobulbar blood flow velocities [Baxter and Williamson, 1995, Kaiser et al., 1997]. The aim of the present study was to assess the functional changes of retinal vessel motion at baseline as well as during flickering light stimulation in chronic smokers compared to non-smoking individuals.

2.3 Hypothesis

Chronic smoking results in an impairment of both systemic and ocular vascular function. These changes could result in abnormal perfusion with blood that could

make the eye more sensitive to various stressors and be at higher risk for ocular vascular pathologies.

2.4 Aims

The aim of this study was to assess the effect of chronic smoking on the retinal vascular reactivity at baseline and during flickering light stimulation in otherwise healthy individuals

2.5 Subjects and methods

2.5.1 Recruitment of healthy individuals

Healthy smoking and non-smoking individuals of all ages have been recruited from volunteers at Aston University, Birmingham. Smoking was identified by self report. In order to be classified as smoker, one had to smoke on a regular basis for at least 6 months prior to the study. No other tests were performed to confirm abstinence from this habit.

2.5.1.1 Inclusion criteria

Subjects of all ages (minimum age 18yrs) were included in the study, no upper age limit applied.

2.5.1.2 Exclusion criteria

Ocular and systemic exclusion criteria are outlined below:

- Patients with a refractive error of more than +/- 3dpt spherical equivalent and more than +/-1 dpt cylindrical equivalent due to the magnification/ minification causing over/ underestimation of retinal diameter measured;
- IOP above 24 mmHg;
- The presence of cataract or any other media opacities;
- Any history of intraocular surgery ;
- Any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system;
- Any history of systemic disease, such as diabetes mellitus, hypertension or any vascular abnormalities such as Raynaud's Syndrome; and

- Any use of vaso-active drugs such as blood thinners and lipid lowering drugs.

2.5.2 Ethical approval

Prior to the study ethical approval was obtained from Aston University Ethics Committee. Written informed consent was received from all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets of the Declaration of Helsinki.

2.5.3 Experimental protocol

Patient preparation All subjects were instructed to refrain from consuming caffeinated products, such as coffee or tea as well as from smoking, and drinking alcohol on the study day. Measurements were obtained between 12 noon and 2pm on all subjects.

2.5.3.1 Intraocular pressure measurement

Due to the study design the subjects could not be seated at a slit-lamp, therefore contact tonometry was performed with the TonopenXL (TonopenXL, Medtronic Solan, PMS Instuments, Maidenhead, UK). All measurements were taken after instillation of one drop of 0.4% benoxinate hydrochloride. The device automatically calculates the mean of 3 consecutive readings along with the coefficient of variance (CV).

2.5.3.2 Blood pressure and pulse assessment

After an acclimatization period of 15-20 minutes in a temperature controlled room of 22-25ºCelsius, SBP, DBP and heart rate were obtained by manual sphygmomanometer (Digital BP Monitor UA-767EX-C, PMS Instruments, Maidenhead, UK). During the measurement the subject was in a sitting position with their arm resting at heart level. The cuff was placed around the right arm in all subjects. SBP and DBP values were measured three times at baseline before the start of retinal vessel measurements to ensure the subject was acclimatized, this was reached when the BP measurements obtained were stable. BP measurements were also performed at 1 minute intervals during retinal vessel assessment.

2.5.3.3 Assessment of retinal vessel dynamics

Measurements were performed in one randomly selected eye of each patient. Retinal vascular dynamics, of both, arteries and veins was assessed with the

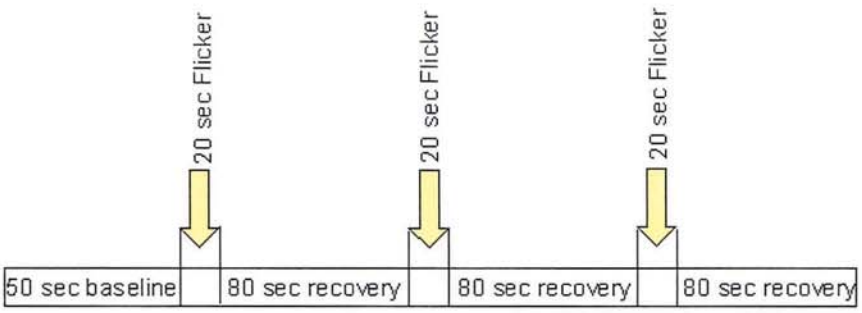

Figure 2.1: Measurement sequence of the retinal diameter assessment

RVA (Imedos GmbH, Germany) as already described in Section 1.3.3.2. After full pupil dilation was reached the measurement was obtained according to the protocol in Figure 2.1 on page 79. More detail on the parameters assessed can be found in Section 1.3.3.2.

As demonstrated in studies on human brain arterioles [Chan et al., 2008, Bauser-Heaton and Bohlen, 2007], we have also hypothesized that a retinal blood vessel has a fixed MD; similar to a rubber band, a vessel with a certain diameter has elastic properties that permit various diameter fluctuations (dilatation and constriction) up to a certain amount. If the vessel is dilated prior to exposure to certain provocation, any further increase in diameter can only occur up to the vessel's maximum capacity to dilate. As retinal vessels have similarities to those in the brain, we could expect a similar type of vascular reactivity as the one described above occurs in retinal arterioles and veins. In order to test this hypothesis, in each tested individual we compared the one-second mean baseline that precedes the flicker stimulation with a measure called dil. Dil was calculated according to following formula:

$$Dil = MD - OneSecondBaselineDiameter \tag{2.1}$$

Retinal vascular stiffness In order to assess the relative stiffness of retinal arterioles we have calculated the ratio between the baseline diameter fluctuation before stimulation and the maximum dilation (MD) after provocation; this ratio was called average peak ratio (APR, see below) and it reflects the degree of retinal arterioles elasticity.

$$APR = MD/BDF \tag{2.2}$$

Body Mass Index The body mass index (BMI) was calculated for each subject according to the formula below:

$$BMI = weight[kg]/(height[m]^2) \tag{2.3}$$

2.5.4 Statistical Analysis

All results are given as the mean +/- standard deviation (SD). Spearman's correlation coefficient R was used to explore the age dependency of dynamic response values to flicker light provocation and to assess any influence of baseline and demographic parameters upon experimental values. Group differences were analysed either by using the Mann-Whitney-U test or an unpaired t-test if normally distributed. When normal distribution was absent, the data analyzed was log transformed. In all other cases it was directly assessed by repeated measurements ANOVA and followed by post-hoc analysis using Tukeys test. Statistical significance was defined at the level of $p<0.05$. When computing multiple comparisons we set the significance level at $p<0.01$ to minimize bias.

2.6 Results

2.6.1 Sample

100 eyes of 45 women and 55 men (mean age: 36 (13) yrs) were included in this study. However, as a result of careful image analysis, 8 subjects whose data had poor image quality, showed poor fixation or had excessive movement during the experiment were excluded from the statistical analysis. Finally, 71 eyes (29 women and 42 men, mean age: 36 (14) yrs) of non-smokers and 21 eyes (13 women and 8 men, mean age: 31(10) yrs) of smokers subjects were included in the final analysis. There was no difference in age between the study groups ($p>0.05$; see Table 2.1 on page 81). In the group of smokers, cigarettes smoked per day ranged from 5 to 15 (cigarettes smoked per day: 9 (3)), and the length of their smoking history varied between 3 and 20 years (smoking history: 10 (5) yrs).

There was no statistically significant difference between groups at the baseline with regards to IOP, DBP, MAP and retinal arteriolar vascular diameter (all $p>0.05$; see Table 2.1 on page 81). However, nonsmokers had significantly higher BMI ($p=0.034$), SBP ($p=0.012$) and smaller venous diameters ($p=0.004$) than smoking individuals. Neither age, systemic BP nor BMI had any impact on the dynamic retinal vessel parameters measured in either smokers or non-smokers (ANCOVA $p>0.05$).

Parameter	Nonsmoker [n=71]	Smoker [n=21]	Mann-Whitney-U p-value [2sided]
	Mean (SD)	*Mean (SD)*	
AGE [yrs]	36 (13)	31 (10)	0.191
BMI [kg/ m²]	25 (3)	23 (3)	**0.034**
IOP [mmHg]	14 (3)	15 (2)	0.158
SBP [mmHg]	120 (12)	112 (10)	**0.012**
DBP [mmHg]	74 (9)	71 (9)	0.153
MAP [mmHg]	89 (9)	85 (9)	0.303
Size(A) [AU]	120.22 (20.10)	122.88 (15.32)	0.369
Size(V) [AU]	149.88 17.88)	161.27 (16.36)	**0.004**

Table 2.1: Group characteristics of the smoking and non-smoking sample; BMI: body mass index, IOP: Intra ocular pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial blood pressure, Size(A): arterial diameter, Size (V): venous diameter

Parameter	Mean (SD)	Spearman R [n=71]	p-value
Age & CV-A-MD [%]	0.013 (0.011)	0.17	0.155
Age & CV-A-MC [%]	0.012 (0.009)	-0.11	0.349
Age & CV-A-DA [%]	0.197 (0.148)	0.26	0.024
Age & CV-V-MD [%]	0.010 (0.008)	-0.07	0.526
Age & CV-V-MC [%]	0.008 (0.006)	-0.01	0.947
Age & CV-V-DA [%]	0.155 (0.106)	0.06	0.614
Age & CV-A-RT [%]	0.315 (0.193)	0.11	0.375
Age & CV-V-RT [%]	0.179 (0.127)	0.05	0.702

Table 2.2: Age effect on the measurements variability in both arteries and veins in non-smoking individuals; CV: Coefficient of variance, A: Artery, V: Vein, MD: Maximum dilation, MC: Maximum constriction, DA: Dilation amplitude, RT: Reaction time

2.6.2 Non-smoking subjects

2.6.2.1 The effect of age on measurement variability in non-smokers

In order to examine the impact of age on the measurement variability, CV-values were also examined in relation to this demographic parameter; however, no significant influence has been found (all p>0.01; see Table 2.2 on page 81).

2.6.2.2 The effect of baseline diameter before flicker stimulation on the amount of vascular dilation after flickering

Effects on the arteries The results listed in Table 2.3 on page 82 and Figure 2.2 on page 82 show a significant negative correlation between the arterial one-second mean baseline diameter immediately before flicker initiation and its corresponding value of dil (cycle one: R=-0.40, p=0.001; cycle two: R=-0.31,

Arteries			Veins	
Parameter	*Mean (SD)*		Parameter	*Mean (SD)*
baseline 1 [AU]	100.03 (1.47)		*baseline 1 [AU]*	99.94 (1.01)
baseline 2 [AU]	100.11 (1.24)		*baseline 2 [AU]*	100.06 (1.25)
baseline 3 [AU]	100.19 (1.36)		*baseline 3 [AU]*	100.03 (1.06)
dil 1 [AU]	4.61 (2.76)		*dil 1 [AU]*	5.88 (2.85
dil 2 [AU]	4.69 (2.83)		*dil 2 [AU]*	5.75 (2.61)
dil 3 [AU]	4.88 (2.86)		*dil 3 [AU]*	6.04 (2.39)

Table 2.3: Arterial and venous baseline diameters and their corresponding "Dil" values for each flicker cycle in the sample of non-smoking individuals; AU: Arbitrary Units

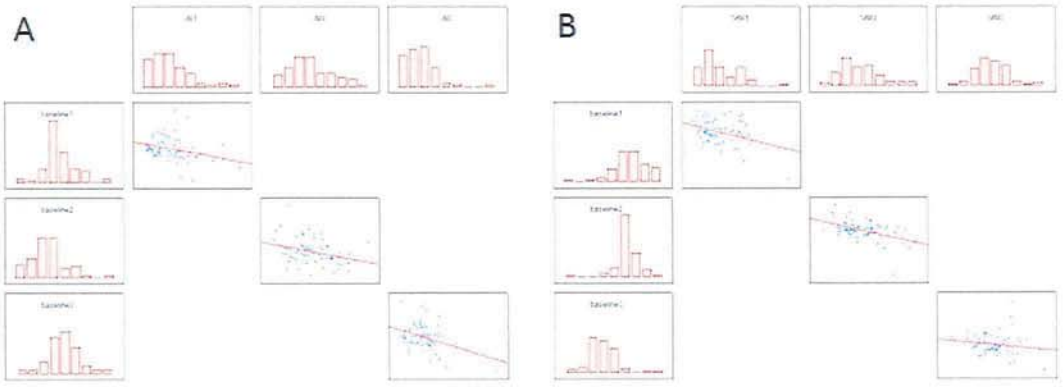

Figure 2.2: A) Correlation of the arterial baseline diameter before flicker stimulation and its corresponding value of "Dil" for each cycle; (B) Correlation venous baseline diameter before flicker stimulation and its corresponding value of "Dil" for each cycle

p=0.009; cycle three: R=-0.26, p=0.029).

Effects on the veins As described above for the arteries, we also examined the relationship between the individual venous baseline diameter values preceding each flicker stimulation and the MD for each cycle separately. There was a significant negative correlation for the first two stimulations but not for the third one (cycle one: R=-0.26, p=0.032; cycle two: R=-0.34, p=0.004; cycle three: R=-0.19, p>0.05).

Effect on arerial dilation amplitude Calculation of the correlation between the mean arterial BDF and the mean arterial DA to test the hypothesis that a high flexibility at baseline, as reflected through a large diameter fluctuation, would yield a greater dilation when stimulated with flickering light in retinal arterioles. There was a strong significant correlation between the average arteriolar BDF and the average arteriolar DA due to flicker light stimulation (average BDF= 4.15 (1.95),

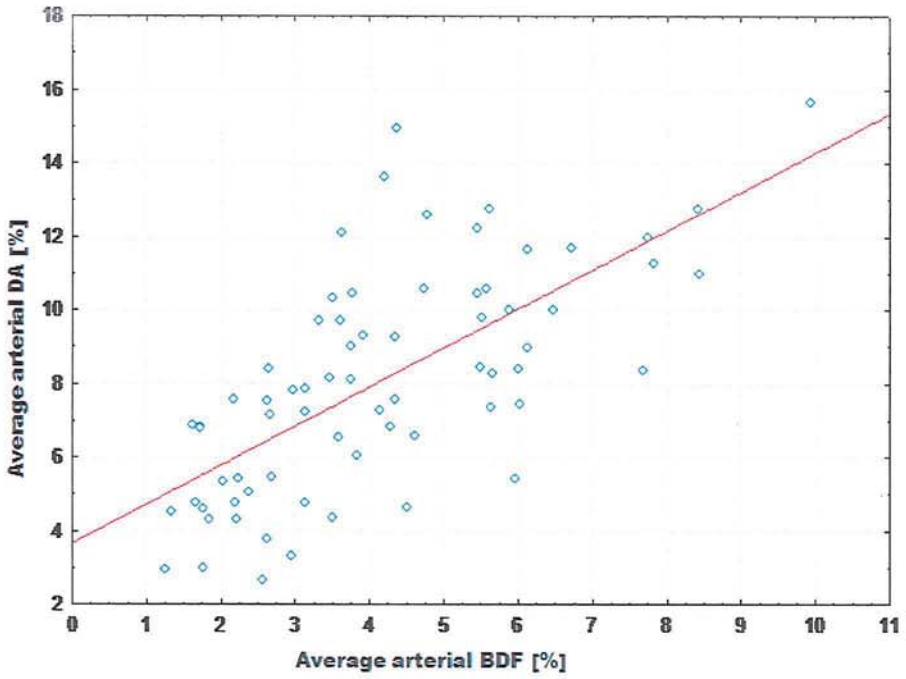

Figure 2.3: Correlation between the average arterial baseline diameter fluctuation (BDF) before flicker stimulation and the average arterial dilation amplitude (DA) due to flicker provocation in non-smokers

average DA= 8.14 (3.05), R=0.70, p<0.0001); Figure 2.3 on page 83.

2.6.3 Chronic smokers

2.6.3.1 The effect of age on measurement variability in smokers

In order to examine the impact of age on the measurement variability, CV-values were also examined in relation to this demographic parameter; however, no significant influence has been found, (all p>0.01; see Table 2.4 on page 83).

Parameter	Mean (SD)	Spearman R [n=21]	p-value
Age & CV-A-MD [%]	0.01 (0.01)	0.05	0.806
Age & CV-A-MC [%]	0.01 (0.01)	0.04	0.861
Age & CV-A-DA [%]	0.15 (0.11)	-0.04	0.852
Age & CV-V-MD [%]	0.01 (0.01)	-0.24	0.29
Age & CV-V-MC [%]	0.01 (0.01)	-0.16	0.487
Age & CV-V-DA [%]	0.20 (0.11)	-0.21	0.348
Age & CV-A-RT [%]	0.38 (0.20)	-0.02	0.93
Age & CV-V-RT [%]	0.18 (0.12)	-0.49	0.022

Table 2.4: Age effect on the measurements variability in both arteries and veins in smoking individuals; CV: Coefficient of variance, A: Artery, V: Vein, MD: Maximum dilation, MC: Maximum constriction, DA: Dilation amplitude, RT: Reaction time

Arteries			Veins	
Parameter	*Mean (SD)*		Parameter	*Mean (SD)*
baseline 1 [AU]	100.06 (1.49)		*baseline 1 [AU]*	100.22 (1.14)
baseline 2 [AU]	100.27 (1.53)		*baseline 2 [AU]*	100.15 (1.61)
baseline 3 [AU]	99.64 (1.92)		*baseline 3 [AU]*	99.54 (0.79)
dil 1 [AU]	5.80 (2.78)		*dil 1 [AU]*	6.37 (2.95)
dil 2 [AU]	5.27 (2.64)		*dil 2 [AU]*	6.92 (2.96)
dil 3 [AU]	6.89 (4.84)		*dil 3 [AU]*	7.05 (3.19)

Table 2.5: Arterial and venous baseline diameters and their corresponding "Dil" values for each flicker cycle in the sample of smoking individuals; AU: Arbitrary Units

2.6.3.2 The effect of baseline diameter before flicker stimulation on the amount of vascular dilation after flickering:

Effects on the arteries As with the non-smoking group, see above, there was a significant correlation present between the one-second mean baseline measurements and the corresponding value of "Dil" (see part A of Figure 2.4 on page 85). However, only the first and last baseline showed significant correlation with the preceding amount of dilation (R=-0.596; p=0.004 and R=-0.767; p=0.0001 respectively) but failed to do so for the second cycle of stimulation (R=-0.30, p>0.05).

Effects on the veins A significant correlation between venous dilation and the individual baseline diameter preceding flicker stimulation was only present for second stimulation cycle (R=-0.52, p=0.014) but failed to do so for the first (R=0.07, p>0.05) and third (R=-0.43, p=>0.05); see individual values in Table 2.5 on page 84 and part B of Figure 2.4 on page 85.

Effect on arterial dilation amplitude Similar to the group of non-smokers, smokers' mean arterial BDF before flicker stimulation of retinal arteries showed a significant correlation with their mean arterial DA due to flicker provocation (R=0.49, p=0.022; see Figure 2.5 on page 85).

2.6.4 Smokers versus non-smokers

There were significant differences between smokers and non-smokers regarding BDF (ANOVA p=0.005), DA (ANOVA p=0.002) and for the BFR (ANOVA p=0.02); see Table 2.6 on page 86. In all cases but one, there were no significant differences between groups regarding the dynamic values measured for all three cycles (seeTable 2.6 on page 86 and Figure 2.6 on page 87). The one exception

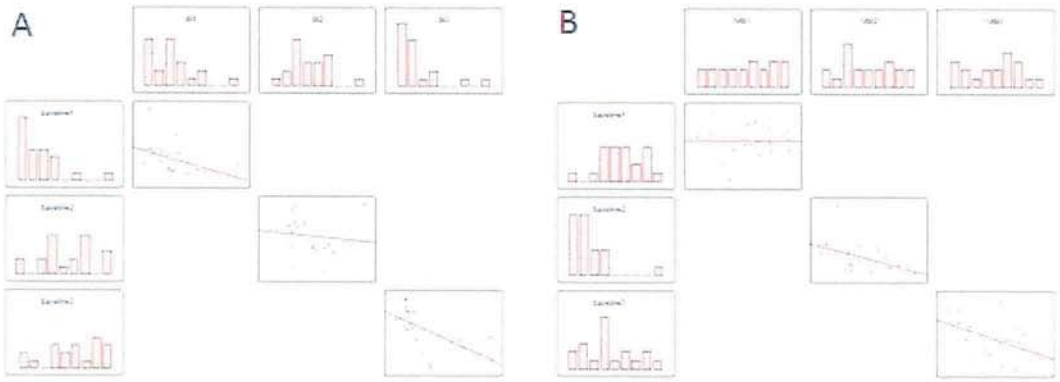

Figure 2.4: (A) Correlation of the arterial baseline diameter before flicker stimulation and its corresponding value of "Dil" for each cycle; (B) Correlation venous baseline diameter before flicker stimulation and its corresponding value of "Dil" for each cycle

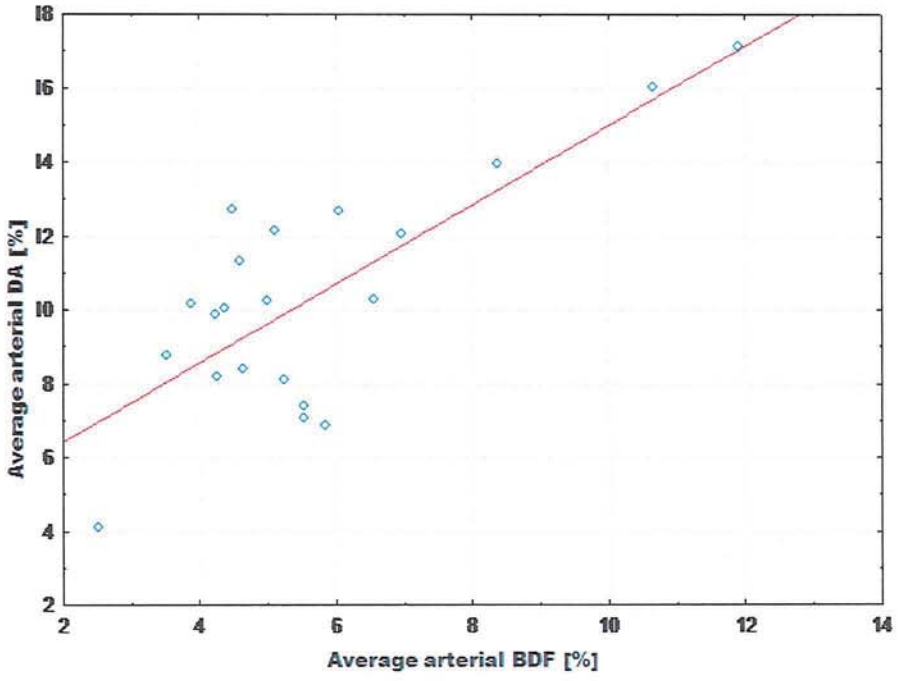

Figure 2.5: Correlation between the average arterial BDF before flicker stimulation and the average arterial DA due to flicker provocation in smokers; BDF: Baseline diameter fluctuation, DA: Dilation amplitude

Parameter	Nonsmokers [n=71]	Smokers [n=21]	ANOVA [p-value]	Post-hoc/ Tukeys [p-value]
	Mean +/-SD	*Mean +/-SD*		
BDF [%]			**0.005**	
Flicker 1	4.04 (2.13)	5.04 (2.63)		0.11
Flicker 2	4.16 (2.13)	6.05 (3.10)		**0.002**
Flicker 3	4.31 (2.66)	5.95 (2.21)		**0.014**
DA [%]			**0.002**	
Flicker 1	7.94 (3.56)	10.69 (2.94)		**0.001**
Flicker 2	8.02 (3.26)	10.26 (3.96)		**0.008**
Flicker 3	8.33 (3.51)	10.14 (4.04)		**0.02**
BFR [%]			**0.02**	
Flicker 1	3.76 (2.64)	5.65 (2.11)		**0.003**
Flicker 2	3.86 (2.69)	4.21 (3.22)		0.636
Flicker 3	4.01 (2.78)	4.20 (3.54)		0.597

Table 2.6: The measured vascular reactivity values in the 2 study groups; BDF: Baseline diameter fluctuation, DA: Dilation amplitude, BFR: Baseline corrected flicker response

is represented by the BFR which was significantly higher for the first stimulation cycle than for the second and the third ones in the smoking subjects only (Friedman's ANOVA p=0.04). This effect could have occurred due to the fact that the first BDF of retinal arterioles of smokers was the lowest of all three cycles while the DA was highest in the first stimulation cycle; Table 2.6 on page 86.

As shown in Table 2.7 on page 87, there was no significant difference in the RT to reach MD after flicker provocation in both retinal arteries and veins within and between groups (ANOVA: p>0.05, p>0.05). Whereas non-smokers reach maximum dilation within similar time for each flicker cycle, reaction in smokers was less predictable for each cycle. For arteries, non-smokers need approximately the same time to reach maximum dilation, whereas smokers reaction is prolonged in the second cycle compared to the first and third. For veins, non-smokers need approximately the same time in each cycle to reach maximum dilation, but smokers react fastest for the second cycle compared to the first and third.

Table 2.8 on page 88 contains the results for the venous response to flicker stimulation as measured for each cycle of smokers and non-smokers. There was no significant difference present in the venous response to flicker stimulation between smokers and non-smokers for the venous MD, the MC and the DA (ANOVA: all p>0.05). In addition, there were no inter-group differences between the three stimulation cycles (all p>0.05).

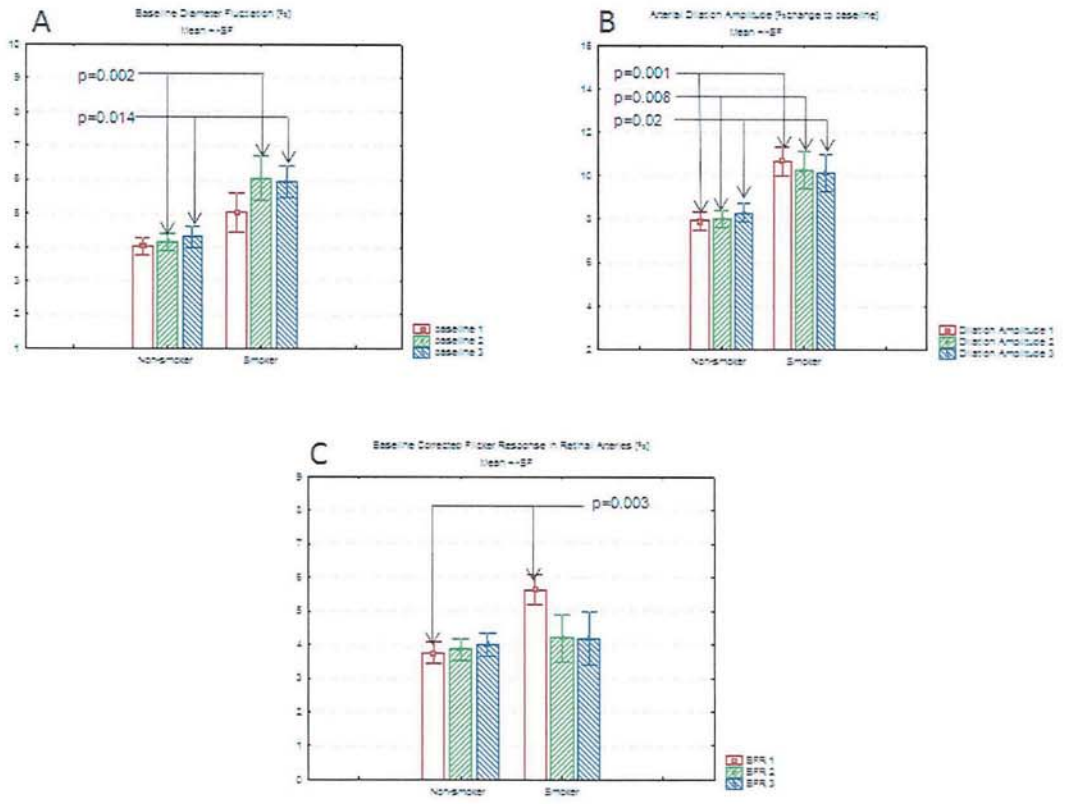

Figure 2.6: Group differences for BDF, DA and BFR in smokers and controls; BDF: Baseline diameter fluctuation, DA: Dilation amplitude, BFR: Baseline corrected flicker response

Parameter	Nonsmokers [n=71]	Smokers [n=21]	ANOVA [p-value]
	Mean +/-SD	*Mean +/-SD*	
RT (Artery) [sec]			0.258
Flicker 1	15.50 (5.28)	13.81 (7.71)	
Flicker 2	15.97 (5.81)	17.10 (7.00)	
Flicker 3	16.90 (6.58)	15.62 (5.15)	
RT (Vein) [sec]			0.546
Flicker 1	19.46 (4.02)	19.67 (4.10)	
Flicker 2	19.47 (5.35)	18.52 (5.06)	
Flicker 3	19.43 (4.11)	19.25 (4.29)	

Table 2.7: Arterial and venous RT to flicker stimulation in smokers and non-smokers; RT: Reaction time

Parameter	Nonsmokers [n=71]	Smokers [n=21]	ANOVA [p-value]
	Mean +/-SD	*Mean +/-SD*	
MD [%]			0.523
Flicker 1	5.80 (2.63)	6.60 (3.15)	
Flicker 2	5.81 (2.29)	7.08 (2.60)	
Flicker 3	6.08 (2.46)	6.56 (2.84)	
MC [%]			0.72
Flicker 1	-1.27 (1.35)	-1.37 (1.24)	
Flicker 2	-1.28 (1.33)	-1.71 (1.29)	
Flicker 3	-1.22 (1.31)	-1.32 (0.86)	
DA [%]			0.23
Flicker 1	7.08 (3.18)	7.98 (3.04)	
Flicker 2	7.09 (2.86)	8.79 (2.84)	
Flicker 3	7.31 (3.03)	7.88 (3.19)	

Table 2.8: Retinal venous diameter response to flicker stimulation in smokers and non-smokers; MD: Maximum dilation, MC: Maximum constriction, DA: Dilation amplitude

2.6.4.1 Retinal vascular stiffness

Smokers showed a significantly decreased APR compared to non-smokers (non-smokers APR: cycle 1=2.21 (1.10), cycle 2=2.18 (0.92), cycle 3=2.43 (1.22) and smokers APR: cycle 1=2.43 (0.94), cycle 2=1.92 (0.83), cycle3=1.81 (0.69); ANOVA p=0.035).

2.7 Discussion

2.7.1 Main findings

The present study assessed the retinal vascular motion at baseline, as well as the response of retinal vessels to flickering light stimulation in a sample of chronic smoking individuals and compared them to age-matched non-smoking controls. Chronic smokers exhibited lower SBP and larger retinal venous size measures compared to their age-matched non-smoking controls; however, none of these measures (BP values and retinal vessel size) had any impact upon dynamic parameters assessed. In the non-smoking sample the MD reached due to flicker provocation was strongly related to the immediate baseline diameter before the start of flickering light stimulation. This relationship was only partially present in chronic smokers. In addition, chronic smokers exhibited a significant change in

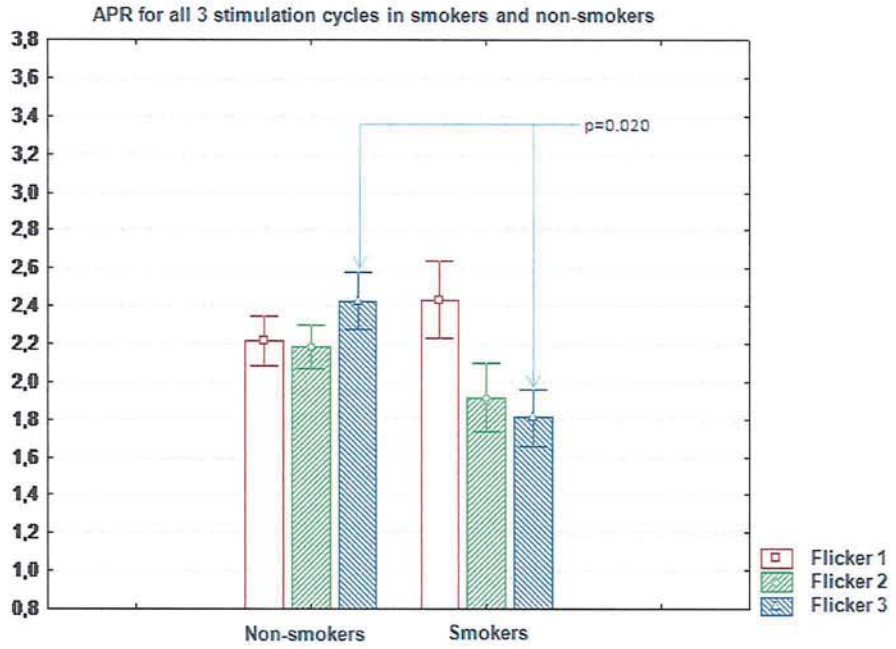

Figure 2.7: Arterial average peak ratio of retinal arterioles for all 3 stimulation cycles for patients and controls; APR: Arterial peak ratio

the reaction pattern of retinal arteries to multiple stimulation with flickering light as well as showing an increase in retinal arteriolar stiffness. No significant changes were observed in any dynamic measurements of retinal veins between the two study groups.

2.7.2 The effect of age on the retinal vascular reactivity

As described earlier in Chapter 1 1.3.3.2, when the vascular system ages, collagen and elastin, part of the blood vessel structure, degrade and smooth muscle cells proliferate causing the blood vessel walls to thicken [O'Rourke, 2007, O'Rourke and Hashimoto, 2007]. At the retinal level age-related changes can be readily observed non-invasively by ophthalmoscopy and fundus photography. Common age-related changes at the retinal level include arteriolar narrowing, loss of reflex and changes in vascular morphology [Kawasaki et al., 2006, Klein et al., 2007, Wong et al., 2006b, 2003b, 2002a].

There was no significant correlation between retinal vascular reactivity and age in both our study groups; in addition, the same was true for the measurement variability of the vascular reactivity parameters assessed. This is in opposition to previous studies that reported age to be associated with structural retinal venular dilation in smokers [Wong et al., 2006b, Wang et al., 2006], possibly relating chronic smoking to premature aging of the vascular system. However, our parameters assessed are mostly functional in nature, therefore, possibly did not pick up this effect in our subjects.

2.7.3 The effect of baseline diameter and baseline diameter fluctuation on retinal vascular reactivity to flickering light stimulation in chronic smoking

In non-smokers a significant correlation between each baseline and its corresponding dilatory response for all three stimulation cycles in retinal arteries and for the first two stimulation cycles in retinal veins was found, see Section 2.6.3.2. However, in the smoking group a similar significance was only found for the first and third stimulation cycle in retinal arteries and for the second cycle in retinal veins.

2.7.4 The effect of chronic smoking

Smoking is the primary risk factor for both CAD and peripheral artery disease (PAD) [Mayhan and Patel, 1997, Klein et al., 2000, Rahman and Laher, 2007a, Winniford, 1990, Heeringa et al., 2006]. It has been associated with an increased risk of arteriosclerotic vascular disease [Klein et al., 2000], hypertension, myocardial infarction, sudden cardiac death and stroke [Winniford, 1990]. However, the exact substances of cigarette smoke which contribute to the pathogenesis of vascular disease remain unclear [Mayhan and Patel, 1997]. A number of studies tried to explore the mechanism behind smoking related changes in various vascular beds. Such changes lead to injury of the vascular endothelium, production of superoxide anions, reduction in production and bioavailability of NO finally lead to endothelial dysfunction, thrombosis, arteriosclerosis, CAD and stroke [Rahman and Laher, 2007a]. Morphological abnormalities arising due to smoking include endothelial swelling, sub-endothelial oedema and blebs in arterial walls [Asmussen and Kjeldsen, 1975, Boyce et al., 1991, Boutet et al., 1980, Lin et al., 1993] which result in altered vascular functionality. These functional changes manifest in an increased permeability to plasma proteins [Barrow et al., 1990, Holden et al., 1990, Jensen et al., 1992, Lin et al., 1993] which therefore, impair NO synthase-mediated vasodilation of resistance vessels [Rubinstein et al., 1991, Rivers and Duling, 1991]. In fact, a decrease in basal as well as substance-P-stimulated NO bioavailability has been described by Barura [Barua et al., 2002b]. Mayhan and Sharpe [Mayhan et al., 1999, Mayhan and Sharpe, 1998] reported impaired reaction due to an increase in the synthesis and release of oxygen free radicals which are known to have a toxic effect upon blood vessels.

2.7.5 Vascular effects: systemic and ocular

In the presented study smokers had significantly lower SBP and larger retinal venular diameter compared to healthy non-smoking controls. Similar findings of

decreased SBP [Kaiser et al., 1997] and larger retinal venous diameter associated with cigarette smoking have been already described in larger population studies [Wang et al., 2006, Wong et al., 2006a,b, Liew et al., 2008]. In addition, SBP and DBP did not have any influence on retinal vascular reactivity in our sample. This is in agreement with earlier studies reporting no effect of a change in MAP upon retinal vessel diameter [Garhofer et al., 2003] and flicker-induced vasodilation [Nagel et al., 2006]. In addition, our results also showed an increased retinal arterial vascular pulsation at baseline, as assessed by BDF, and a significantly higher arterial DA due to flicker stimulation in smokers compared to non-smokers. However, taking into account the BDF and comparing the pure reaction to flicker light stimulation to healthy non-smoking controls, a significant difference was only apparent for the first stimulation cycle. No differences were found between smokers and nonsmokers for the reactivity of retinal veins. One could now hypothesize that the diffuse endothelial impairment and decreased bioavailability of NO associated with chronic smoking may have had an "exhaustive" effect on vascular reactivity to provocation. In support of this theory comes the difference found between groups in the RT to the three cycles of stimulation that we applied during this study. Non-smokers react to every flicker provocation within approximately the same time. In contrast, smokers seem to have an increased arterial and decreased venous reaction time for the second cycle compared to the first and third ones; however, this was not statistically significant. This tendency to opposite reaction in the two study groups was found during the measurement of the one-second mean baseline before flicker stimulation; in this case a relationship between the vascular baseline diameter preceding flicker stimulation and the following amount of dilation (Dil), was in arterioles only present for the first and third stimulation cycle, whereas in the vein a relationship was only present for the second stimulation cycle. This may well reflect a change in micro-vascular function at the retinal level in smokers: this could be the result of a disequilibrium between endothelial vaso dilatory and vaso constrictory molecules [Wimpissinger et al., 2004, 2005]. Chronic hypoxia caused by smoking may also play a role [Williams and Pearce, 2006].

Another possible explanation to why smokers exhibited a somehow different vascular reactivity than non smokers could be offered by the measurement technique itself. As described earlier in Section 1.3.3.2, the vascular diameter was measured by frame to frame analysis and is highly dependent upon the contrast and vertex distance. Any change in reflectance and vertex distance would result in an increased or decreased diameter measured. Retinal arteries exhibit a generally lower contrast than retinal veins, this is because of their difference in vessel wall composition and highly oxygenated arterial blood is brighter in colour than deoxygenated blood in veins. In addition, changes in vascular wall compo

sition can result in an altered change of reflex . In fact, Riva et al (2005) have suggested an influence of reflectance due to flicker stimulation [Riva et al., 2005]. Wimpissinger and co workers (2003) have demonstrated increased fundus pulsation amplitude in smokers after inhalation of carbogen. This effect could have contributed to increased dilation amplitude measured in our sample, as flicker stimulation has a similar effect as carbogen inhalation: causing NO release.

2.7.6 Conclusion

In summary, retinal vessel reactivity to flickering light stimulation is less predictable in smokers compared to non-smokers. The retinal arteries of smokers exhibit a change in reaction pattern when stimulated with flickering light, manifesting in a tendency to constrict with increased stimulation frequency. It can be concluded that this could reflect an abnormal vascular endothelial function at the level of the retina. Age did not have any effect on any of the reported changes in either smokers or non-smokers. Future studies on larger samples and including more measures are needed to further explore this subject.

Chapter 3

The acute and chronic effect of smoking on retinal vascular reactivity

3.1 Abstract

Purpose: To evaluate the acute effect of cigarette smoke on retinal vessel reactivity in otherwise healthy smoking and non-smoking individuals.

Methods: 10 chronic smokers and 13 non-smokers were involved in the study. After an initial baseline recording of systemic blood pressure (BP), intra ocular pressure (IOP) and retinal vessel reactivity measurement of the superior temporal artery and vein using the Retinal Vessel Analyser (RVA) flicker test, all subject were given one cigarette of the same brand to smoke. All measurements were repeated immediately after smoking.

Results: In both smokers and non-smokers systolic blood pressure (SBP) and diastolic blood pressure (DBP) increased significantly after smoking (smokers: SBP $p<0.001$, DBP $p<0.001$; non-smokers: SBP $p<0.0001$, DBP $p<0.001$). Pre-smoking, smokers exhibited higher arterial pulsation amplitude before flicker provocations than controls (ANOVA $p=0.007$) but this effect was absent after smoking. In addition, smokers showed greater arterial dilation to flicker stimulation pre-smoking compared to controls (ANOVA $p=0.011$), but again this effect was absent after smoking ($p>0.05$). The RT to reach maximum vascular dilation to flicker stimulation in the artery and vein were comparable between smokers and non-smokers for each condition (pre- and post-smoking, $p>0.05$). The venous reactivity was also comparable between the two study groups pre- and post-smoking ($p>0.05$).

Conclusions: A chronically compromised endothelial vascular function and structure in smoking individuals is leading to a vaso-constrictory response when applying multiple stimulation during the assessment of retinal vascular reactivity.

3.2 Introduction

Smoking has long been identified as one of the major risk factors for CVD and peripheral vascular disease (PVD), see Chapter 2. Cigarette smoke contains circa 4000 different substances, and an average cigarette has about 1mg of nicotine when inhaled. Nicotine is a potent neurotoxin able to cross the blood brain barrier in about seven seconds. Nicotine effects last up to two hours, having a half life of approximately 12 hrs and is metabolized by the liver to cotinine which stays in the blood stream for a further 48 hrs [Feng et al., 2007]. Smoking promotes the release of acetylcholine, norepinephrine, epinephrine, vasopressin, arginine, dopamine and beta-endorphine. It further alters the expression of ET cell gene encoding for vaso-active and thrombogenic factors such as eNOS, angiotensin I converting enzyme, tissue-type plasminogen activator, prasminogen activator inhibitor I, vWF and VCAM [Zhang et al., 2001]. In both, animals and humans, cigarette smoke reduces ET-dependent vaso-dilation, and NO generation, [Barua et al., 2001, Wright et al., 1999], and although increased expression of eNOS, it results in decreased eNOS activity [Barua et al., 2001]. Synergistic effects of all these mechanisms eventually change the equilibrium between vaso-constrictive and vaso-dilatory factors in smokers which can lead to susceptibility towards vaso-constriction as well as to changes in auto-regulatory responses when challenged. In fact, smokers have been shown to suffer from epicardial coronary (ET) dysfunction attributed to increased levels of inflammatory biomarkers and oxidative stress, while microvascular endothelial function was still preserved [Lavi et al., 2007]. The ocular circulation is highly susceptible to changes in vascular tone, making it vulnerable to any changes to its auto-regulatory system. The acute and chronic effect of smoking on the ocular circulation has only been assessed mainly to elucidate the effect of nicotine on ocular blood flow [Robinson et al., 1985, Wimpissinger et al., 2005, 2003, 2004, Steigerwalt et al., 2000]. The mechanisms of acute cigarette smoking as well as the chronic effect upon vascular reactivity and auto-regulatory velocity are, however, still not well understood. To find out more about the change in vascular function and reactivity of the ocular blood vessels in smokers we assessed a group of non-smokers and smokers pre- and post- smoking of one cigarette of the same brand.

3.3 Hypothesis

Acute smoking results in signs of vascular functional impairment that is stronger in chronic smokers than in non-smoking individuals.

3.4 Aims

The aim of this study was to elucidate the acute effects of cigarette smoking on endothelial-dependent vaso-dilation of retinal vessels in otherwise healthy smoking and non-smoking individuals.

3.5 Subjects and methods

3.5.1 Recruitment of healthy subjects

Subjects were recruited from Aston University staff, students and other volunteers.

3.5.1.1 Inclusion criteria

In order to be included in the present study, subjects had to fulfill the following criteria:

- Free from any vascular disease (e.g. diabetes mellitus, hypertension);
- Normal ocular findings;
- VA 6/9 or better; and
- Refractive error <+/-3dpt and astigmatism of less than 1 dpt. Volunteers with a refractive error of more than +/- 3dpt spherical equivalent and more than +/-1 dpt cylindrical equivalent were excluded due to the magnification/ minification causing over/ underestimation of retinal diameter measured;

To be classified as smokers, individuals habitually smoking on a daily basis for more than two years were included.

3.5.1.2 Exclusion criteria

Individuals suffering from any cardiovascular, vascular disease, diabetes mellitus, hypertension or not fitting any of the above outlined inclusion criteria were excluded from the study.

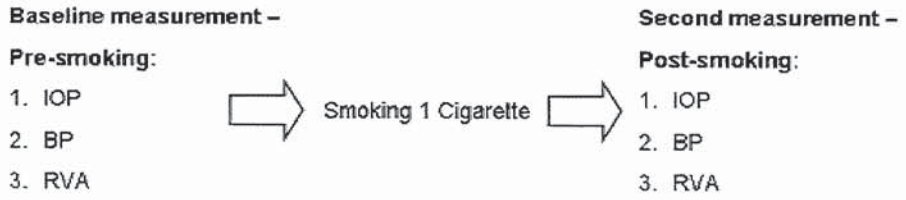

Figure 3.1: Measurement sequence

3.5.2 Ethical approval

Prior to the study ethical approval was obtained from Aston University Ethics Committee. Written informed consent was received from all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets of the Declaration of Helsinki.

3.5.3 Experimental protocol

After full ocular examination to rule out any abnormalities, all subjects were measured according to the protocol sequence described below:

Patient preparation All subjects were instructed to refrain from consuming caffeinated products, such as coffee or tea, smoking, and drinking alcohol for 12 hrs prior the appointment. All measurements were obtained between 3pm and 4pm on all subjects in a temperature controlled room of 23-25ºC.

3.5.3.1 Intraocular Pressure

Due to the study design the subject could not be seated at a slit-lamp, therefore contact tonometry was performed with the TonopenXL (TonopenXL, Medtronic Solan, PMS Instuments, Maidenhead, UK), a hand held device to measure IOP. All measurements were taken after instillation of one drop of 0.4% benoxinate hydrochloride. This device automatically calculates the mean of three consecutive readings along with the coefficient of variance (CV).

3.5.3.2 Systemic blood pressure

After an acclimatization period of 15-20 minutes in a temperature controlled room of 22-25ºCelsius, systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were obtained by manual sphygmomanometer (Digital BP Monitor UA-767EX-C, PMS Instruments, Maidenhead, UK). During the measurement subjects were in a sitting position with their arm resting at heart level. The cuff

was placed around the right arm in all subjects. SBP and DBP values were measured three times at baseline before the start of retinal vessel measurements to ensure the subject was acclimatized; this was reached when the blood pressure (BP) measurements obtained were stable. During retinal vessel assessment BP was obtained at one minute intervals (three measurements) and then averaged for statistical analysis.

3.5.3.3 Retinal vessel analysis

Measurements were performed in one randomly selected eye of each patient. Retinal vascular dynamics, of both, arteries and veins was assessed with the RVA (Imedos GmbH, Germany) as described earlier in Section 1.3.3.2. After full pupil dilation was reached the measurement was obtained according to the protocol described earlier, see Figure 3.1 on page 96. Only measurements that complied with our quality criteria as described in Section 1.3.3.2 were included for the statistical analysis .

3.5.3.4 Smoking

All subjects were given one single cigarette of the same brand containing 0.6 mg nicotine and eight milligram tar. This was done so as to ensure the same conditions for each subject.

3.5.4 Statistical analysis

All results are given as the mean +/- standard deviation (SD). For analysis of demographic data we used an unpaired t-test and the Mann-Whitney-U test, depending on data distribution. For all within group analysis of demographic data we applied either a paired t-test of the Wilcoxon test. The effect of demographic data upon dynamic values measured was assessed using ANCOVA. Dynamic retinal vessel reactivity parameters were assessed using ANOVA after log transforming all data in an attempt to normalize it. Statistical significance was defined at the level of $p<0.05$.

3.6 Results

3.6.1 Sample

23 eyes of 10 chronic smokers and 13 non-smokers were included in the present study. No data set had to be excluded for statistical analysis. The chronic smokers' group consisted of six women and four men with a mean age of 29 (5) and

	Non-smoker [n=13]	Smoker [n=10]	
Parameter	*Mean +/-SD*	*Mean +/-SD*	*p-value*
Age [yrs]	28 (5)	29 (5)	0.767
pre-smoking			
IOP [mmHg]	14 (3)	14 (3)	0.891
SBP [mmHg]	116 (11)	111 (13)	0.377
DBP [mmHg]	72 (9)	69 (11)	0.389
HR [beats per minute]	68 (10)	73 (12)	0.255
size A [AU]	130 (17)	125 (16)	0.474
size V [AU]	154 (20)	166 (16)	0.094
post-smoking			
IOP [mmHg]	14 (3)	15 (2)	0.805
SBP [mmHg]	126 (11)	122 (11)	0.409
DBP [mmHg]	79 (7)	78 (8)	0.725
HR [beats per minute]	74 (16)	83 (10)	0.134
size A [AU]	128 (16)	125 (16)	0.559
size V [AU]	156 (21)	167 (14)	0.121

Table 3.1: Demographic data of both study groups at baseline and after smoking; IOP: Intra ocular pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HR: Heart rate, size A: arterial diameter, size V: venous diameter, AU: Arbitrary Units

the non-smoking group included seven women and six men having a mean age of 28 (5) ($p>0.05$), see Table 3.1 on page 98. There were no statistically significant differences between the two study groups with regards to IOP, SBP, DBP, pulse, arterial and venous size during both measurement conditions ($p>0.05$, Table 3.1 on page 98).

3.6.2 The effect of acute smoking: chronic smokers vs non-smokers

3.6.2.1 The effect on systemic BP, IOP and vascular diameter

Chronic smokers showed a significant increase in SBP, DBP and HR after smoking one cigarette ($p<0.001$, $p<0.001$ and $p=0.016$ respectively). IOP values, as well as retinal arterial and venous size were also comparable pre-smoking and post-smoking (all $p>0.05$); see Table 3.1 on page 98 and Table 3.2 on page 99.

Non-smokers showed a significant increase in SBP ($p<0.0001$) and DBP ($p<0.001$). HR and IOP values as well as retinal arterial and venous size (all $p>0.05$) were also comparable pre-smoking and post-smoking (all $p>0.05$); see Table 3.2 on page 99.

	Non-smoker [n=13]		
Parameter	*Mean +/-SD*	*Mean +/-SD*	*p-value*
	pre-smoking	***post-smoking***	
IOP [mmHg]	14 (3)	14 (3)	0.999
SBP [mmHg]	116 (11)	126 (11)	**<0.0001**
DBP [mmHg]	72 (9)	79 (7)	**<0.001**
HR [beats per minute]	68 (10)	74 (16)	0.069
size A [AU]	130 (17)	128 (16)	0.156
size V [AU]	154 (20)	156 (21)	0.071
	Smoker [n=10]		
Parameter	*Mean +/-SD*	*Mean +/-SD*	*p-value*
	pre-smoking	***post-smoking***	
IOP [mmHg]	14 (3)	15 (2)	0.397
SBP [mmHg]	111 (13)	122 (11)	**<0.001**
DBP [mmHg]	69 (11)	78 (8)	**<0.001**
HR [beats per minute]	73 (12)	83 (10)	**0.016**
size A [AU]	125 (16)	125 (16)	0.541
size V [AU]	166 (16)	167 (14)	0.405

Table 3.2: Pre- and post-smoking data in both study groups; IOP: Intra ocular pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HR: Heart rate, size A: arterial diameter, size V: venous diameter, AU: Arbitrary Units

3.6.2.2 The effect of retinal vessels function

Pre-flicker Smokers showed significantly higher arterial BDF as measured at baseline, pre smoking, before flicker stimulation than non-smokers (ANOVA p=0.007; see Table 3.3 on page 100); however, after smoking, the arterial BDF at baseline prior to flicker provocation was comparable between smokers and non-smokers (ANOVA p=0.299; see Table 3.3 on page 100 and Figure 3.3 on page 101). In addition, the change in arterial BDF prior to flicker stimulation when comparing pre to post smoking conditions, was significantly different between groups (ANOVA p=0.007; see Table 3.3 on page 100): smokers BDF prior to flicker decreased after smoking one cigarette whereas non-smokers BDF increased (ANOVA p=0.007; see Table 3.3 on page 100 and Figure 3.3 on page 101)

After stimulation with flickering light While the arterial DA due to flicker stimulation was significantly higher in smokers than controls before smoking (ANOVA p=0.011; see Table 3.3 on page 100 and Figure 3.2 on page 101) but not after smoking (ANOVA p=0.126; see Table 3.3 on page 100 and Figure 3.2 on page 101), the venous dilation was comparable between the two groups for both pre- and post-smoking (all p>0.05; see Table 3.3 on page 100).

	Non-smoker [n=13]			Smoker [n=10]			
	pre-smoking	*post-smoking*	*Intergroup difference*	*pre-smoking*	*post-smoking*	*Intergroup difference*	*Between group difference*
Parameter	*Mean +/- SD*	*Mean +/- SD*	*p-value [ANOVA]*	*Mean +/- SD*	*Mean +/- SD*	*p-value [ANOVA]*	*p-value [ANOVA]*
Arterial Reaction							
BDF							
Flicker 1	3.81 (1.37)	4.26 (2.15)	>0.05	6.39 (4.94)	4.86 (2.27)	>0.05	**0.007 (change)**
Flicker 2	3.44 (1.76)	4.46 (2.77)		7.22 (3.89)	4.52 (1.44)		**0.007 (pre-smoking)**
Flicker 3	4.02 (1.99)	4.06 (1.73)		6.44 (3.66)	5.06 (2.15)		0.299 (post-smoking)
DA							
Flicker 1	6.82 (3.19)	7.63 (3.55)	>0.05	11.73 (5.54)	9.56 (2.68)	>0.05	0.605 (change)
Flicker 2	6.95 (2.96)	8.27 (2.81)		11.93 (7.96)	10.05 (3.36)		**0.011 (pre-smoking)**
Flicker 3	6.72 (3.26)	8.25 (3.45)		11.46 (5.02)	10.60 (3.09)		0.126 (post-smoking)
RT							
Flicker 1	15 (8)	16 (7)	>0.05	19 (4)	15 (6)	>0.05	0.367 (pre-smoking)
Flicker 2	18 (4)	17 (5)		18 (6)	19 (3)		0.629 (post-smoking)
Flicker 3	17 (7)	17 (7)		17 (7)	17 (6)		
Venous Reaction							
DA							
Flicker 1	6.54 (2.02)	6.43 (3.57)	>0.05	7.58 (3.80)	8.22 (3.21)	>0.05	0.79 (pre-smoking)
Flicker 2	6.56 (2.31)	7.26 (2.76)		7.45 (3.06)	7.50 (2.92)		0.398 (post-smoking)
Flicker 3	7.01 (2.26)	6.97 (2.92)		7.31 (3.83)	8.06 (2.65)		
RT							
Flicker 1	22 (5)	20 (5)	>0.05	18 (6)	20 (4)	>0.05	0.105 (pre-smoking)
Flicker 2	20 (5)	19 (5)		18 (7)	20 (5)		0.915 (post-smoking)
Flicker 3	21 (7)	19 (4)		18 (4)	18 (6)		

Table 3.3: Intergroup and between group comarison of the dynamic retinal vessel parameters assessed pre and post smoking in both groups; BDF: Baseline Diameter Fluctuation, DA: Dilation Amplitude, RT: Reaction Time

No difference between smokers and non-smokers at both conditions, pre- and post-smoking, was observed for the arterial and venous RT to flicker stimulation (all p>0.05; see Table 3.3 on page 100).

3.7 Discussion

3.7.1 Main findings

At the retinal arteries level, vascular pulsation amplitude at baseline before flicker provocation was significantly higher in chronic smokers than in controls. The chronic effect of smoking upon vascular reactivity parameters as measured with the RVA has been described and discussed in more detail in Chapter 2. It has been previously shown that chronic smoking reduces ocular vascular reactivity to hyperoxia as measured by SLDF and RVA [Morgado et al., 1994, Wimpissinger et al., 2005]. This effect has been attributed to the vaso-constrictive effects of nicotine through sympathetic stimulation [Morgado et al., 1994] and release of catecholamines from sympathetic nerve endings [Grilli et al., 2005, Jacobs et al., 2002]. We were able to demonstrate in this smaller sample once more that chronic smoking results in an increase of the arterial vascular pulsation amplitude at both baseline and when stimulated with flickering light. However, after smoking one cigarette, the pulsation amplitude decreased in chronic smokers and increased in controls to approximately similar magnitude, levelling the differ-

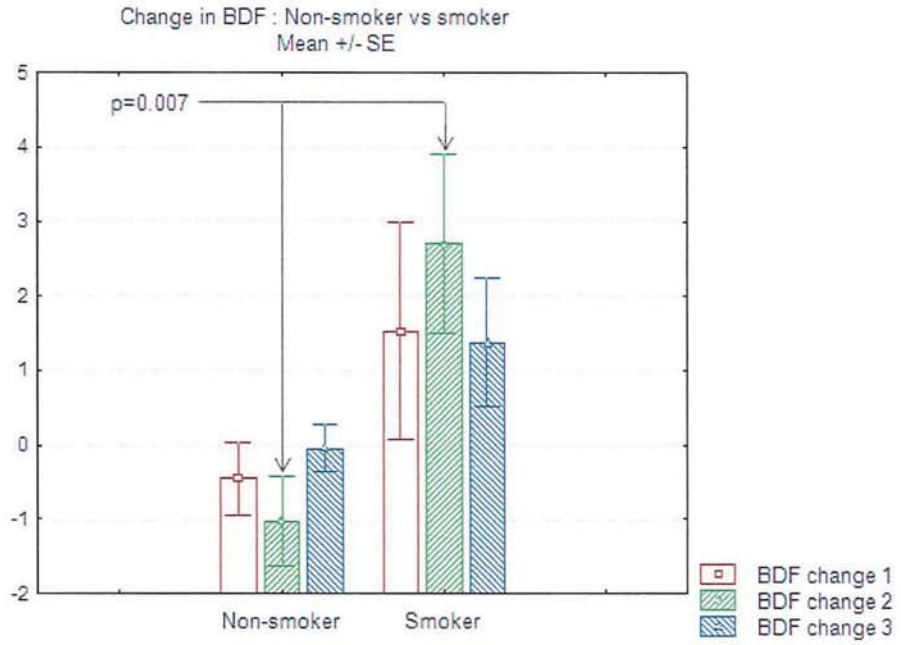

Figure 3.2: Change in BDF after smoking in both groups; BDF: Baseline diameter fluctuation

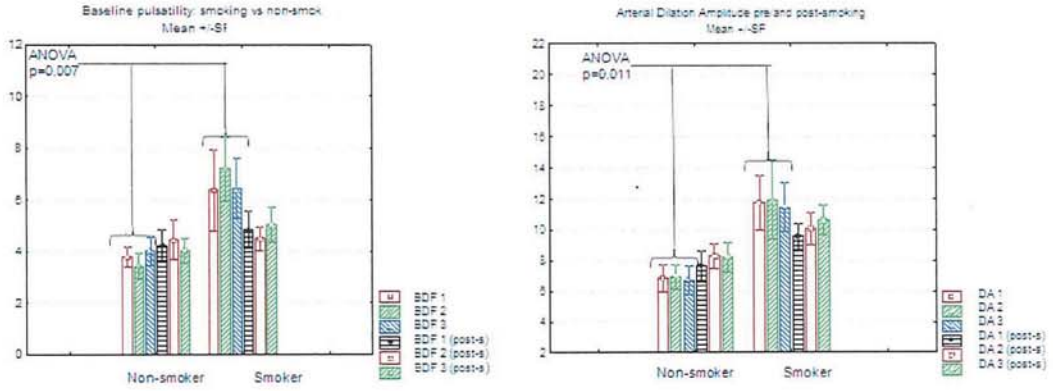

Figure 3.3: Comparison of arterial BDF and DA pre and post smoking for both study groups; BDF: Baseline diameter fluctuation, DA: Dilation amplitude

ence between the two groups. In addition, smokers were showing significantly higher DA to flicker stimulation pre- but not post-smoking compared to controls. This type of reactivity was not observed at the venous level, where both chronic smokers and non-smokers showed comparable reactivity to flicker light stimulation. The RT to stimulation with flicker light, to achieve MD in both arteries and veins, was comparable between the two groups and not altered by smoking.

3.7.2 The effect of acute smoking

IOP was unaffected by the acute effect of smoking in our sample which is in agreement with previous studies [Tamaki et al., 1999]. However, smoking increased SBP and DBP significantly compared to baseline values (=before smoking) as observed in other studies [Tamaki et al., 1999, Robinson et al., 1985]. HR was significantly increased in the chronic smoking group, but not in controls.

The chronic effect of smoking on the vascular system results in an abnormal vascular function due to loss of balance between vasodilation and vasoconstriction [Wimpissinger et al., 2005]. Chronic smoking also result in changes of the vascular structure, such as calcium overload in the arteries (mainly the internal elastic membrane) that further contribute to the vascular dysfunction; as a consequence, there is an abnormal communication between ET-derived vasodilatory substances, such as NO, and vascular smooth muscle cells [Kugiyama et al., 1996]. We have demonstrated that all these changes might also be present at the retinal vessels level resulting in abnormal reactivity at baseline and after flicker provocation as well as and when acutely exposed to cigarette smoke.

When considering our protocol, we have taken into consideration the mechanism showing how nicotine is absorbed after acute inhalation, with peak levels being obtained within 10 minutes of smoking one cigarette [Morgado et al., 1994]. The shifted vasomotion in chronic smokers towards a greater vaso-constriction during acute exposure of cigarette smoke could be down to a series of interacting mechanisms. Chronic smokers have been shown to suffer from "chronic vasodilation" due to the alteration of vaso-active substances [Wimpissinger et al., 2005]. In addition, chronic smokers also exhibit diminished eNOS activity leading to a decrease in NO bioavailability. Nicotine therefore acts via both pathways to cause vaso-constriction: directly through changing the equilibrium of vaso-constrictive and vaso-dilatory substances and their activity as well as via the sympathetic nerve endings. In addition, chronic smokers also exhibit higher plasma levels of ET-1 immediately after smoking [Goerre et al., 1995, Haak et al., 1993, 1994] but have lower basal ET-1 levels [Ahlborg and Lundberg, 2001] than non-smokers. This abnormal increase of ET-1 could also contribute to the abnormal retinal vascular reactivity observed at both baseline and after smoking. And least but not last, chronic hypoxia due to chronic exposure to cigarette smoke can also result in

vascular structural changes; chronic hypoxia can alter both the reactivity and morphology of smooth muscle and ET cells as shown before, e.g. in the pulmonary circulation [Faller, 1999, Kourembanas, 2002, Murata et al., 2001]. Williams JM and Pearce WJ were able to show similar changes in the ET cell and smooth muscle cells from ovine adult and fetal cranial arteries [Williams and Pearce, 2006].

In conclusion, chronic hypoxia caused by long standing smoking leads to an imbalance between vasodilatory and vasoconstrictoy factors. It can be hypothesised that in these subjects, the requirement for supplementary vasodilation in the event of an additional stressor, overstretch the vasoregulatory system, therefore, resulting in additional functional disturbances that can be observed at various levels, including the retina.

3.7.3 Conclusion

Chronically compromised vascular function and structure in smokers leads to abnormal reactivity when exposed to further challenges.

3.7.4 Limitations

As we did not collect blood samples we can only base our explanations on results published in previous studies as detailed above. However, our findings were in agreement with previously published work as well as supporting the theory of a vaso-constrictive shift in the auto-regulatory response of smokers. In order to get meaningful data from blood collection it would have been necessary to collect blood samples both at baseline and after smoking which would have posed the patient at un-necessary risk compared to the purpose of the study.

Chapter 4

Ocular Blood Flow and Retinal vessel reactivity in patients suffering from coronary artery disease compared to healthy subjects

4.1 Abstract

Purpose: To assess retro-bulbar blood flow, retinal blood flow and the retinal vessel reactivity to flicker light stimulation in patients suffering coronary artery disease compared to age matched healthy individuals.

Methods: Twenty four patients suffering from coronary artery disease (CAD) (established by coronary angiography) and 30 healthy age-matched controls were included in the study. After routine ocular examination to rule out any ocular abnormalities in both groups, retro-bulbar blood flow velocities of the ophthalmic artery (OA), central retinal artery (CRA), medial and temporal posterior ciliary arteries (PCAs) were assessed using colour Doppler Ultrasound. Retinal perfusion was measured using the Heidelberg Retinal Flowmeter (HRF) and retinal vessel reactivity to flicker light stimulation was evaluated by Retinal Vessel Analyser (RVA) measurements. Systemic blood pressure (BP) has been continuously measured throughout the study day with an ambulatory BP unit (Cardio Tens-01, Meditech Ltd., Hungary) furthermore, intra ocular pressure (IOP) values were obtained by contact tonometry using the TonopenXL (TonopenXL, Medtronic Solan, PMS Instuments, Maidenhead, UK).

Results: CAD patients showed significantly higher body mass index (BMI) (p=0.023) than age and gender matched controls. Retro-bulbar blood flow (BF) as measured by colour Doppler imaging (CDI) showed no difference between patients and controls, however, the spectral wave form was significantly different when comparing the two groups for the OA and the CRA (p=0.01 and p<0.001, respectively). Furthermore, patients exhibited comparable values for flow, volume and velocity as assessed using HRF in all four quadrants of the retina while controls had maximum volume in quadrant 1: Q1 (p=0.024) and the minimum in quadrant 2: Q2 (p=0.023). No significance in retinal vessel dynamics was found for maximum dilation (MD), maximum constriction (MC), dilation amplitude (DA) and venous reaction time (RT) (all p>0.05) as assessed using RVA. However, arterial RT significantly increased for each stimulation cycle within the patient group, but not the control group (ANOVA, p=0.017).

Conclusions: CAD patients exhibit an impaired endothelial vascular response at the level of retinal arterioles, manifesting through a gradual increase in RT when stimulated with flickering light.

4.2 Introduction

Atherosclerosis is a major cause of CAD; moreover, atherosclerotic vascular changes affect vascular beds throughout the body and has been extensively examined by large population studies, such as the MARS, CARDIA, ARIC and the Dublin outcome study, [Cheung et al., 2007c]. Due to those vascular alterations, vital functions of the vasculature are compromised, more precisely, its auto regulatory mechanism [Harris and Matthews, 2004, Verdecchia et al., 2006, O'Rourke, 2007, O'Rourke and Hashimoto, 2007]. Atherosclerotic plaque formation has a direct impact on the blood flow by decreasing lumen size and compromising the vascular endothelial cells function at multiple levels, including the retina [Klein et al., 2002, Taylor and Lightman, 2003, Werner et al., 2001, van Hecke et al., 2006, Nagaoka et al., 2005]. As the general vascular elasticity in patients suffering from CAD is altered and arterial stiffness is increased, ocular blood flow in general as well as retinal vascular reactivity parameters are also likely to be compromised. Indeed, Nagel et al. has shown a decreased reactivity of retinal vessels is in patients suffering from systemic HT compared to age matched healthy controls [Nagel et al., 2004]. In addition,[Fujioka et al., 2006] showed that in diabetic patients suffering from CAD, the spectral wave shape of the colour Doppler measurements at the retrobulbar vessels level exhibited a flattening of the systolic phase. It is known that vascular stiffness is increased not only in the hypertensive and pre-hypertensive [Gilani et al., 2007] patient but also in patients

suffering from CAD [Dolan et al., 2006b, Duprez and Cohn, 2007]; however, to date no research has been done to assess retinal BF, retinal vascular reactivity as well as the relationship between structural and functional vascular parameters at the retinal level in patients suffering from CAD. Therefore, the aim of the present study is to fill this lack of knowledge.

4.3 Hypothesis

Vascular reactivity and ocular blood flow is altered in patients suffering CAD due to atherosclerotic changes in their vascular system. As atherosclerosis manifests as an increase in vascular rigidity and stiffness, a delay in reactivity as well as subsequent change in blood flow variables, functional changes could be present before visible vascular structural alterations occur.

4.4 Aims

The aim of this study was to assess if compared to healthy controls, patients suffering from CAD present:

- Abnormalities of the retrobulbar and/or retinal perfusion;
- An altered retinal vascular response to flicker stimulation;

Moreover, we would also analyse the relationship between static and dynamic retinal vascular measures in this type of patients.

4.5 Subjects and methods

4.5.1 Patient recruitment

Patients were recruited from the cardiac rehabilitation unit at City Hospital, Birmingham. No upper age limit was applied, however, patients had to be at least 18 yrs of age to be included in the study. Age-matched healthy individuals were recruited from patients spouse and other volunteers.

4.5.2 Inclusion criteria

Patients were included in the study if they met all the following:

- Established diagnosis of CAD confirmed by angiography showing abnormalities of coronary arteries
- No upper age limit applid (patients had to be at least 18yrs of age)

4.5.3 Exclusion criteria

Exclusion criteria are outlined below:

- Diabetes mellitus
- Hypertension, as defined see Section 1.7.1, see Table 1.3 on page 62;
- Atrial fibrillation;
- History of any ocular disease;
- Patients with a refractive error of more than +/- 3dpt spherical equivalent and more than +/-1 dpt cylindrical equivalent due to the magnification/ minification causing over/ underestimation of retinal diameter measured;
- History of neurological diseases associated with loss of visual function
- Any type of ocular surgery; and
- Normal left ventricular function (LV Function); as defined by depressed left ventricular ejection fraction (LVEF). LVEF represents stroke volume/LV end-diastolic volume, whereas the normal LVEF equals approximately 60%, but impaired LVEF is generally defined as less than 45%.

In addition to the exclusion criteria above, healthy controls had to be free from any form of systemic and vascular disease, including diabetes mellitus (DM), hypertension (HT), and CAD.

4.5.4 Ethical approval

Prior to the study ethical approval was obtained from NHS Sandwell and West Birmingham Ethics Committee and Aston University Ethics Committee. Written informed consent was received from all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets the of Declaration of Helsinki.

4.5.5 Experimental protocol

After approaching the prospective patient/ control subject, a full ocular history and examination took place to ensure the patients were free from any systemic and ocular disease as outlined in the exclusion criteria above. After this initial examination the patient was booked in for the research appointment. All subjects were instructed to refrain from consuming caffeinated products, such as coffee or tea, chocolate, drinking alcohol and smoking on the study day. Additionally, patients

were asked to abstain from their usual medication 24 hrs prior to the appointment in an attempt to minimize the effect of medication; this is in accordance to previous studies and practice in CVD research [Lind, 2007]. Subjects were examined based on the protocol stated below:

Day 1: Systemic circulation 24hr BP and ECG monitor fitted

Day 2: Ocular circulation

1. CDI measurements;
2. HRF measurements;
3. Instillation of one drop of benoxinate (0.4%) and IOP assessment;
4. Instillation of one drop of tropicamide (0.5%);
5. Retinal vessel assessment; and
6. Fundus photography.

All ocular measurements were taken from the same randomly selected eye of each participant. The above measurements are detailed in the following sections below.

4.5.5.1 Intra-ocular Pressure (IOP)

Due to the study design the subject could not be seated at a slit-lamp, therefore, contact tonometry was performed with the Tonopen (TonopenXL, Medtronic Solan, PMS Instuments, Maidenhead, UK). The device automatically calculates the mean of three consecutive readings along with the CV. All measurements were taken after instillation of one drop of 0.4% benoxinate hydrochloride.

4.5.5.2 Systemic Blood Pressure

Systemic BP was measured throughout the study day using an ambulatory BP and ECG unit (Cardio Tens-01, Meditech Ltd., Hungary). The instrument and procedure were described in detail in Section 1.7.2.1. The device was programmed to take BP measurements at 15 minute intervals during day and night. Additional measurements were manually triggered when assessing OBF parameters and during retinal vascular reactivity assessment.

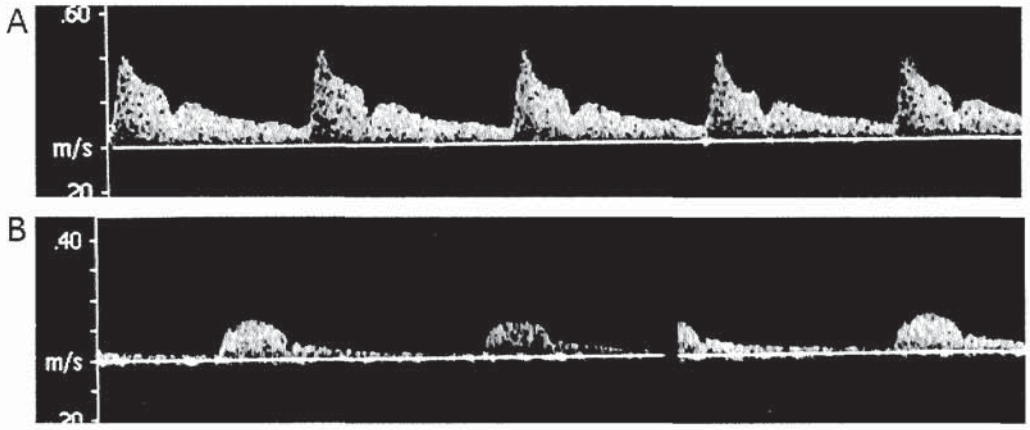

Figure 4.1: (A) Normal spectral wave shape of a the OA; (B) Abnormal spectral wave shape of the OA, showing a depressed systolic phase

4.5.5.3 Ocular blood flow assessments

Retrobulbar blood flow velocities During the measurements, the patient was laying down with the head resting on a pillow. After a 15 min acclimatisation period necessary to reach stable BP measurements, blood flow velocities were measured at the level of OA, CRA and PCAs using the Acuson Sequoia (Siemens, UK) . More details about the machine and technique can be found in Section 1.3.1. Peak systolic velocity (PSV) and end diastolic velocities (EDV) of each, OA,CRA medial and temporal PCAs were measured using callipers on the measured spectral pulse wave. Resistive indices (RI) were calculated according to the formula given in Section 1.3.1. Furthermore we examined the spectral wave form of the blood flow velocities measured in the OA and CRA, as described by Fujioka and co workers [Fujioka et al., 2006]. A change in the spectral wave's appearance, reflects a change in transference properties of the assessed blood vessel. Based on the appearance of the spectral wave, we grouped the subjects into either normal or abnormal according to their wave shape; a flattened systolic phase was used for the definition of "abnormal" (flattened systolic phase wave=abnormal) for both, the OA and CRA. , please see two examples showing a normal and abnormal spectral wave form.

Peripapillary retinal perfusion The peripapillary retinal blood flow, velocity and volume were measured using the HRF (Heidelberg Retinal Flow Meter, Heidelberg Ltd., Germany) according to a method already described in Section 1.3.2. Due to the spatial heterogeneity of the retinal microcirculation and pulsation, the flow rate within the scanned area is not constant [Bohdanecka et al., 1998]. In order to overcome this problem and minimize the error of flow rate differences arising due to pulsation, we applied a technique introduced by Hosking et al [Hosking et al., 2001] which applies a search strategy to determine minimum and maxi-

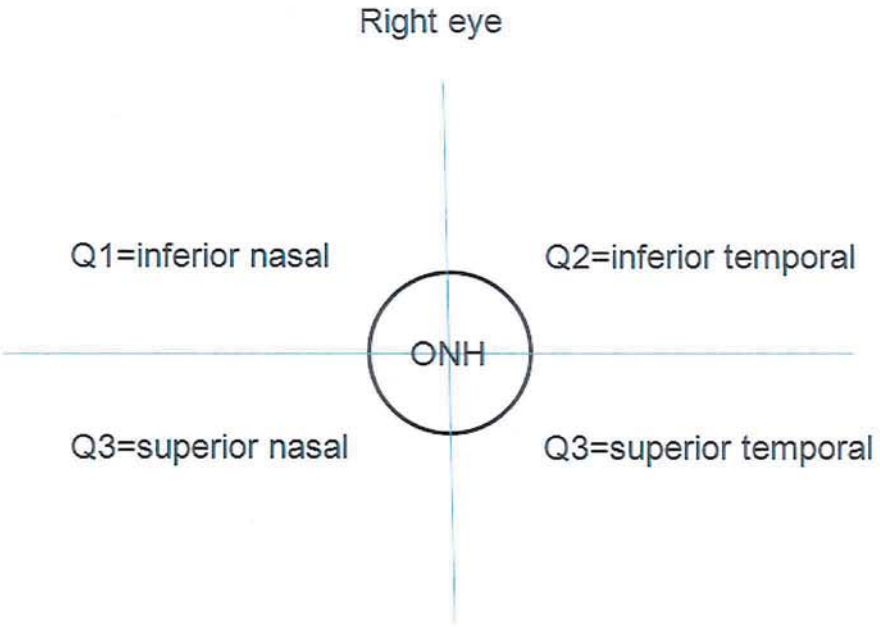

Figure 4.2: Schematic of quadrant division of the peri-papillary retina; ONH: optic nerve head

mum values for flow, volume and velocity in a set measurement window with a size of 15 * 15 pixel in all four quadrants (Q1, Q2, Q3 and Q4) in close proximity to the ONH. The parameters HRF parameters "flow", "volume" and "velocity" were then obtained by applying the above described search strategy. Only images with sufficient illumination and a signal to noise ratio between of 80 < DC < 200 were included in the analysis.

Retinal Vessel Reactivity Retinal vascular dynamics, of both, arteries and veins was assessed using the RVA as described earlier in Section 1.3.3.2. After full pupil dilation was reached the measurement was performed according to the protocol described in Figure 2.1 on page 79. Furthermore, values for MD, MC and DA, arterial and venous reaction time to flicker provocation and slope of both veins and arteries were obtained according to the equations outlined in Section 1.3.3.3.

Static retinal vessel assessment Black and white fundus images were obtained from each subject, at an 30 degree angle and with the ONH centered, using the inbuilt Zeiss 450F fundus camera (Zeiss GmbH, Germany) of the RVA (Imedos GmbH, Germany). Great care was taken to ensure good contrast and sufficient illumination of the photographs obtained. AVR, CRAE and CRVE were calculated by using the VesselImap software (Imedos GmbH, Germany) according to the protocol described in Section 1.3.3.1.

4.5.6 Statistical Analysis

All results are given as the mean +/- SD. Normally distributed data was assessed using an unpaired t-test and repeat measures ANOVA. If normal distribution was not present, the data was either evaluated using a non-parametric test (Mann-Whitney-U, Spearmans R) or log transformed in an attempt to normalise it. Differences in the shape of the spectral waves of OA and CRA were assessed using the CHI-square-test within and between the two study groups. Significant results for ANOVA evaluation was followed up by post-hoc analysis using Tukeys test. Statistical significance was defined at the level of $p<0.05$. When performing multiple comparisons, the level of p was set at $p<0.01$ to minimize bias.

4.6 Results

4.6.1 Sample

60 eyes of 11 women and 49 men were assessed during this study. However, as a result of careful image analysis, six subjects whose data had poor image quality, showed poor fixation or had excessive head movement during the experiment were excluded from the final statistical analysis. Fifty-four data sets remained, comprising of 24 eyes (3 women and 21 men) of CAD patients and 30 eyes (7 women and 23 men) of healthy controls.

4.6.1.1 General characteristics of the study groups

All patiets and controls were of Caucasian origin. There were no statistically significant differences between patients and controls with regards to age, gender , BP and IOP (all $p>0.05$); however, CAD patients showed a significantly higher BMI than controls (BMI: 28 (4) vs 26 (3) kg/m2; $p=0.023$, see Table 4.1 on page 112). BMI was calculated according to the Equation (2.3).

4.6.2 Ocular blood flow assessments

4.6.2.1 Retrobulbar blood flow velocities

There were no statistically significant differences in the measured parameters in either OA, CRA or PCAs between patients and controls (all $p>0.05$, see Table 4.2 on page 113). In the patient group, a flattened spectral wave shape of the OA and the CRA was significantly more common than in controls (OA: patients: 16 out of 24 and controls: 8 out of 30, $p=0.01$ and CRA: patients: 20 out of 24 and controls: 4 out of 30, $p<0.001$).

Parameter	Controls [n=30]	CAD [n=24]	P-value
	Mean +/-SD	*Mean +/-SD*	
Age [yrs]	53 (9)	56 (9)	0.162
IOP [mmHg]	13 (3)	14 (4)	0.698
BMI [kg/m²]	26 (3)	28 (4)	**0.023**
Gender	7 F (23 M)	3 F (21 M)	0.437
SBP [mmHg]	120 (12)	121 (13)	0.341
DBP [mmHg]	75 (9)	73 (12)	0.883
IOP [mmHg]	13 (3)	14 (4)	0.697

Table 4.1: General characteristics of CAD patients and controls; IOP: Intra ocular pressure, BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, CAD: Corornary artery disease

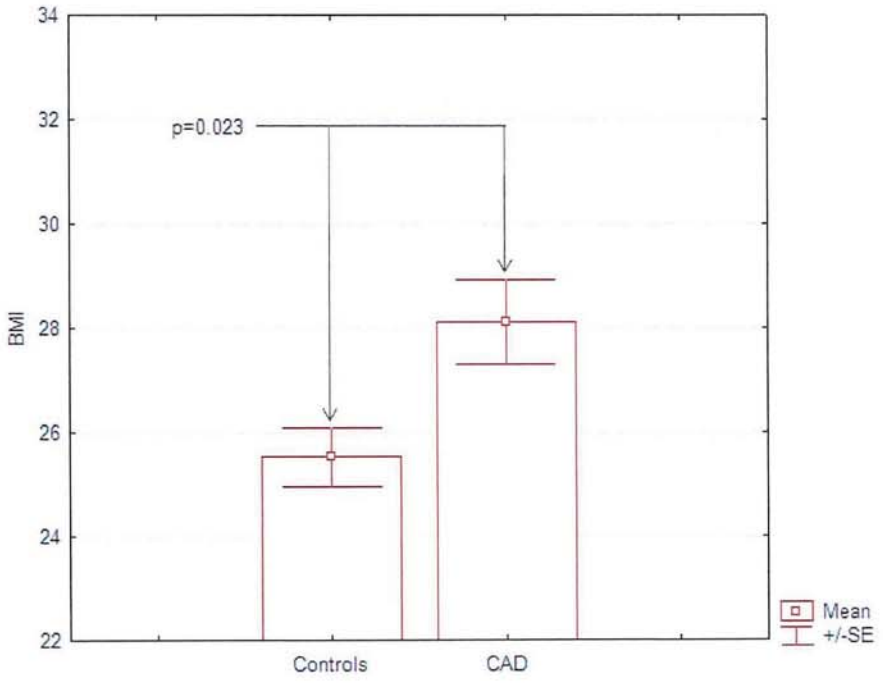

Figure 4.3: Difference in BMI between CAD patients and controls; BMI: Body mass index, CAD: Cornonary artery disease

Parameter	Controls [n=30]	CAD [n=24]	Mann-Whitney-U [2-sided]
	Mean +/-SD	*Mean +/-SD*	p-value
OA(PSV) [m/s]	0.362 (0.085)	0.401 (0.094)	0.107
OA(EDV) [m/s]	0.093 (0.040)	0.096 (0.033)	0.371
OA(RI)	0.747 (0.058)	0.761 (0.066)	0.654
CRA(PSV) [m/s]	0.114 (0.028)	0.116 (0.034)	0.969
CRA(EDV) [m/s]	0.034 (0.012)	0.067 (0.151)	0.611
CRA(RI)	0.696 (0.076)	0.682 (0.100)	0.622
CRV(MAX) [m/s]	0.064 (0.030)	0.073 (0.035)	0.275
CRV(MIN) [m/s]	0.042 (0.020)	0.049 (0.029)	0.302
tPCA(PSV) [m/s]	0.107 (0.026)	0.110 (0.026)	0.74
tPCA(EDV) [m/s]	0.043 (0.011)	0.043 (0.013)	0.779
tPCA(RI)	0.601 (0.060)	0.610 (0.071)	0.58
mPCA(PSV) [m/s]	0.105 (0.028)	0.107 (0.031)	0.776
mPCA(EDV) [m/s]	0.038 (0.012)	0.037 (0.011)	0.891
mPCA(RI)	0.634 (0.064	0.644 (0.083)	0.334
SBP [mmHg]	120 (12)	121 (13)	0.938
DBP [mmHg]	75 (9)	73 (12)	0.856
IOP [mmHg]	13 (3)	14 (4)	0.694

Table 4.2: Results for all parameters assessed using CDI in CAD patients and controls; CDI:Colour Doppler Imaging, OA: Ophthalmic artery, CRA: Central retinal artery, CRV: Central retinal vein, tPCA: temporal posterior ciliary artery, mPCA: medial posterior ciliary artery, PSV: Peak systolic velocity, EDV: End diastolic velocity, MAX: maximum, MIN: minimum, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, IOP: Intra ocular blood pressure, CAD: Coronary Artery Disease

	Controls [n=30]				CAD patients [n=24]			
Parameter								
IOP [mmHg]	13 (3)				14 (4)			
SBP [mmHg]	125 (15)				123 (13)			
DBP [mmHg]	79 (10)				76 (13)			
	Quadrant 1	*Quadrant 2*	*Quadrant 3*	*Quadrant 4*	*Quadrant 1*	*Quadrant 2*	*Quadrant 3*	*Quadrant 4*
Max Volume [au]	23.33 (6.77)	22.50 (4.72)	20.03 (6.85)	20.43 (6.14)	22.64 (8.15)	22.51 (7.10)	21.83 (6.03)	21.21 (5.24)
Max Flow [au]	374.63 (131.37)	353.77 (86.22)	316.28 (122.41)	313.99 (116.20)	353.20 (141.70)	335.89 (126.67)	329.26 (112.39)	330.91 (115.65)
Max Velocity [au]	1.28 (0.39)	1.22 (0.28)	1.11 (0.39)	1.09 (0.34)	1.21 (0.43)	1.15 (0.36)	1.15 (0.36)	1.16 (0.39)
Min Volume [au]	21.00 (6.06)	19.46 (4.21)	18.16 (6.92)	18.12 (5.79)	19.97 (8.17)	20.32 (5.70)	19.56 (6.23)	19.12 (4.89)
Min Flow [au]	306.98 (92.47)	279.35 (75.52)	273.08 (123.99)	262.39 (88.32)	304.00 (141.80)	276.13 (101.36)	276.17 (116.30)	278.85 (104.05)
Min Velocity [au]	1.07 (0.29)	0.98 (0.26)	0.96 (0.39)	0.93 (0.28)	1.05 (0.44)	0.96 (0.31)	0.97 (0.38)	0.99 (0.35)

Table 4.3: Retinal blood flow parameters as measured using the HRF; HRF: Heidelberg Retinal Flowmeter, IOP: Intra ocular pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, AU: Arbitraty units, CAD: Coronary artery disease

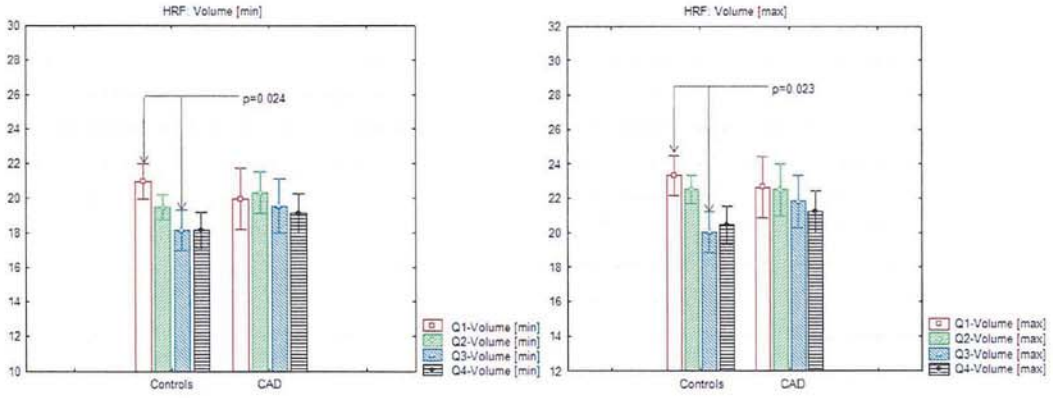

Figure 4.4: HRF values obtained for minimum and maximum volume of all 4 retinal quadrants in CAD patients and controls; HRF: Heidelberg Retinal Flowmeter

4.6.2.2 Peripapillary retinal perfusion

Flow, volume and velocity values were determined through analysis of HRF scanning images for each quadrant of one eye of each subject according to the search strategy described earlier. Prior to the statistical analysis, all HRF data was log transformed in an attempt to normalize it. Groups were then compared using a repeat measure ANOVA model with one factor being the location (i.e. quadrant measured) and the other being the group. Group means and standard deviations are shown in Table 4.3 on page 114.

There were no significant differences present between patients and controls in any of the four quadrants analysed for flow, volume and velocity ($p>0.05$). However, while CAD patients had similar values in all 4 quadrants for maximum and minimum flow, volume and velocity ($p>0.05$; see Table 4.3 on page 114 and Figure 4.4 on page 114), in healthy controls maximum and minimum volume were highest in Q1and lowest in Q3 as comparing to CAD patients ($p=0.024$ and $p=0.023$ respectively; see Table 4.3 on page 114 and Figure 4.4 on page 114).

	Arterial Dilation to Flicker							
	Controls [n=30]				CAD [n=24]			
	Maximum Dilation	*Maximum Constriction*	*Dilation Amplitude*	*BFR*	*Maximum Dilation*	*Maximum Constriction*	*Dilation Amplitude*	*BFR*
Flicker 1 [%]	5.8 (4.1)	-2.5 (2.3)	8.4 (4.9)	4.3 (3.7)	5.9 (3.3)	-2.4 (1.3)	8.3 (4.0)	3.7 (3.7)
Flicker 2 [%]	6.0 (3.4)	-2.4 (1.4)	8.4 (3.8)	4.4 (3.4)	4.9 (3.4)	-1.8 (1.4)	6.7 (3.4)	3.0 (3.0)
Flicker 3 [%]	6.0 (3.2)	-1.8 (1.7)	7.9 (3.0)	3.9 (2.7)	5.7 (3.3)	-2.2 (1.6)	7.9 (3.7)	3.3 (3.2)
ANOVA p-value [within groups]	0.472	0.061	0.779	0.565	0.165	0.229	0.065	0.051
ANOVA p-value [between groups]					0.677	0.590	0.668	0.329

Table 4.4: Retinal arterial reactivity parameters MD, MC, DA and BFR and results for within and between group ANOVA; MD: Maximum dilation, MC: Maximum constriction, DA: Dilation amplitude, BFR: Baseline corrected flicker response, CAD: Coronary artery disease

	Venous Dilation to Flicker					
	Controls [n=30]			CAD [n=24]		
	Maximum Dilation	*Maximum Constriction*	*Dilation Amplitude*	*Maximum Dilation*	*Maximum Constriction*	*Dilation Amplitude*
Flicker 1 [%]	6.3 (2.7)	-1.4 (1.1)	7.7 (3.3)	5.0 (2.1)	-1.5 (1.4)	6.4 (2.2)
Flicker 2 [%]	5.7 (1.8)	-1.7 (1.1)	7.4 (2.4)	5.4 (1.7)	-1.7 (1.4)	7.1 (1.9)
Flicker 3 [%]	6.1 (1.9)	-1.1 (1.2)	7.2 (2.3)	5.6 (1.5)	-1.5 (1.6)	7.1 (1.7)
ANOVA p-value [within groups]	0.062	0.163	0.553	0.956	0.368	0.264
ANOVA p-value [between groups]				0.168	0.841	0.285

Table 4.5: Retinal venous reactivity parameters MD, MC and DA and results for within and between group ANOVA; MD: Maximum dilation, MC: Maximum constriction, DA: Dilation amplitude, BFR: Baseline corrected flicker response, CAD: Coronary artery disease

4.6.3 Retinal vessel reactivity

4.6.3.1 Diameter Response

All dynamic response variables (maximum dilation (MD) and constriction (MC), dilation amplitude DA and BFR) of retinal arteries and veins were independent of SBP, DBP and IOP (ANCOVA $p>0.05$). There were no statistically significant differences within and between groups for MD, MC, DA and the BFR in retinal arterioles (all $p>0.05$), see Table 4.4 on page 115.

No significant difference was present within and between groups for the MD, MC and DA of retinal veins to repeated flicker stimulation (all $p>0.05$; see Table 4.5 on page 115).

	Time couse of retinal arterial dilation	
	Controls [n=30]	CAD [n=24]
	Mean +/- SD	*Mean +/- SD*
Flicker 1 [sec]	16.8 (5.2)	15.6 (6.2)
Flicker 2 [sec]	16.6 (6.2)	18.8 (6.2)
Flicker 3 [sec]	18.1 (6.3)	21.5 (10.5)
ANOVA p-value [within groups]	0.568	**0.017**
ANOVA p-value [between groups]		0.100

Table 4.6: Retinal arterial RT to flickering light stimulation of all 3 cycles in CAD patients and controls; RT: Reaction Time, CAD: Coronary Artery Disease

4.6.3.2 Time course to reach max dilation (RT)

The time needed to reach maximum dilation for both the measured artery and vein was calculated for each of the 3 stimulation cycles separately. We have defined this as the reaction time (RT, in seconds) measured from flicker initiation to the point when MD was reached as previously described in chapter 1.3.3.2. There was no significant difference between the three stimulation cycles for the RT of retinal arterial dilation in the control group (ANOVA p>0.05, Table 4.6 on page 116). However, in CAD patients, the RT to reach arterial MD increased at the rate of approximately three seconds for each cycle, reaching a maximum in the last cycle (p=0.016; cycle 1: 15.6 +/-6.2 sec, cycle 2: 18.8 +/-6.2 sec and cycle 3: 21.5 +/-10.5 sec).

There was no statistically significant difference between patients and controls in the time course of retinal venous dilation (ANOVA p=0.374; Table 4.7 on page 117). Furthermore, the time needed to reach maximum venous dilation was comparable for each cycle within groups (p>0.05; and p>0.05 respectively).

4.6.3.3 Slope

Analysis of slope unites time and diameter analysis to some degree, giving a better understanding at what rate the vascular diameter increases. Slope values were determined for each stimulation cycle separately for retinal arteries and veins as described in chapter 1.3.3.3 and see Figure 4.6 on page 118 below.

There were no statistically significant differences between and within groups for arterial slopes (p>0.05; see Table 4.8 on page 118). Although the arterial slope during the first flicker stimulation compared to the second and third was reduced in patients compared to controls, this did not reach statistical significance.

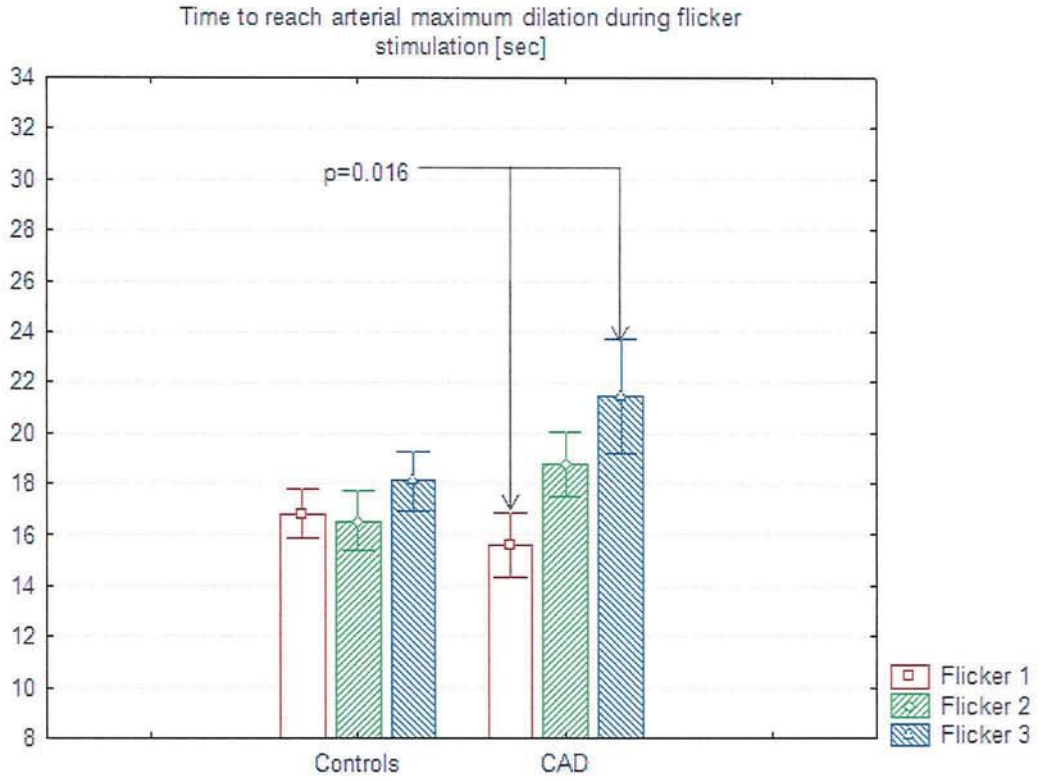

Figure 4.5: RT of retinal arterioles to flickering light stimulation for all 3 cycles in CAD patients and controls; RT: Reaction Time, CAD: Coronary Artery Disease

	Time couse of retinal venous dilation	
	Controls [n=30]	**CAD [n=24]**
	Mean +/- SD	*Mean +/- SD*
Flicker 1 [sec]	19.5 (3.8)	21.3 (4.6)
Flicker 2 [sec]	19.3 (5.8)	20.0 (4.7)
Flicker 3 [sec]	18.4 (4.1)	21.1 (5.7)
ANOVA p-value [within groups]	0.142	0.896
ANOVA p-value [between groups]		0.374

Table 4.7: Retinal venous RT to flickering light stimulation of all 3 cycles in CAD patients and controls; RT: Reaction Time, CAD: Coronary Artery Disease

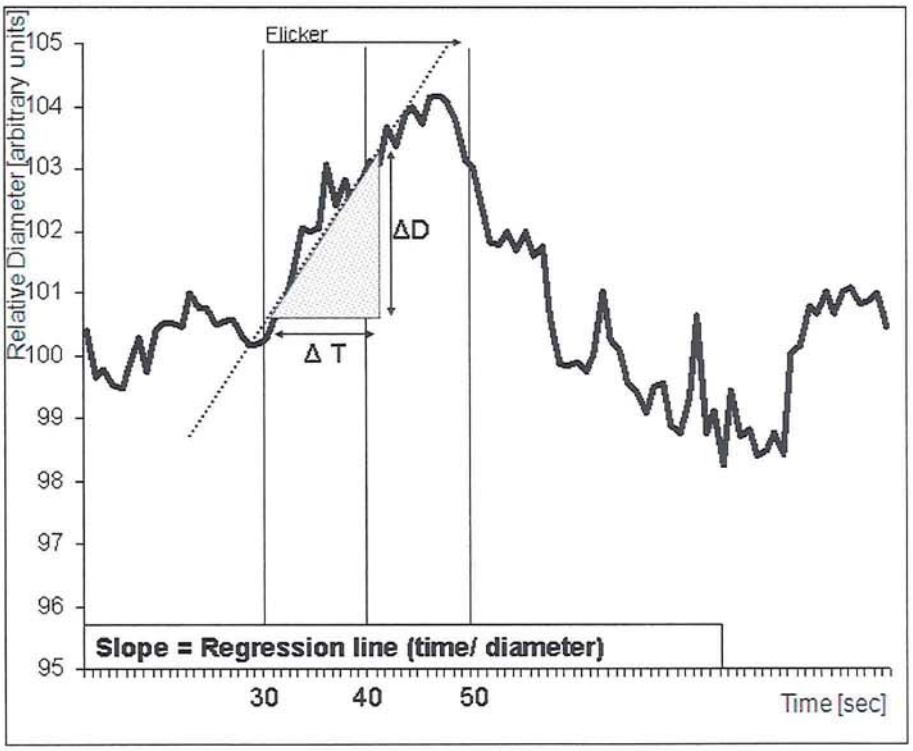

Figure 4.6: Graphical determination of retinal vascular slope

	Slope (artery)	
	Controls [n=30]	CAD [n=24]
	Mean +/- SD	*Mean +/- SD*
Flicker 1	0.48 (0.25)	0.35 (0.31)
Flicker 2	0.46 (0.29)	0.47 (0.29)
Flicker 3	0.48 (0.23)	0.48 (0.22)
ANOVA p-value [within groups]	0.867	0.200
ANOVA p-value [between groups]		0.392

Table 4.8: Retinal arterial slopes for each flicker cycle in CAD patients and controls; CAD: Coronary Artery Disease

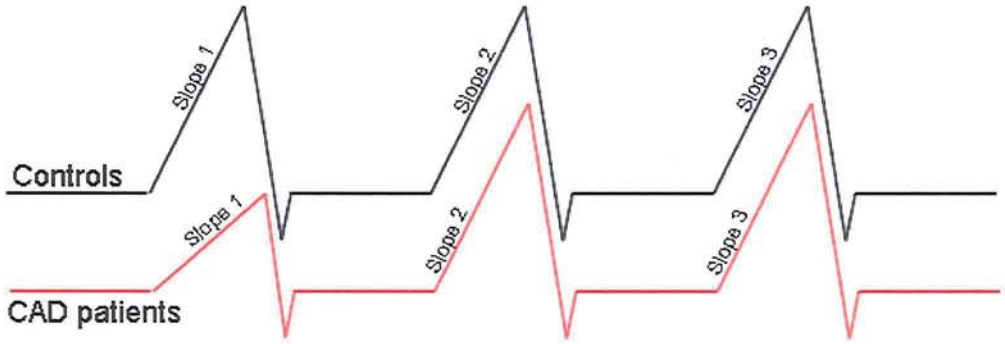

Figure 4.7: Schematic of arterial slopes during flicker assessment in CAD patients and controls; CAD: Coronary Artery Disease

	Slope (vein)	
	Controls [n=30]	CAD [n=24]
	Mean +/- SD	*Mean +/- SD*
Flicker 1	0.72 (0.12)	0.41 (0.30)
Flicker 2	0.61 (0.23)	0.60 (0.24)
Flicker 3	0.63 (0.25)	0.62 (0.26)
ANOVA p-value [within groups]	0.574	**0.022**
ANOVA p-value [between groups]		**0.001**

Table 4.9: Retinal venous slopes for each flicker cycle in CAD patients and controls; CAD: Coronary Artery Disease

Venular slope values were significantly different in patients compared to controls (p=0.001; see Table 4.9 on page 119). Patients exhibited significantly lower rate of diameter increase for the first stimulation cycle than controls. Furthermore, venular slopes were significantly different within the 3 cycles in patients (p=0.022), but not controls (p>0.05; see Table 4.9 on page 119).

4.6.3.4 Arterio-venous coupling

We also correlated the slope values obtained for each flicker cycle of retinal arteries and retinal veins separately. The results show a strong positive relationship/ coupling between arterial and venular reactivity in controls for all three stimulation cycles (p=0.017, p=0.015 and p=0.002, respectively, Table 4.10 on page 120). However, in the patient group such relationship was only found for the first and third stimulation cycle (p=0.027 and p=0.039), but not for the second cycle (p=0.070).

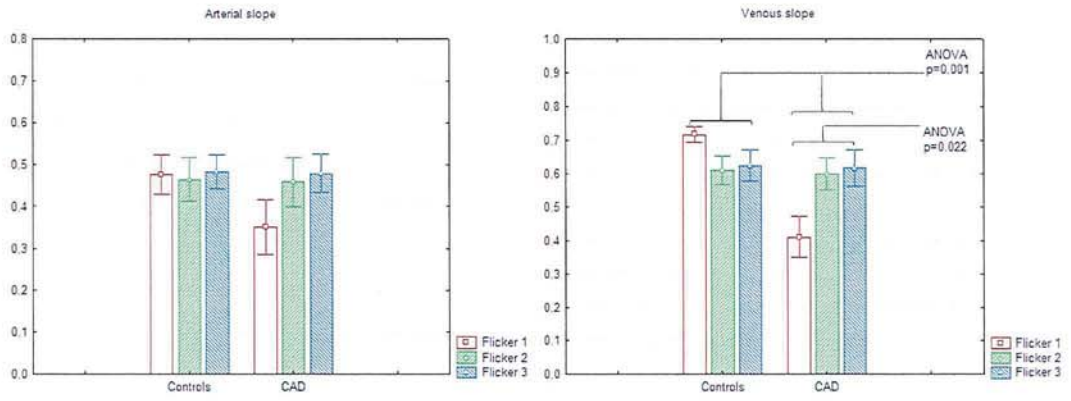

Figure 4.8: Retinal arterial and venous slopes for each flicker cycle in CAD patients and controls; CAD: Coronary Artery Disease

Parameter	Controls [n=30]		CAD [n=24]	
	Spearmans R	*p-value*	*Spearmans R*	*p-value*
slopeA1 & slopeV1	0.43	**0.017**	0.44	**0.027**
slopeA2 & slopeV2	0.44	**0.015**	0.37	0.070
slopeA3 & slopeV3	0.55	**0.002**	0.43	**0.039**

Table 4.10: Correlation between arterial and venous slopes for each flicker cycle in CAD patients and controls; CAD: Coronary Artery Disease

4.6.4 Static retinal image analysis

No significant difference was present between patients and controls for AVR, CRAE, BP, IOP and the size of veins and arteries undergoing dynamic measurement, $p > 0.05$ (see Table 4.11 on page 121). However, CRVE was significantly higher in patients (251+/-26 µm) compared to controls (232+/26 µm; p=0.041).

4.6.5 Relationship between static and dynamic vascular variables

No significant correlation was found between RI parameter measured at the CRA level and CRAE in both patients and controls (p>0.05). However, the RI measurement correlated significantly and negatively with the average APR parameter in patients but not in controls (patients: r=-0.43, controls: r=0.12; see Table 4.12 on page 122). However, PSV and EDV correlated significantly and negatively with the average APR this time in the control group but not the patient group (controls: r=-0.60 and r=-0.50, patients: r=0.07 and r=0.01; see Table 4.12 on page 122).

Parameter	Controls [n=30]	CAD [n=24]	p-value
	Mean +/-SD	*Mean +/-SD*	
IOP [mmHg]	13 (4)	14 (4)	0.698
fRVA(SBP) [mmHg]	121 (13)	126 (14)	0.34
fRVA(DBP) [mmHg]	76 (9)	76 (12)	0.886
sizeA [AU]	114 (18)	118 (16)	0.471
sizeV [AU]	147 (17)	156 (22)	0.061
A/V-ratio	0.85 (0.09)	0.83 (0.09)	0.923
CRAE [AU]	193 (26)	201 (21)	0.33
CRVE [AU]	232 (26)	251 (26)	**0.041**

Table 4.11: Static Retinal parameters of both CAD patients and controls; CAD: Coronary Artery Disease, IOP; Intra Ocular Pressure, fRVA(SBP) and fRVA(DBP): Systolic and Diastolic Blood Pressure measured during static assessment, sizeA: retinal arterial size, sizeV: retinal venous size; A/V-ratio: Arterio Venous ratio, CRAE: Cenral Retinal Artery Equivalent, CRVE: Central Retinal Venous Equivalent

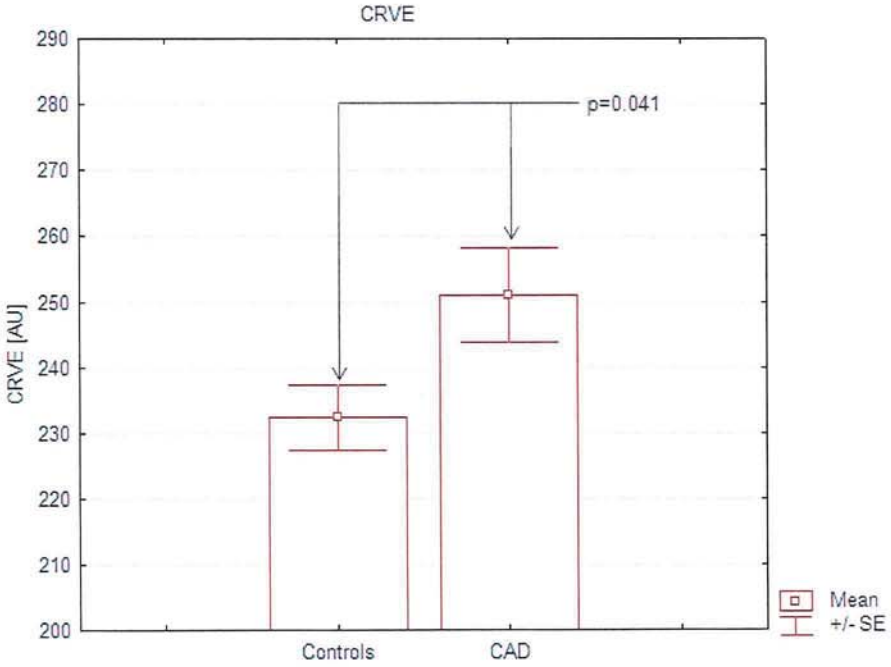

Figure 4.9: CRVE of CAD patients and controls; CRVE: Central Retinal Artery Equivalent, CAD: Coronary Artery Disease

	Controls [n=30]			CAD [n=24]		
Parameter	*Mean +/-SD*			*Mean +/-SD*		
CRAE [AU]	193 (26)			201 (21)		
CRA(RI)	0.70 (0.08)			0.68 (0.10)		
CRA(PSV) [m/s]	0.114 (0.027)			0.116 (0.151)		
CRA(EDV) [m/s]	0.035 (0.012)			0.067 (0.100)		
average APR	2.20 (0.81)			1.94 (0.95)		
		Spearman R	*p-value*		*Spearman R*	*p-value*
CRA(RI) & CRAE		0.07	0.713		-0.08	0.788
CRA(RI) & average APR		0.12	0.522		-0.43	**0.037**
CRA(PSV) & average APR		-0.60	**<0.001**		0.07	0.748
CRA(EDV) & average APR		-0.50	**0.005**		0.31	0.146

Table 4.12: Correlations between static and dynamic variables measured at the ocular vascular level in CAD patients and controls; CAD: Coronary Artery Disease, CRAE: Central Retinal Artery Equivalent, CRA(RI): Restistivity Index of the Central Retinal Artery, CRA(PSV): Peak Systolic Velocity of the Central Retinal Artery, CRA(EDV): End Diastolic Velocity of the Central Retinal Artery, average APR: average Arterial Peak Ratio

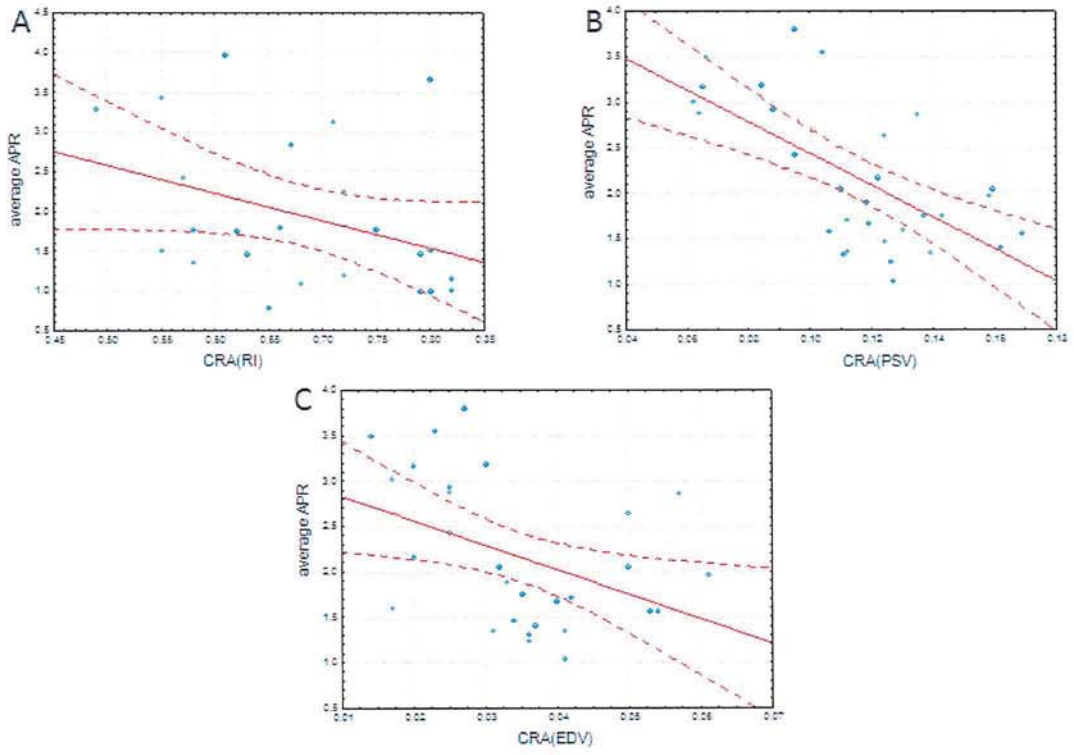

Figure 4.10: (A) Correlation between average APR and CRA(RI) in controls; (B) Correlation between average APR and CRA(PSV) in CAD patients; (C) Correlation between average APR and CRA(EDV) in CAD patients; average APR: average Arterial Peak Ratio, CRA(RI): Restistivity Index of the Central Retinal Artery, CRA(PSV): Peak Systolic Velocity of the Central Retinal Artery, CRA(EDV): End Diastolic Velocity of the Central Retinal Artery, CAD: Coronary Artery Disease

4.7 Discussion

4.7.1 Main findings

The aim of the present study was to assess various dynamic and static ocular vascular parameters and the relationship between them in patients suffering from CAD as compared to age-matched healthy controls. In agreement to previously published work [Wong et al., 2006a] the presented study found that CAD patients exhibited higher CRVE values than healthy controls. In addition, CAD patients showed an increased RT to reach MD to flicker light stimulation of retinal arterioles.

4.7.2 The effect of coronary artery disease on ocular blood flow at all levels of its vascular tree

CAD is affecting vascular beds throughout the body. Similar to the cerebral circulation [Patton et al., 2005] the retina is a highly perfused tissue and very vulnerable to alterations in blood flow rate and pulsatility [Pournaras et al., 2008]. It is very likely that ATS vascular diseases as well as changes in blood perfusion and blood rheology result in various alterations at the ocular level [Klein et al., 2000, Woodward et al., 2007].

4.7.2.1 Retrobular blood flow alterations

There have been reports showing an increased prevalence of CRA and CRV occlusion in those suffering from CAD [Wilson et al., 1979, Wong et al., 2005]. Preliminary sign of such occlusive event could be a change in retrobulbar blood flow velocity as measured using CDI technology. However, there was no measureable difference in flow velocities and resistivity indices in the presented sample, but a difference between groups in regard to the spectral waves of the OA and CRA. A similar finding, of changed spectral waves, has been shown by Fujioka and co workers in 2006 when assessing diabetic patients without diabetic retinopathy suffering from CAD [Fujioka et al., 2006]. Spectral wave shape alterations could occur due to age-related vascular wall changes . Nevertheless, in our study, this finding was age-independent and present only in patients suffering from CAD but not in the age-matched control group. As our patients were free of diabetes, this change could reflect a true effect of the general vascular changes associated with CAD.

4.7.2.2 Retinal blood flow

However, the autoregulatory mechanisms could vary according to vessel's location. It has been shown that the inferior retina is less responsive to carbogen inhalation compared to superior retina [Chung et al., 1999]. There is also a regional variability amongst the 4 quadrants in retinal blood flow variables and their reactivity to hypercapnia, with the most pronounced difference between superior and inferior quadrants [Roff et al., 1999]. This regional variability is also measurable when assessing visual fields [Roff Hilton et al., 2003] reflecting a different auto-regulation of superior versus inferior retina. All the afore mentioned studies support our finding, showing a difference between the superior and inferior quadrants in controls but not CAD patients. This further supports our hypothesis of a compromised vascular regulation at the ocular level in the presence of CVD.

4.7.2.3 Retinal vascular reactivity

Endothelial function is an independent marker of vascular disease progression and cardiovascular mortality [Constans and Conri, 2006, Spiel et al., 2008]. Endothelial function, a measure of auto-regulatory properties, can be assessed in many different ways as described [Nagel and Vilser, 2004b, Blum et al., 1999, Woodward et al., 2007, Chong et al., 2004, van Hecke et al., 2006, Felmeden and Lip, 2005]. Most techniques have in common a baseline measurement, followed by various stimulations/ provocations (see chapter 1) that mainly works through manipulation of systemic BP, and a recovery time. Unlike other vascular beds, blood vessels of the retina can be observed non-invasively; therefore, in our study endothelial function at the retinal level was assessed using a RVA and flickering light stimulation as described in Section 1.3.3.2. This technique has the advantage that it directly and exclusively stimulates the retinal tissue instead of indirect stimulation via BP increase/ decrease, therefore, minimising the risk of measurement bias due to co- variables. We were able to demonstrate a delayed response to flicker stimulation in retinal arteriols as well as in the retinal veins. This time delay could be the result of either atherosclerotic vessel wall changes [Wong et al., 2001b] or an abnormal auto-regulatory response as a result of increased arterial stiffness [Dolan et al., 2006b, 2007, Hansen et al., 2006, Cohn et al., 2005, Duprez and Cohn, 2007]. However, this remains to be verified by assessing the relationship between systemic markers of ATS and ocular parameters as described.

In order to insure a stable blood flow, arterial and venous vasomotion has to be coupled, meaning e.g. an increase in arterial diameter should lead to an increase in venous diameter to ensure adequate "outflow". Another factor contributing to their "synchronisation" is the fact that arteries and veins travel in close

vicinity to each other, therefore causing tissue movement within their embedding that further contributes to this coupled vasomotion. However, it is not possible to relate one point along an artery to correspond to exactly one point along a vein. Furthermore, one would always expect a delay in the venous motion as it relies on arterial input. Gugleta and co workers 2006 [Gugleta et al., 2006a] calculated arterio-venous phase delays using fast Fourier transformation (FFT); however, the frequencies had been obtained using average diameter values which bears a high bias due to interference. In addition, one cannot assume the existence of two corresponding points on both the artery and vein. To give a better insight into this coupling/ synchronisation mechanism we correlated the arterial and venous slopes obtained during each stimulation cycle separately; in this way we could possibly have a marker for assessing autoregulatory integrity at the retinal level. In controls a strong relationship between arterial and venous responses was present, but this significantly decreased in magnitude in CAD patients, in fact being completely absent in the second stimulation cycle. This could represent a sign of endothelial dysfunction in patients suffering from CAD.

4.7.2.4 Structural changes

Common changes of retinal vessels due to CAD and those at risk are changes in retinal vascular calibre of both arteries and veins and has been confirmed in large multi ethnic population based studies around the world [Klein et al., 2000, Cheung et al., 2007b]. The findings of this study show an increased venular diameter in patients compared to controls are in agreement with the current research. However, venular dilation is also a common feature in those at risk of CVD in particular in subjects smoking [Wang et al., 2006]. Therefore, this finding is not solely attributable to the presence of CAD but could more so be a pre-existing vascular alteration due to previous smoking and life style, e.g. alcohol intake, high fat diet and the lack of exercise. Indeed, 19 of our patients have smoked sometime during their life.

4.7.2.5 The relationship between structural and functional parameters of retinal vessels

We have found various significant relationships between average APR parameter and velocity parameters (RI, PSV, EDV) measured at the CRA level in patients and controls. This finding further supports the hypothesis that in disease, numerous loss of vascular elasticity is manifest along the vascular tree but in health such relationship is absent.

4.7.3 Conclusion

In summary, retro-bulbar and retinal blood flow is preserved in the presented sample of CAD patients that are regularly attending cardiovascular exercise programs. However, reaction to flicker stimulation is delayed in patients compared to controls. It can be concluded that, despite various treatments for heart disease, an endothelial vascular dysfunction at the retinal level still exist in these patients.

4.7.4 Limitations

Based on previous studies and our hypothesis, we thought to find a much greater difference in retinal blood flow and reactivity variables than described above. However, our CAD patients were under various systemic medications. Moreover, they also undergone a cardiac rehabilitation program at the time of recruitment. It has been shown that cardiac rehabilitation improves significantly endothelial function in patients suffering CAD [Lee et al., 2006, Hambrecht et al., 2000b, Linke et al., 2008, Erbs et al., 2006, Hambrecht et al., 1999, 2004, Linke et al., 2006]. This effect, along with the effect of systemic medication may have led to an improvement in endothelial function in our sample of patients, reflected in the "normal" values obtained during our measurements. Although similar to other studies [Lind, 2007] we tried to minimise the effect of medication upon our measurements by discontinuing it for 24 hrs prior the appointment it is possible that the combined effect of the drugs taken and the impact on their half lifes due to the combination had an effect on our results. Ideally patients newly diagnosed would be a better group to assess, but the fact that those newly diagnoses are most common unstable CAD patients, excluded their participation immediately, therefore we relied on patients that had been newly diagnosed but undergone cardiac intervention and cardiac rehabilitation prior to the research appointment to ensure stability and minimise cross variable bias.

Chapter 5

Nail-fold capillary blood flow and its relationship to autonomic nervous system function in coronary artery disease patients that have undergone cardiovascular rehabilitation

5.1 Abstract

Purpose: To assess the autonomic nervous system function and peripheral blood flow and reactivity in patients suffering from coronary artery disease (CAD) compared to healthy controls.

Methods: Twenty-eight patients suffering from CAD and 30 age and sex matched controls underwent ambulatory 24 hr blood pressure (BP) and electrocardiogram (ECG) monitoring using Cardiotens-01 (Meditech Ltd., Hungary). Ambulatory blood pressure values were used to calculate the ambulatory arterial stiffness index (AASI) as previously explained in chapter 1.7.2.2. HRV parameters frequency domain parameters low-frequency (LF), high-frequency (HF) and LF/HF ratio were also measured. In addition, peripheral blood flow and reactivity at the nail-fold capillaries were assessed in both patients and controls.

Results: AASI was significantly higher in CAD patients (patients: 0.35 (0.15) and controls: (0.24 (0.09); p=0.005). HF values was significantly lower during night time in patients as compared to controls (p=0.047); moreover, circa-

dian changes in HF, LF and the LF/HF-ratio were significantly different in patients in compared to controls (HF p= 0.006, LF p=0.004 and LF/HF-ratio p=0.027). Although there was no difference between groups for the baseline capillary flow (BCF) at the nail-fold capillaries level (p=0.064), patients exhibited significantly longer recovery time (RT) after cold provocation (p=0.009).

Conclusions: Patients suffering from CAD showed a significantly impaired autonomic nervous system (ANS) and peripheral vascular function despite undergoing cardiovascular rehabilitation (CVR).

5.2 Introduction

Systemic BP was long used as the sole marker for risk assessment of cardiovascular disease (CVD) and its progression [Martin et al., 2008]. However, over the decades more evidence emerged that not only the BP measurement but also assessments of both the ANS and endothelial function (EF) complement the risk and progression indices for this type of diseases [Harris and Matthews, 2004, Abrams, 1997]. CAD patients frequently suffer from coronary spasm due to atherosclerotic vessel wall changes and endothelial impairment [Abrams, 1997, Harris and Matthews, 2004]. However, it is unclear if this vasospastic behavior manifests only at the peripheral level or is a more general occurrence. Normal vascular function is relying on a healthy endothelium as much as on an intact ANS due to the complex relationship between those two systems [Linke et al., 2008]. ANS neurotransmitters are able to diffuse across the vascular smooth muscle cells to act on the endothelium [Shepherd, 1995] and basal release of NO by the vascular endothelium attenuates sympathetic nervous system (SNS) initiated vaso-constriction [Vanhoutte and Miller, 1989, Owlya et al., 1997]. A high SNS activity, or a suppressed parasympathetic nervous system (PSNS) function could represent a sign of impaired ANS activity [Harris and Matthews, 2004, Dekker et al., 2000, Vardas et al., 1996]; moreover, this abnormal ANS function can result in up-regulation of vascular endothelial response activity to a degree where the endothelium can not cope adequately anymore. However, different vascular beds work different and endothelium-related factors can initiate various responses depending on both the stimulus and the stimulation pathway used [Troseid et al., 2006].

Recent research has identified that arterial stiffness can result in impairments of vascular function either with age or appearance of atherosclerosis [Dolan et al., 2007, Nigam et al., 2003, Duprez et al., 2005, Duprez and Cohn, 2007, Cohn et al., 2005, Dolan et al., 2003, Cheung et al., 2007c,a]. This factor, named arterial stiffness can be quantified by using various methodologies; a simple way

of assessing it is by using ambulatory BP values as described earlier in Chapter 1.7.2.2. The AASI index obtained in this way offers information on the elastic properties of larger arteries (conduit arteries) but is not useful in determining the elasticity of small arteries. Therefore, the use of capillary microscopy for the assessment of peripheral vascular reactivity could represent a novel way of measuring the function of such small vessels in both health and disease.

All patients participating in the present study were recruited from the CRU and had undergone a minimum of three months exercise training (two sessions of 45 min each per week). A number of previous studies [Lee et al., 2006, Deligiannis, 2000, McAllister and Laughlin, 2006] have shown the positive impact of exercise training on autonomic and endothelial function. However, all of these studies measured endothelial or ANS function using techniques aimed at larger arteries and therefore neglecting peripheral vascular function. The peripheral vascular function is important in the systemic regulation as it provides a measure of vascular resistance which plays a major part in BP regulation which in turn affects EF within the regulatory feedback loop potentially relating to damage elsewhere [Cheung et al., 2007a]. Patients suffering from CAD undergoing exercise training may show gross improvement of function at the large vessels level ; however, peripheral vessels activity might still be impaired.

5.3 Hypothesis

The function of compromised large vessels of patients suffering from CAD may be largely restored to some degree by cardiovascular rehabilitation; however some degree of impairment may still be present at peripheral capillary level. This feature may serve as a marker for disease progression, regression and possible for treatment monitoring.

5.4 Aims

The aim of this study was to assess endothelial and ANS function at macro- and microvascular level in CAD patients undergoing cardiac rehabilitation compared to healthy controls.

5.5 Subjects and methods

5.5.1 Patient recruitment

Patients were recruited from the cardiac rehabilitation unit (CRU) at City Hospital, Birmingham. The cardiac rehabilitation program consisted of a number of educational sessions and individual consultations helping the patient to further understand their condition and assist them in psychological and socioeconomic questions as well as helping managing their changes in everyday life such as work and diet. Furthermore, all patients were enrolled in a 12 week monitored exercise program consisting of 2 one hourly sessions per week. These sessions were custom designed for each individual and reassessed during the course of the program. A typical routine was made up of an initial BP check, followed by a 10 minute warm up walk, cycling, rowing and strength building exercise with different weights, ending with a 10 min walk to cool down. After successful completion of the program most patients kept coming for further sessions twice a week. The advantage compared to exercising at a local gym is given by two reasons: for one, nurses and training staff on site can give expert advice to the patient at all times, and second, volunteers helping with the facilitation of the program are a vital support at the psychosocial and emotional level to the patient as all of them have the condition and went through the program themselves too.

5.5.1.1 Study's inclusion criteria

- Patients diagnosed with CAD (abnormal coronary arteries verified by coronary angiography)
- No upper age limit applid (patients had to be at least 18yrs of age)

5.5.1.2 Study's exclusion criteria

- Diabetes mellitus
- Hypertension, as defined see Section 1.7.1; see Table 1.3 on page 62;
- Atrial fibrillation;
- History of any ocular disease;
- History of neurological diseases associated with loss of visual function
- Any type of ocular surgery; and

- Normal left ventricular function (LV Function); as defined by depressed left ventricular ejection fraction (LVEF). LVEF represents stroke volume/LV end-diastolic volume, whereas the normal LVEF equals approximately 60%, but impaired LVEF is generally defined as less than 45%.

5.5.2 Recruitment of healthy individuals

Healthy individuals were recruited from patients spouse and other volunteers. In addition to the exclusion criteria above, healthy controls had to be free from any form of vascular and heart disease.

5.5.3 Ethical approval

Prior to the study ethical approval was obtained from NHS Sandwell and West Birmingham Ethics Committee and Aston University Ethics Committee. Written informed consent was received by all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets of Declaration of Helsinki.

5.5.4 Experimental protocol

5.5.4.1 Patient preparation

Subjects were asked to refrain from consuming caffeinated products such as coffee and chocolate, as well as from drinking alcohol and smoking on the study day. In addition, patients were instructed to abstain from their usual medication for 24hrs prior to the research appointment.

5.5.4.2 Ambulatory BP and ECG monitoring

Each patient and control was fitted with a computer-operated ambulatory blood pressure and ECG monitor (Cardiotens-01, Meditech Ltd., Hungary) for 24 hrs, between 9 and 10 am. A detailed description of the monitor used is offered in Section 1.7.2.1. All subjects were given a diary to fill in the following:

- Any use of medication along with dosage and time;
- Any physical activities, e.g. walking or exercising; and
- The time they went to sleep and got up.

The diary recordings were later used to calculate true wake and sleep times for data analysis.

24hr BP profile The monitor was programmed to measure BP every 15 minute during the daytime and every 30 minutes during the night time. Day and night time periods were estimated based on true sleep and wake up times for each individual included in the study. For the final data analysis, a minimum of 80% of the programmed recordings was required. Outliers of BP values measured were rejected on a method based on PP determination. According to Graham et. al [Graham et al., 1995], a PP of less than 10mmHg with the SBP below 100mmHg or of less than 10% of the SBP if higher than 100mmHg was considered non-physiological and data was not included in the analysis. Nocturnal BP dip was calculated as described in Section 1.7.1.3 Furthermore we calculated the circadian changes of BP according to the equation below:

$$CircardianBPchange = activeperiodBP - passiveperiodBP \tag{5.1}$$

for each: SBP, DBP and MAP for every subject individually.

Ambulatory arterial stiffness index SBP and DBP recordings were used to obtain AASI for each patient and calculated according to the Equation (1.9) as explained in Section 1.7.2.2.

Heart rate variability HRV analysis is a measure of ANS function as described in Section 1.7.3. All HRV values were calculated using the Cardiovision 1.7.2 software (PMS Instruments Ltd., Maidenhead, UK). For evaluation of HRV we applied the frequency domain analysis as described earlier in Section 1.7.3. Values for LF, HF and LF/HF-ratios were obtained for 24hrs, day time, and night time. Circadian changes were calculated using the equation outlined below:

$$CircadianchangeHRV = activeperiodHRV - passiveperiodHRV \tag{5.2}$$

for each LF, HF and LF/HF-ratios of each subject.

5.5.4.3 Peripheral blood flow

Prior to capillaroscopy measurements, all patients and controls were asked the following two questions:

1. Do you suffer from cold hands and feet even during summer? ;
2. Do other people tell you that you have cold hands and feet?

Positive answer was interpreted as sign of peripheral vasospasm and recorded. According to the protocol established by Mahler, Saner and Marth in 1983 [Mahler

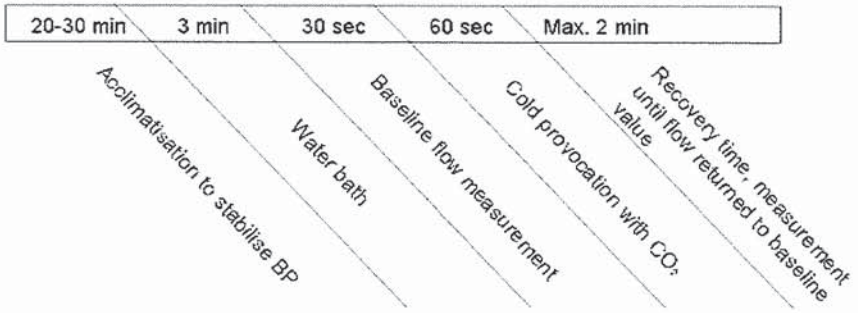

Figure 5.1: Measurement protocol of nail-fold capillary assessment

et al., 1983] all subjects were seated in a temperature controlled room (for more detail see chapter 1.4.3.2) for 20 to 30 minutes prior to the measurement. This ensured stabilization of the patients' blood pressure before the measurement was initiated. Immediately before capillary flow was measured, both hands of each subject were immersed in 40 º C warm water for 3 minutes to maximise blood flow and to therefore minimise any influence of seasonal temperature variations on flow variables [Gasser and A, 1991, Gasser and Dubler, 1996].

Baseline measurement After image focusing, a baseline measurement of at least 30 seconds was obtained by means of laser Doppler flowmetry as described in Section 1.4.3.2.

Cold provocation with Carbon dioxide The nail-fold area was cooled by rapidly decompressing carbon dioxide for 60 seconds immediately after the baseline measurement. Stream temperature was calibrated at -15 ºC in order to cool the nail fold area down to +15ºC. This technique has been described in more detail in Section 1.4.3.2.

Recovery time Recovery time (RT) was defined as the time needed (in seconds) for capillary flow to return to baseline values as measured before cold provocation.

Assessment of peripheral vasospasm Peripheral vasospasm was assessed according to an established protocol by Saner et al. in 1987 [Saner et al., 1987b,a]. In order to establish vasospasm, the recordings had to be of good quality; the assessment has beeb performed off-line from the recorded sequence. For the analysis the first row of capillaries was observed . Any interruption of the blood flow for a period longer than 12 sec during cold exposure was classified as peripheral vasospasm.

Parameter	Controls [n=26]	CAD [n=23]	p-value
	Mean +/-SD	*Mean +/-SD*	
Age [yrs]	53 (9)	57 (9)	0.193
BMI [kg/m²]	26 (3)	28 (4)	**0.01**
Hours of sleep [hrs]	7.76 (1.08)	7.92 (1.09)	0.604

Table 5.1: Group characteristics; BMI: Body mass index, CAD: Coronary artery disease

5.5.5 Statistical analysis

All results are given as the mean +/- SD. Depending on the data distribution either the Mann-Whithey-U test, unpaired or paired t-tests were used for statistical analysis. Within group differences of AASI were analysed by using a one-way ANOVA model followed by Tukey's test for unequal n. Tukey's test for unequal n is a posthoc test used when the groups assessed have significant different sample sizes (i.e. number of participants). Statistical significance was defined at the level of $p<0.05$.

5.6 Results

5.6.1 Sample

Twenty-eight CAD patients (4 women and 24 men) and thirty control subjects (8 women and 22 men), all of Caucasian origin, were assessed for this study. Nine subjects (5 patients) were excluded from the statistical analysis, due to multiple artefacts, electrode loss or insufficient BP recordings. The final sample consisted of 23 patients (3 women and 20 men) and 26 (5 women and 21 men) controls. Demographic data of the sample analysed is listed in Table 5.1 on page 134. Although there was no statistical significant difference in age between the study groups (patients: 57 (9) yrs and controls: 53 (9) yrs; $p>0.05$), patients had significantly higher BMI (patients: 28 (4) and controls: 26 (3); $p=0.010$, see Table 5.1 on page 134) than controls.

5.6.2 Systemic blood pressure

BP values as measured during the 24hrs, day time, night time periods as well as circadian changes are all listed in Table 5.2 on page 135. There were no significant differences in BP values between patients and controls (all $p>0.05$). Numbers of patients with absent nocturnal BP dip (patients: 7 out of 23 and controls: 6 out of 26: $p>0.05$), normal nocturnal BP dip (patients: 10 out of 23 and controls: 13 out of 26; $p>0.05$) and over-dippers (patients: 5 out of 23 and

Parameter	Controls [n=26]	CAD [n=23]	p-value
	Mean +/-SD	*Mean +/-SD*	
SBP-24hr [mmHg]	120 (10)	117 (10)	0.469
DBP-24hr [mmHg]	72 (7)	68 (8)	0.114
MAP-24hr [mmHg]	88 (7)	84 (8)	0.192
SBP-Day [mmHg]	125 (11)	120 (15)	0.381
DBP-Day [mmHg]	76 (8)	73 (9)	0.369
MAP-Day [mmHg]	92 (9)	91 (14)	0.446
SBP-Night [mmHg]	110 (10)	106 (14)	0.311
DBP-Night [mmHg]	63 (7)	62 (9)	0.531
MAP-Night [mmHg]	79 (8)	80 (19)	0.423
Circardian SBP [mmHg]	14 (10)	14 (10)	0.531
Circardian DBP [mmHg]	13 (8)	11 (7)	0.227
Circardian MAP [mmHg]	13 (9)	11 (10)	0.381

Table 5.2: Blood pressure characteristics of the study groups; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial blood pressure; Circadian: Circadian variation = Day – Night values, CAD: Coronary artery disease

controls: 7 out of 26; $p>0.05$) were also comparable between groups; this was assessed using the Chi-square test.

5.6.2.1 Ambulatory arterial stiffness index

CAD patients had significantly higher AASI than controls (patients: 0.35 (0.15) and controls: 0.24 (0.09); $p=0.005$).

Subgroup analysis Subjects exhibiting normal nocturnal BP dip have been shown previously to have lower AASI when compared to non-dippers [Baumann et al., 2008]. In order to examine if this was the case in our sample, we performed a sub-group analysis. First we assessed within group differences of non-dippers, dippers and over-dippers using one-way ANOVA after which we performed a MANOVA with two factors: one factor was the dipping status the other the patient group. All results with p-values are listed in Table 5.3 on page 136. Within the patient group over-dippers, dippers and non-dippers had comparable AASI (non-dippers: 0.36 (0.17), dippers: 0.38 (0.15) and over-dippers 0.26

Parameter	Controls [n=6]	CAD [n=7]	ANOVA p-value [between groups]
Nondipper	*Mean +/-SD*	*Mean +/-SD*	
AASI	0.31 (0.11)	0.36 (0.17)	0.116
Parameter	**Controls [n=13]**	**CAD [n=10]**	
Dipper	*Mean +/-SD*	*Mean +/-SD*	
AASI	0.25 (0.08)	0.38 (0.15)	
Parameter	**Controls [n=7]**	**CAD [n=5]**	
Over-dipper	*Mean +/-SD*	*Mean +/-SD*	
AASI	18 (0.04)	0.26 (0.10)	
ANOVA p-value [within groups]	**0.027**	0.299	

Table 5.3: Subgroup analysis of AASI for controls and CAD patients; AASI: Ambulatory arterial stiffness index, CAD: Coronary artery disease

(0.10); ANOVA p=0.299). However, within the control group, subjects had significantly different AASI depending on their dipping status (non-dippers: 0.31 (0.11), dippers 0.25 (0.08) and over-dippers 0.18 (0.04); ANOVA p=0.027). Post-hoc test using Tukey's test for unequal n revealed comparable AASI for non-dippers and dippers (p=0.278) but a significant difference between non-dippers and over-dippers (p=0.027), see Table 5.3 on page 136 and Figure 5.2 on page 137. However, there was no significant difference between non-dippers, dippers and over-dippers when compared individually between groups (MANOVA p=0.116).

5.6.3 Heart rate variability

No significant differences were present for LF, HF and LF/HF-ratio during the 24 hr period and the day time between CAD patients and controls (all p>0.05), see Table 5.4 on page 137. However, night time HF was significantly reduced in patients compared to controls (patients: 25 (9) and controls 31 (12); p=0.047). Furthermore, circadian changes in HRV were significantly different between patients and controls. Patients had significantly different circadian variations in LF (patients: -2.9 (11) NU and controls 7.9 (10) NU; p=0.004), HF (patients:1.0 (10.0) NU and controls: -8.3 (9.3) NU; p=0.006) and LF/HF-ratio (patients: -0.3 (2.1) NU than controls: 0.2 (0.1) NU; p=0.027) compared to controls.

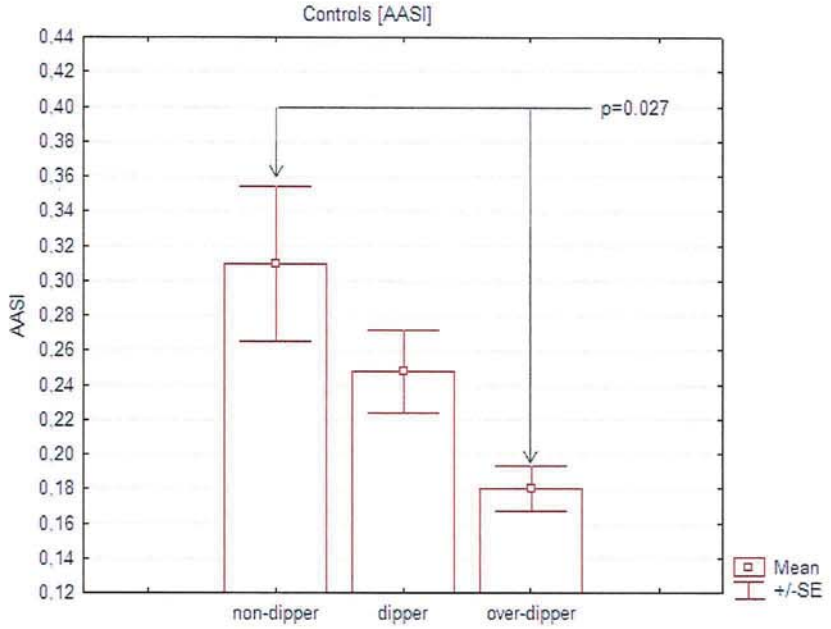

Figure 5.2: Difference between non-dippers, dippers and over-dippers of AASI in healthy controls; AASI: Ambulatory arterial stiffness index

Parameter	Controls [n=26]	CAD [n=23]	Mann-Whitney-U [2-sided] p-value
	Mean +/-SD	*Mean +/-SD*	
LF-24hr [NU]	72 (10)	71 (12)	0.654
HF-24hr [NU]	26 (9)	25 (8)	0.944
LF/HF-24hr	3.2 (1.8)	3.3 (1.5)	0.976
LF-Day [NU]	75 (8)	70 (10)	0.067
HF-Day [NU]	23 (7)	26 (8)	0.205
LF/HF-Day	3.8 (1.7)	3.2 (1.6)	0.114
LF-Night [NU]	67 (13)	73 (10)	0.092
HF-Night [NU]	31 (12)	25 (9)	**0.047**
LF/HF-Night	2.8 (1.9)	3.5 (1.8)	0.069
Circadian LF [NU]	7.9 (10)	-2.9 (11)	**0.004**
Circadian HF [NU]	-8.3 (9.3)	1.0 (10.0)	**0.006**
Circadian LF/HF	0.2 (0.1)	-0.3 (2.1)	**0.027**

Table 5.4: HRV parameters measured in the study groups; HRV: Heart rate variability, LF: Low frequency component of the HRV; HF: High frequency component of the HRV; NU: Normalised units

	Controls [n=26]		Paired t-test p-value	CAD [n=23]		Paired t-test p-value
	Mean +/-SD			*Mean +/-SD*		
Parameter	LF-Day [NU]	LF-Night [NU]		LF-Day [NU]	LF-Night [NU]	
	75 (8)	67 (13)	**<0.001**	70 (10)	73 (10)	0.225
Parameter	HF-Day [NU]	HF-Night [NU]		HF-Day [NU]	HF-Night [NU]	
	23 (7)	31 (12)	**<0.0001**	26 (8)	25 (9)	0.652

Table 5.5: Group difference in circadian changes of LF and HF values obtained for CAD patients and controls; HRV: Heart rate variability, LF: Low frequency component of the HRV; HF: High frequency component of the HRV; NU: Normalised units, CAD: Coronary artery disease

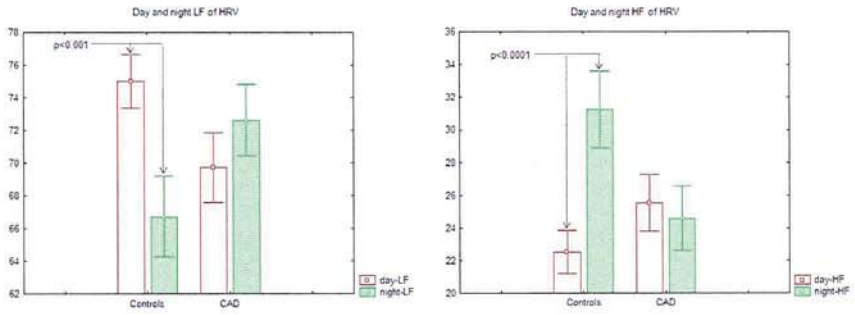

Figure 5.3: Difference in circadian changes of LF and HF components of HRV between CAD patients and controls; HRV: Heart rate variability, LF: Low frequency component of the HRV, HF: High frequency component of the HRV; CAD: Coronary artery disease

Parameter	Controls [n=30]	CAD [n=24]	t-test
	Mean +/- SD	*Mean +/- SD*	*p-value*
SBP [mmHg]	120 (13)	123 (12)	0.475
DBP [mmHg]	77 (11)	76 (11)	0.819
BCF [mm/sec]	0.23 (0.11)	0.18 (0.06)	0.064
RT [sec]	21 (11)	35 (22)	**0.009**

Table 5.6: Results of the nail-fold capillary assessment; BCF: baseline capillary flow; RT: recovery time, CAD: Coronary artery disease

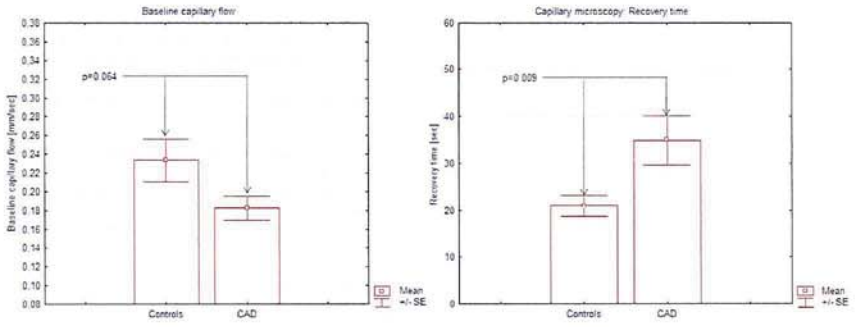

Figure 5.4: Difference in baseline capillary flow and recovery time between CAD patients and controls

5.6.4 Peripheral blood flow assessment

A significantly higher number of patients reported that they suffer from cold hands and feet (patients: 19 out of 24 and controls: 2 out of 30; $p<0.001$).

SBP and DBP during peripheral blood flow assessment were comparable between groups ($p=0.475$ and $p=0.819$, respectively), see Table 5.6 on page 139. Baseline capillary flow (BCF) as measured by Doppler technique was comparable between patients and controls (patients: 0.18 (0.06) mm/sec and controls: 0.23 (0.11) mm/sec; $p=0.064$), while the recovery time (RT) was significantly longer in the patient group (patients: 35 (11) sec and controls: 21 (11) sec; $p=0.009$).

5.6.5 Correlations

In order to examine the relationship between systemic circulation and peripheral circulation we correlated AASI with BCF and RT. While in the patient group no relationship between BCF and AASI (Spearmans $R=0.01$, $p>0.05$) or RT and AASI (Spearmans $R=0.06$, $p>0.05$) was evident (see Table 5.7 on page 140), controls exhibited a significant correlation between RT and AASI (Spearmans $R=0.414$, $p=0.049$) but not BCF and AASI (Spearmans $R=0.27$, $p=0.180$).

Parameter	Controls [n=30]		CAD [n=24]	
	Mean +/- SD		*Mean +/- SD*	
BCF [mm/sec]	0.23 (0.11)		0.18 (0.06)	
RT [sec]	21 (11)		35 (22)	
AASI	0.24 (0.09)		0.35 (0.15)	
	Spearmans R	*p-value*	*Spearmans R*	*p-value*
BCF & AASI	-0.27	0.18	0.01	-0.957
RT & AASI	**-0.414**	**0.049**	0.06	0.803

Table 5.7: Correlations between nail-fold capillaroscopy parameters (BCF, RT) and AASI; BCF: Baseline capillary flow, RT: Recovery time, AASI: Ambulatory arterial stiffness index, CAD: Coronary artery disease

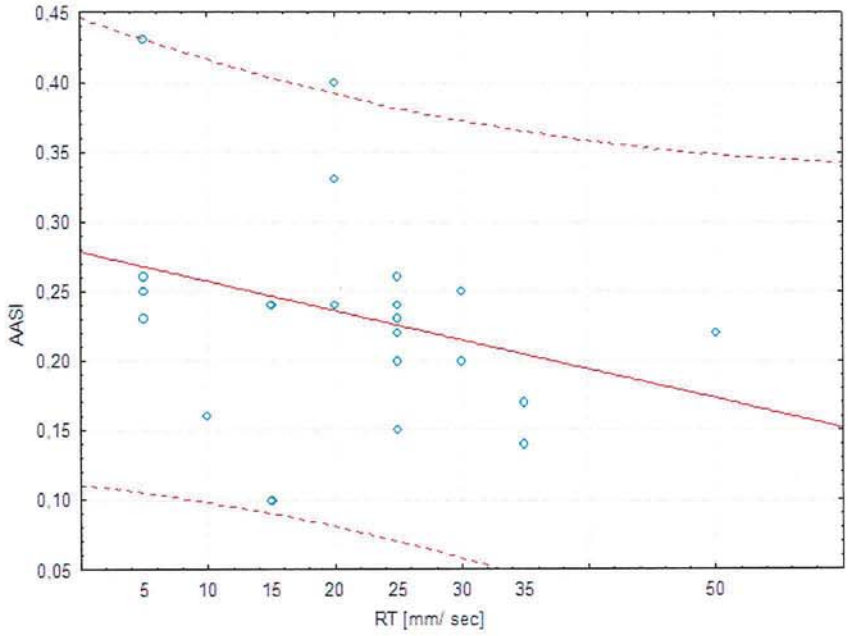

Figure 5.5: Correlation between RT and AASI in controls. RT: Recovery time; AASI: Ambulatory arterial stiffness index

5.7 Discussion

5.7.1 Main findings

Systemic BP values in patients and controls were comparable throughout the 24-h monitoring period. Patients had significantly higher AASI than controls; however, as opposed to normal controls, when we further divided the patients in subgroups based on the presence or absence of nocturnal BP dip, the AASI values were comparable. Parameters reflecting circadian ANS activity in patients were significantly different compared to healthy controls for both sympathetic and parasympathetic function. Moreover, patients exhibited abnormal peripheral blood flow reactivity to cold exposure. While both groups showed similar BCF, patients needed significantly longer to recover to the level of pre-stimulation values than the control group.

5.7.2 Circadian blood pressure and arterial stiffness

All measured BP values, were comparable between groups in our sample. This is not surprising, as patients and controls were normo-tensive. However, the patient group showed a significantly higher AASI than controls independent of their dipping status. This finding is in agreement with other studies showing increased AASI in CAD patients [Dolan et al., 2006b]. The explanation for this finding could be the fact that the underlying systemic arthrosclerosis present in CAD patients results in an increase in arterial stiffness. In addition, obesity has also been linked to increased arterial stiffness [Miyaki et al., 2008, Mizia-Stec et al., 2008]. Indeed, the BMI was significantly elevated in our patient group compared to controls, therefore further supporting this theory.

5.7.3 Autonomic function

The assessment of HRV is a reliable non-invasive measure of the ANS function. A number of studies have demonstrated that CAD patients exhibit an abnormal HRV [Kochiadakis et al., 2000, Hayano et al., 1991, Bellwon et al., 1996, Kupari et al., 1993, Vardas et al., 1996, Huikuri et al., 1994]; however, circadian changes in HRV have been rarely looked at in this context. Abnormal circadian variations in HRV could reflect an abnormal adaptation to changes in mental and physical activity that occur during the day-to-day life. In healthy individuals, the PSNS activity is higher during sleep compared to wake times (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996) . However, in our sample, patients exhibited a decreased parasympathetic activation during sleep compared to controls. Furthermore, circadian changes in all

measured HRV parameters were in the opposite direction compared to controls. A constant high sympathetic tone can lead to an abnormal HRV [Korpelainen et al., 1996, 1997, Preckel and von Kaenel, 2004]; it can also be an indicator of increased oxygen demand in various tissues [Remme, 1998] and results in a low ischaemic threshold in all organs; therefore, these patients are at risk for further ischaemic event at the heart level but also in other organs [Gherghel et al., 2007].

5.7.4 Peripheral blood flow

While BCF was comparable in both groups, patients needed significantly longer to restore flow after cold exposure. This increase in the RT parameter could be partly due to an increased arterial stiffness that is evident also at the peripheral level. Increased stiffness and vascular rigidity along with atherosclerotic vessel wall alterations can potentially directly influence vascular relaxation. Another possible explanation could be offered by the existence of vascular endothelial dysfunction. We have examined a small number of patients and failed to show a decreased BCF; however, we still found a strong trend towards a reduction of peripheral capillary flow in CAD patients as compared to controls. Moreover, the high incidence of positive history for peripheral vasospasm in CAD patients strongly supports the idea of peripheral blood flow measurements as an additional tool for the diagnosis and risk assessment in subjects in danger of developing CVD [Pries et al., 2008, Portig and Maisch, 2004].It is worth mentioning that most patients noticed this effect starting prior to their heart condition being diagnosed, without normalisation after the cardiac treatment/ intervention. The existence of peripheral vasospasm has been attributed partially to high activation of the SNS [Miller, 1995, Di Franco et al., 2007]. Indeed, we were able to demonstrate in our patients group an abnormal HRV with high sympathetic and low parasympathetic activity levels during both day and night; this finding could represent and explanation for the significantly increased RT as a measure of peripheral vasospastic reactivity at the nailfold capillaries level when an additional stressor (cold) has been applied.

Various differences in maco-and microvascular physiology could be accounted for our observations. More research is necessary to clarify the relationship between ANS malfunction and endothelial function measured in various vascular beds in patients suffering from CAD.

5.7.5 The effect of cardiac rehabilitation on endothelial and ANS function in CAD

Regular exercise, such as cardiac rehabilitation, is a well established intervention in prevention and treatment of CVD [Linke et al., 2008, Hambrecht et al., 2000b,a,

1999, Jolliffe et al., 2000, 2001]. Moreover, HRV parameters [Deligiannis, 2000] as well as endothelial makers of vascular health [Lee et al., 2006] showed significant improvement after moderate aerobic exercise training independent of home based or clinic based sessions. Previous studies with patients recruited from the same rehabilitation unit showed significant decrease of endothelial function as measured by flow mediated dilation of the brachial artery (FMD), 24 BP monitoring and circulating markers of endothelial function [Lee et al., 2006]. Other authors also investigated the effect and dose of exercise [Iwasaki et al., 2003] on HRV variables as a measure of ANS activity and psychosocial stress. These studies showed that exercise improved PSNS activity as represented by the HF band of HRV [Iwasaki et al., 2003, Buchheit et al., 2004b]. The graphic relationship between exercise load/ intensity and HRV is bell-shaped for the HF band [Iwasaki et al., 2003, Buchheit et al., 2004a], explaining why HRV indices return to basal values when exercising but decrease again if exercise load is prolonged/ more intense in both athletes and sedentary subjects [Iwasaki et al., 2003]. Exercise induces a number of mechanisms helping to restore endothelial function (EF) and therefore achieving a better balance of ANS activity and EF as illustrated below.

This improvement of EF and ANS activity is achieved through a cascade of molecular, adaptive, structural and functional improvements within the vascular beds and its regulatory bodies.

5.7.6 Conclusion

Although being subjected to a CVR programme, CAD patients included in this study have still exhibited an abnormal ANS and peripheral vascular function. At this point we want to stress the need of more non-invasive assessment of ANS and peripheral function to complement the diagnosis, risk assessment and treatment monitoring of patients suffering from CAD.

5.7.7 Limitations

Patients with thick skin, cuticle trauma due to manual work or nail manicure are difficult to assess as in these individuals capillaries can not be visualized to a sufficient degree, making the measurement impossible. Apart from those technical problems, patient recruitment was another factor contributing to the low numbers assessed. Due to strict exclusion criteria outlined in the methods, we have been limited as to the number of patients suitable for inclusion. Most CAD patients are commonly suffering from either HT of diabetes or both. Furthermore, patients and controls had to have healthy eyes and a refractive error within a certain limit, which added to the difficulties explained above. However, these stringent measures were necessary in order to be able to examine solely the effect of CAD on

our measurements.

Chapter 6

Visual function in CAD

6.1 Abstract

Purpose: To assess visual function in patients with a known vascular dysfunction: coronary artery disease (CAD), compared to age and gender matched healthy controls.

Methods: Twenty-eight patients suffering from CAD (mean age: 57 (9) yrs) and 32 healthy controls (mean age: 54 (9)yrs) were assessed for this study. All subjects underwent visual function measurement as follows: visual acuity (VA), contrast sensitivity (CS) measured with the CS-1000-E (Vector Vision, PMS Instruments, UK), colour vision evaluation using Waggoner pseudoisochromatic test plates and visual field assessment using Octopus flicker perimetry (Octopus 301, Haag-Streit, Switzerland) and 30-2 program of the frequency doubling technology (FDT) (Humphrey Matrix, Zeiss, Germany).

Results: Visual acuity and colour vision were comparable in both groups ($p>0.05$). However, patients showed a marked decrease of CS as measured at three CPD compared to controls ($p<0.001$). Parameters assessed by visual field testing were comparable for both groups (parameters assessed: FDT: mean deviation (MD), pattern standard deviation (PSD), mean sensitivity (MS) full field, MS superior and inferior field, total deviation (TD) full field, TD superior and inferior field; Octopus: MS, MD, loss variance (LV), MS full field, MS superior and inferior field), but measurement variability for the Octopus flicker perimetry was significantly increased for both, the inferior and superior visual field in the patient group ($p=0.003$ and $p=0.007$ respectively). Subgroup analysis of the visual field parameter of FDT assessment revealed decreased sensitivity in CAD patients with greater measurement variability.

Conclusions: Visual function in patients suffering CAD is grossly normal. However, when filtering those with greater coefficient of variance (CV) values which represent a decrease in reliability, these patients appear to have an impaired visual function which could be artifactual in origin due to measurement compliance and variability.

6.2 Introduction

The vascular regulation of patients suffering from CAD is compromised manifesting as autonomic nervous system (ANS) and endothelial impairment as shown in chapter 4. Minor changes in perfusion, pulse wave transmission and blood flow rate can have a major impact on the retinal function resulting in impaired visual function. A number of recent studies aimed to explore the effects and mechanisms of changes in perfusion pressure, blood flow and oxygen supply using carbogen and oxygen inhalation techniques. Roff-Hilton et al. were able to show an impairment of visual function as measured by visual field in subjects inhaling carbogen and normal room air [Roff Hilton et al., 2003], Chung and co-workers [Chung et al., 1999] suggest that the superior retina is less responsive to vasodilatory stimuli, and more sensitive to vaso-constrictive agents. It follows that the inferior retina may be more vulnerable to changes in ocular perfusion pressure, arterial oxygen content and increases in metabolic demand. Erb et al [Erb et al., 2000] found diffuse visual field loss in patients suffering from CAD. Furthermore, colour vision could be impaired in this patient group due to systemic medication. Synergistic effects of endothelial dysfunction and medication could lead to impaired visual function as measurable using VF assessment, CS and colour vision techniques. Early detection of a compromised visual function is very important in order to prevent the development of sight threatening chronic ocular disease.

6.3 Hypothesis

Due to the high sensitivity of the ocular circulation to flow disturbances and changes in vascular perfusion as well as the effects of systemic medication, visual function could be compromised in CAD patients.

6.4 Aim

The aim of this study was to assess visual function in patients suffering from CAD but free of any ocular complications at the time of measurement.

6.5 Subjects and recruitment

6.5.1 Patient recruitment

Patients were recruited from the cardiac rehabilitation centre at City Hospital, Birmingham. Healthy individuals were recruited from patients spouse and other volunteers.

6.5.1.1 Inclusion criteria

Patients were included in the study if they met all the following:

- Established diagnosis of CAD confirmed by angiography showing abnormalities of coronary arteries;
- No upper age limit applied (patients had to be at least 18 yrs of age).

6.5.1.2 Exclusion criteria

Exclusion criteria are outlined below:

- Diabetes mellitus;
- Hypertension, as defined in Section 1.7.1; see Table 1.3 on page 62;
- Atrial fibrillation;
- History of any ocular disease;
- Patients with a refractive error of more than +/- 3dpt spherical equivalent and more than +/-1 dpt cylindrical equivalent due to the magnification/ minification causing over/ underestimation of retinal diameter measured;
- History of neurological diseases associated with loss of visual function;
- Any type of ocular surgery; and Normal left ventricular function (LV Function); as defined by depressed left ventricular ejection fraction (LVEF). LVEF represents stroke volume/LV end-diastolic volume, whereas the normal LVEF equals approximately 60%, but impaired LVEF is generally defined as less than 45%.

In addition to the exclusion criteria above, healthy controls had to be free from any form of systemic vascular disease, including diabetes mellitus, hypertension or CAD.

6.5.2 Ethical approval

Prior to the study ethical approval was obtained from NHS Sandwell and West Birmingham Ethics Committee and Aston University Ethics Committee. Written informed consent was received by all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets of Declaration of Helsinki.

6.5.3 Experimental protocol

Patient preparation Patients were asked to refrain from caffeinated products, drinking alcohol, eating chocolate and cigarette smoking on the study day. In addition, patients were instructed to abstain from their usual medication for 24hrs prior to the research appointment. One randomly selected eye was chosen to undergo visual function testing. If one eye had a significantly worse VA, the best eye was chosen for assessment.

6.5.3.1 Colour vision assessment

Colour vision assessment was performed using the Waggoner colour vision test (4th edition). For more detail about this test and its usage we refer to Section 1.5.1.

6.5.3.2 Contrast sensitivity assessment

CS was assessed using the CS1000-E CS test unit with retro illumination for a testing distance of 2.5m. CS was measured for 3,6,12 and 18 cycle per degree (CPD) in each subject, wearing their habitual distance prescription. A detailed description of this equipment is given in Section 1.5.3. One randomly chosen eye was assessed while the fellow eye was occluded. Each patient was instructed to indicate the circles they thought to contain the grating, after an initial trial session to aid compliance.

6.5.3.3 Visual Field assessment

Visual fields were assessed using two tests. One test using frequency doubling illusion with the Zeiss Matrix FDT 30-2 test, the other using the Octopus flicker test to measure critical flicker fusion (CFF) on the same 30 degree visual field. Test sequence was randomised to minimise bias.

Zeiss Matrix (FDT) For FDT testing patients and controls were seated with the fellow eye un-occluded. After adjusting height and patient fixation the room lights

were switched off. Each patient was given one practise session. After room acclimatisation of approximately 10 minutes to ensure constant retinal light adaptation in each subject, the test was performed on the chosen eye.

Octopus (flicker perimetry) For the Octopus flicker test patients and controls were seated with the fellow eye occluded and the room lights switched off after adjustment of patient fixation and height. Similar to the FDT all patients were given one practise session after which the test was performed. A stimulus of Goldman size III with a 1000 ms presentation duration was chosen.

Note: Mean deviation (MD) assessed using FDT is given as a positive/negative value, depending on the patient achieving better/worse sensitivity results than expected for his/her particular age group. However, the MD value obtained in flicker perimetry corresponds only to a decrease in sensitivity compared to controls and lacks the information to quantify an increase in sensitivity, e.g. quantification of a defect is possible but not of above normal results. Therefore these two parameters can not be directly compared.

6.5.4 Statistical Analysis

To assess group mean differences either the Mann-Whitney-U test or an unpaired t-test, depending on data distribution was applied. Within group differences were assessed by using the Wilcoxon test or a paired t-test. Pearson's correlation coefficient was used to assess the relationship between MS as measured by FDT and flicker perimetry for the superior and inferior visual fields. The chi-square-test was used to assess differences in colour vision function between patients and controls.

6.6 Results

6.6.1 Sample

All patiets and controls were of Caucasian origin. After careful evaluation of the test results obtained, six patients (two women) and eight controls (four women) had been excluded due to unreliable results as quantified by fixation error and false positive and negative count. Cut off for inclusion was set at >33% of either fixation losses, false positive or negative errors, based on previous studies [Johnson et al., 2002]. The remaining 22 patients (two women) and 22 controls (four women) included in the data analysis were age and gender matched (patients: 57 (9) yrs and controls: 55 (9) yrs; $p>0.05$).

Parameter	Controls [n=22]	CAD [n=22]	Mann-Whithney-U [2-sided] p-value
	Mean +/-SD	*Mean +/-SD*	
3 CPD	1.56 (0.32)	1.10 (0.44)	**<0.001**
6 CPD	1.62 (0.31)	1.49 (0.31)	0.164
12 CPD	1.08 (0.29)	0.98 (0.34)	0.382
18 CPD	0.57 (0.38)	0.58 (0.36)	0.862

Table 6.1: Contrast sensitivity results for coronary artery disease patients and controls; CAD: Coronary artery disease, CPD: Cycles per degree

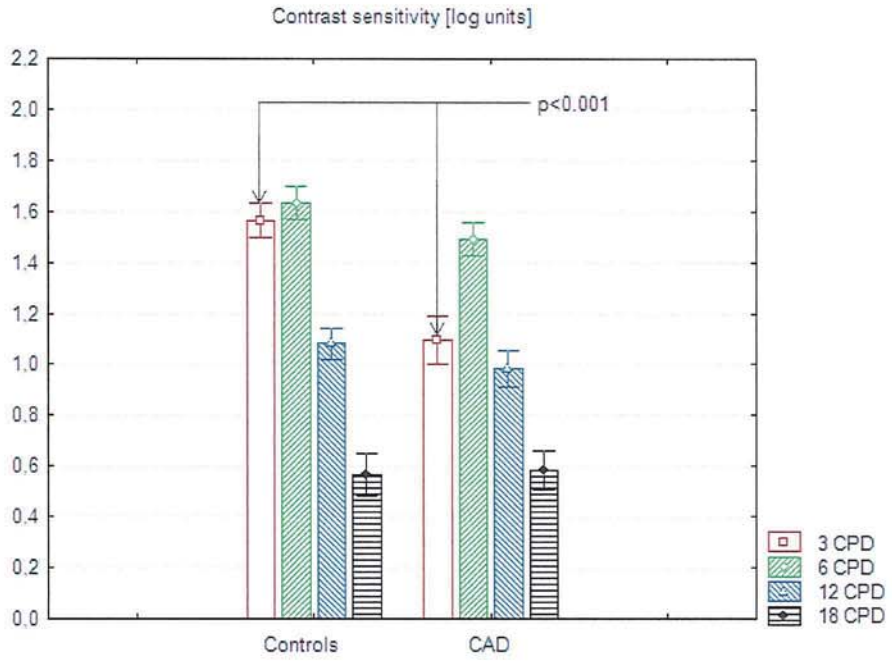

Figure 6.1: Difference in contrast sensitivity between coronary artery disease (CAD) patients and controls

6.6.2 Contrast sensitivity

Patients showed significantly decreased CS values as measured at 3 cycles per degree (CPD) compared to controls (patients: 1.10 (0.44) log units and controls: 1.56 (0.32) log units; $p<0.001$). Contrast sensitivity levels at all other special frequencies, 6 CPD, 12 CPD and 18 CPD were comparable between groups (all $p>0.05$), see Table 6.1 on page 150 and Figure 6.1 on page 150.

6.6.3 Visual field assessment

6.6.3.1 Frequency doubling technology

CAD patients and controls showed similar results in regard of test duration, fixation losses, false negative and false positive errors (all $p>0.05$; see Table 6.2 on

Parameter	Controls [n=26]	CAD [n=23]	p-value
	Mean +/-SD	*Mean +/-SD*	
FDT test duration [min]	6.3 (0.3)	6.3 (0.4)	0.854
Fixation losses [%]	9.4 (11.6)	11.1 (12.1)	0.652
False positive errors [%]	1.2 (3.3)	1.7 (3.)	0.759
False negative errors [%]	0 (0)	1.4 (6.9)	0.806
MD (dB)	-0.09 (2.76)	-0.11 (2.18)	0.982
PSD (dB)	2.85 (0.80)	3.03 (0.91)	0.450
FDT(threshold)fovea [dB]	30 (4.00)	29 (3.00)	0.518
FDT(TD)fovea [dB]	-1.32 (3.88)	-1.52 (3.31)	0.848
FDTmean sensitivity (superior field) [dB]	27 (3.00)	27 (2.00)	0.856
FDTmean sensitivity (inferior field) [dB]	27 (3.00)	27 (2.00)	0.800
FDTmean sensitivity difference (inf.-sup. field) [dB]	0.56 (1.56)	0.24 (1.33)	0.443
FDT(TD) [dB]	0.22 (2.46)	0.26 (2.15)	0.954
FDT(TD)superior field [dB]	0.07 (2.46)	0.19 (2.05)	0.855
FDT(TD)inferior field [dB]	0.38 (2.65)	0.31 (2.42)	0.933
FDT(TD)difference (inf.-sup. field) [dB]	0.30 (1.38)	0.12 (1.30)	0.639

Table 6.2: Results for the frequency doubling technology visual field assessment of coronary artery disease patients and controls; FDT: Frequency doubling technology, MD: Mean defect, PSD: Pattern standard deviation, TD: Total deviation, dB: decibel; CAD: coronary artery disease.

page 151). Furthermore there was no significant difference between patients and controls for any of the visual field parameters assessed (all p>0.05; see Table 6.2 on page 151). Patients and controls were comparable in MD, PSD, foveal threshold, MS of the superior and inferior visual field, sensitivity difference between superior and inferior visual field, TD, superior and inferior TD and the difference in TD between superior and inferior visual field (all p>0.05; see Table 6.2 on page 151).

6.6.3.2 Flicker perimetry

CAD patients needed significantly longer to complete the Octopus flicker test than their age-matched controls (CAD patients: 15.2 (5.4) sec and controls: 12.4 (2.0) sec; p=0.03). Futhermore, CAD patients exhibited a significantly higher number in "Questions", a measure reflecting the repetition of stimuli presentation, than controls (CAD patients: 375 (188) "Questions "and controls: 263 (87) "Questions"; p=0.003; see Table 6.3 on page 152 and Figure 6.3 on page 153). No significant difference between patients and controls was observed for all parameters measured with flicker perimetry (MS full field, MS superior and inferior field, MD and LV; all p>0.05; see Table 6.3 on page 152). Patients and controls showed similar test results for MS (global, superior and inferior visual field), MD and LV as was the difference in MS between superior and inferior visual field (VF) (all p>0.05).

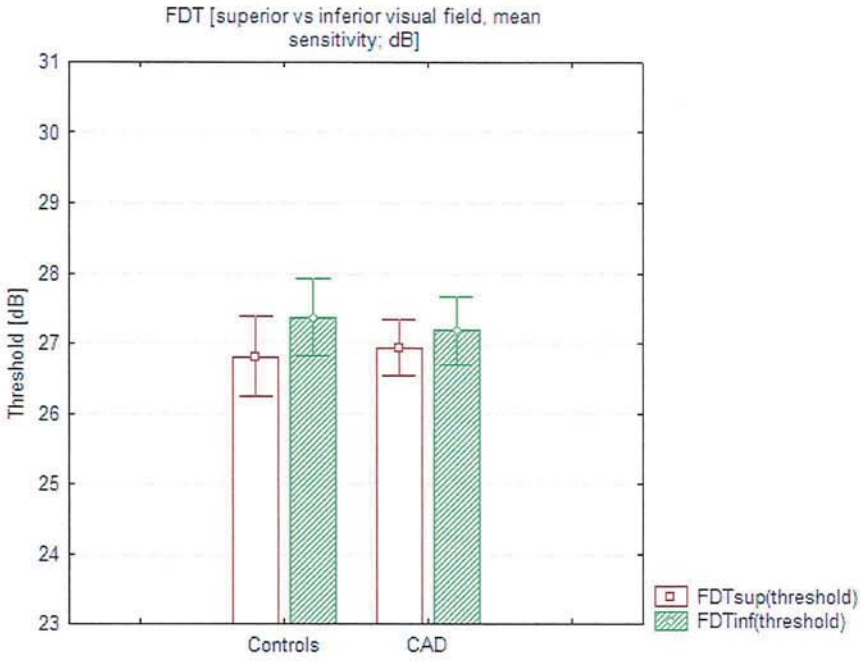

Figure 6.2: Mean sensitivity of the superior and inferior visual field as measured using FDT in CAD patients and controls; FDT: Frequency doubling technology, CAD: Coronary artery disease

Parameter	Controls [n=22]	CAD [n=22]	p-value
	Mean +/-SD	*Mean +/-SD*	
Octopus test duration [min]	12.4 (2.0)	15.2 (5.4)	**0.033**
Questions	263 (87)	375 (188)	**0.003**
False positive errors [%]	13.6 (12.7)	20.0 (20.0)	0.287
False negative errors [%]	0.9 (2.4)	1.8 (5.2)	0.789
MS [Hz]	37 (3)	37 (5)	0.898
MD [Hz]	-1.61 (2.89)	-1.62 (5.26)	0.952
LV [Hz²]	22 (10)	26 (8)	0.097
MS superior visual field [Hz]	38 (3)	37 (5)	0.972
MS inferior visual field [Hz]	37 (2)	37 (5)	0.843
MS difference (inf.-sup. field) [Hz]	-0.76 (1.52)	-0.37 (1.73)	0.667

Table 6.3: Results for the flicker perimetry of CAD patients and controls; CAD: Coronary artery disease, MS: Mean sensitivity, MD: mean defect, LV: Loss variance, Hz: Hertz

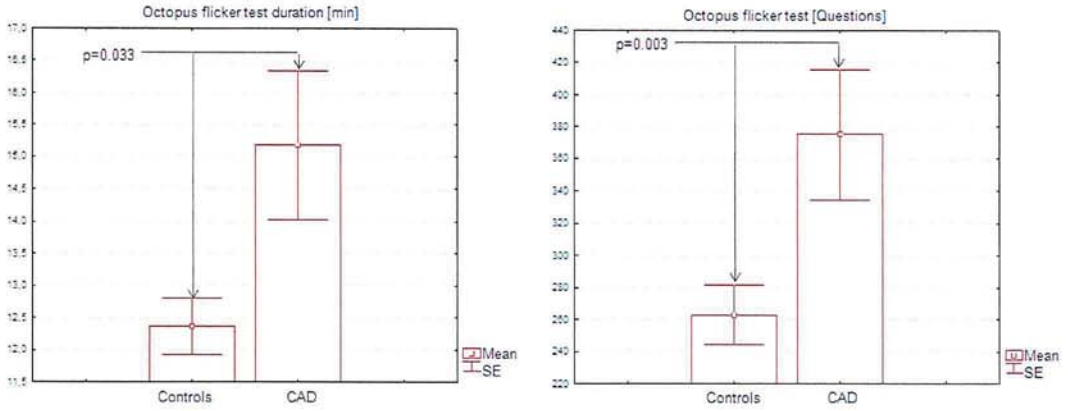

Figure 6.3: Comparison of Octopus flicker test duration and "Questions" for CAD patients and controls; CAD: Coronary artery disease

Parameter	Controls [n=22]		CAD [n=22]	
	Pearson R	*p-value*	*Pearson R*	*p-value*
FDT [full-field threshold] & flicker perimetry [full-field threshold]	0.13	0.561	0.22	0.332
FDT [superior-field threshold] & flicker perimetry [superior-field threshold]	0.26	0.239	0.17	0.449
FDT [inferior-field threshold] & flicker perimetry [inferior-field threshold]	0.01	0.951	0.10	0.648
FDT [diff] & flicker perimetry [diff]	0.36	0.097	-0.08	0.718

Table 6.4: Correlation between parameters of FDT and flicker perimetry; FDT: Frequency doubling technology, CAD: Coronary artery disease

6.6.3.3 Comparison: Frequency doubling technology vs flicker perimetry

In order to compare the results obtained from VF assessment by FDT and Octopus flicker test, the frequency measurement units of flicker perimetry were converted into a decibel scale using the following equation:

$$[Hz]\text{to } [dB] = 20 \times Log_{10}Hz \tag{6.1}$$

The sample assessed was normally distributed. Group MS for the superior visual field, inferior visual field and superior-inferior sensitivity difference was compared between the patient and control group. There was no significant correlation between the two visual field tests using Pearson's correlation coefficient, R (all p>0.05; see Table 6.4 on page 153).

6.6.3.4 Visual field variability

Within group variability The coefficient of variance (CV) for each subject was calculated for the superior and inferior VF of each test (FDT and flicker perimetry)

	Controls [n=22]		
Parameter	*Octopus superior field CV [%]*	*Octopus inferior field CV [%]*	**Wilcoxon-test p-value**
Mean +/-SD	10 (2)	10 (5)	0.375
Parameter	*FDT superior field CV [%]*	*FDT inferior field CV [%]*	
Mean +/-SD	12 (4)	11 (3)	0.212

	CAD [n=22]		
Parameter	*Octopus superior field CV [%]*	*Octopus inferior field CV [%]*	**Wilcoxon-test p-value**
Mean +/-SD	16 (16)	18 (21)	0.904
Parameter	*FDT superior field CV [%]*	*FDT inferior field CV [%]*	
Mean +/-SD	13 (5)	12 (5)	0.778

Table 6.5: Within group comparison of the measurement variability of the inferior ans superior visual field for FDT and flicker perimetry; CV: Coefficient of variance, FDT: Frequency doubling technology

separately using the following equation:

$$CV = SD[MS]/Mean[MS] \tag{6.2}$$

For statistical analysis, the group mean CV and corresponding SD were computed. There was no significant difference in the variability of MS measurements in the superior and inferior visual fields within groups (all p>0.05; see Table 6.5 on page 154).

Between group variability The group mean CV of the MS measured for the superior and inferior VF was significantly different between groups for flicker perimetry (superior visual field: p=0.007 and inferior visual field: p=0.003; see Table 6.6 on page 155 and Figure 6.4 on page 155) but not the FDT (superior visual field: p>0.05 and inferior visual field: p>0.05; Table 6.6 on page 155).

6.6.4 Visual field - sub group analysis

6.6.4.1 Frequency doubling technology

For the subgroup visual field analysis the following selection criteria for grouping was applied: patients exhibiting inferior threshold CV values outside the 5%-limit of those determined for controls were classified as "true abnormals" which then entered the subgroup analysis. These subjects were compared to the normal cohort again as described above for the whole group comparisons. When doing so, patients were significantly different from controls as measured by the FDT full field MS (patients: 25 (2) dB and controls: 27 (3) dB; p=0.045), FDT inferior field

Parameter	Controls [n=22]	CAD [n=22]	Mann-Whitney-U [2-sided] p-value
	Mean +/-SD	*Mean +/-SD*	
Flicker perimetry superior field CV [%]	10 (2)	16 (16)	**0.007**
Flicker perimetry inferior field CV [%]	10 (5)	18 (21)	**0.003**
FDT superior field CV [%]	12 (4)	13 (5)	0.599
FDT inferior field CV [%]	11 (3)	12 (5)	0.456

Table 6.6: Comparison of the measurement variability of FDT and flicker perimetry in CAD patients and controls; CV: Coefficient of variance, FDT: Frequency doubling technology, CAD: Coronary artery disease

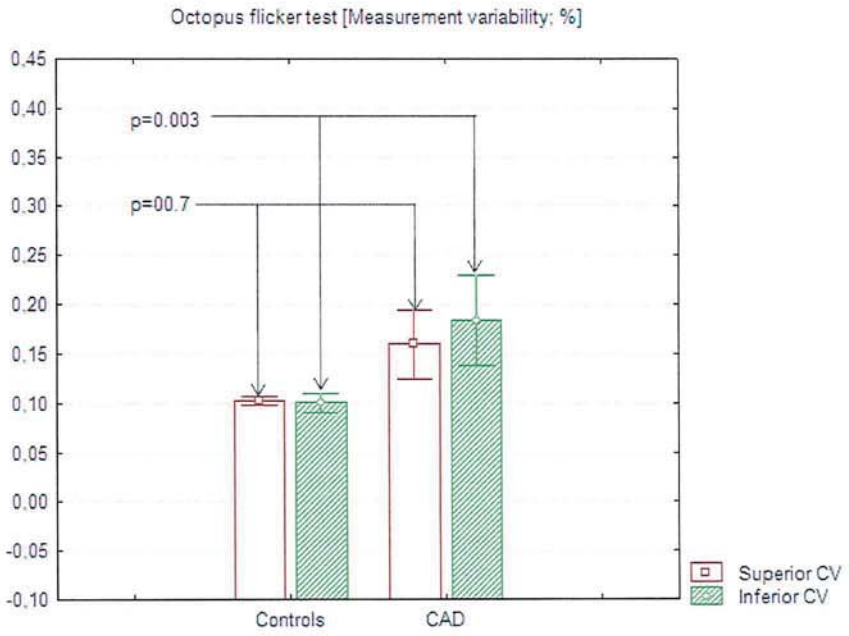

Figure 6.4: Between group comparison of flicker perimetry CV; CV: Coefficient of variance, CAD: Coronary artery disease

Parameter	Controls [n=26]	CAD [n=6]	p-value
	Mean +/-SD	*Mean +/-SD*	
Age [yrs]	53 (9)	56 (8)	0.334
MD (dB)	-0.09 (2.76)	-2.23 (2.11)	0.086
FDT-full field threshold [dB]	27 (3)	25 (2)	**0.045**
FDTmean sensitivity (superior field) [dB]	27 (3)	25 (2)	0.149
FDTmean sensitivity (inferior field) [dB]	27 (3)	24 (2)	**0.017**
FDTmean sensitivity difference (inf.-sup. field) [dB]	1 (2)	-1 (2)	0.077
FDT(TD) [dB]	0 (2)	-2.21 (2)	**0.033**
FDT(TD)superior field [dB]	0 (2)	-2 (2)	0.083
FDT(TD)inferior field [dB]	0 (3)	-3 (2)	**0.019**
FDT(TD)difference (inf.-sup. field) [dB]	0 (1)	-1 (2)	0.131
FDT(TD)fovea [dB]	-1 (4)	-2 (5)	0.981
FDT(threshold)fovea [dB]	30 (4)	27 (5)	0.575
PSD (dB)	3 (1)	4 (1)	0.144

Table 6.7: Results for the FDT visual field sub group analysis of CAD patients and controls; FDT: Frequency doubling technology, MD: Mean defect, PSD: Pattern standard deviation, TD: Total deviation, dB: decibel

Parameter	Controls [n=22]	CAD [n=3]	p-value
	Mean +/-SD	*Mean +/-SD*	
Age [yrs]	53 (9)	53 (10)	0.093
MS [Hz]	37 (5)	31 (7)	0.206
MD [Hz]	-1.62 (5.26)	4.23 (6.72)	0.177
MS superior visual field [Hz]	37 (5)	36 (8)	0.446
MS inferior visual field [Hz]	37 (5)	35 (9)	0.398
MS difference (inf.-sup. field) [Hz]	-0.37 (1.73)	-0.84 (1.43)	0.969

Table 6.8: Results for the flicker perimetry sub group analysis of CAD patients and controls; CAD: Coronary artery disease, MS: Mean sensitivity, MD: mean defect, LV: Loss variance, Hz: Hertz

MS (patients: 24 (2) dB and controls: 27 (3) dB; p=0.017), FDT TD (patients: -2.21 (2) dB and controls: 0 (2) dB; p=0.033) and FDT TD inferior (patients: -3 (2) dB and controls: 0 (3) dB; p=0.019); see Table 6.7 on page 156. All other variables as measured with the using FDT were not different to controls (all p>0.05; see Table 6.7 on page 156).

6.6.4.2 Flicker perimetry

The same selection criteria as described above for the subgroup analysis of the FDT assessment were applied to the flicker perimetry. However, CAD patients and controls had comparable results (all p>0.05; see Table 6.8 on page 156).

6.7 Discussion

6.7.1 Main findings

Patients with CAD had significantly decreased CS as measured at 3 CPD, but comparable results for CS at 6,12 and 18 CPD. Visual field assessment revealed similar results for MS, PSD and hemi-field MS, however for patients exhibiting significantly higher CV values in the inferior visual field as controls a sub-group analysis was performed. This revealed significantly lower MS and TD values for both the full field and the inferior visual field in CAD patients than age-matched controls. Furthermore, measurement variability of the MS as measured using the Octopus flicker test was significantly increased in CAD patients for both, the superior and inferior visual field. Flicker perimetry showed significant higher variability than FDT, this increase in variability is coherent to those of previous studies [Bernardi et al., 2007].

6.7.2 Contrast sensitivity

Contrast sensitivity is a measure of foveal function. Patients with CAD yielded significantly lower scores for log-values of CS at 3 CPD whilst at 6 CPD CS was still decreased but failed to show statistical significance. Contrast sensitivity has been shown previously to decrease with age [Bernardi et al., 2007, Matsuo et al., 2002] and decreased media transmission [Sherman et al., 1988]. However, all patients underwent dilated fundus and slit-lamp examination prior to the research appointment to rule out any lens opacities, using the LOCS scale to ensure clear media [Chylack et al., 1993a,b]. Furthermore, CS values obtained did not show any significant correlation with age, neither in patients nor controls. In addition, patients and controls had comparable VA and both groups were instructed to wear their best distance prescription for the CS assessment to ensure maximum VA. The most plausible reason for this finding of clinically decreased CS in patients with CAD could be due to a disturbance in the vascular autoregulatory ability. The macula in particular is very sensitive to changes in perfusion [Cornut et al., 2008] and therefore, functional changes could manifest earlier than any other part of the retina. However, another more likely possibility is the one of vascular ageing [Dasch et al., 2005, Dallinger et al., 1998, Harris et al., 2000, O'Rourke, 2007, O'Rourke and Hashimoto, 2007]. Even though the measured values showed no correlation with age amongst patients, atherosclerotic vascular changes along with changes of vascular autoregulation could have lead to a premature ageing and therefore lead to subnormal values for this patient group. This is of particular interest, since in age related macular degeneration (AMD), a disease affecting mainly the central vision, premature aging of the vascular system is thought to be

a major contributor in the developement and onset of the disease. This premature aging of the vascular system is thought to be accelerated in subjects smoking, which is also a major risk factor in developing CVDs.

6.7.3 Visual fields

Overall scores of the global visual field indices in patients with CAD were comparable to controls for MD, full field MS, superior and inferior visual field sectors for both flicker perimetry and FDT. Similarly, the global indices which assess focal visual field loss, PSD and LV, Total Deviation probability and foveal thresholds were also comparable between groups. However, it can be hypothesised that individuals exhibiting larger CV-values than those obtained from controls could be classed as 'truly' abnormal. In order to test this hypothesis individuals exhbiting CV-values outside the 5%-limit of those obtained for controls were selected for subgroup analysis. When doing so, the six patients (FDT subgroup analysis) qualifying for this analysis exhibited a significant decrease in visual field sensitivity for the full and the inferior visual field parameters as measured by FDT. The superior retina, corresponding to the inferior visual field, has been shown previously to have a reduced vascular reserve and therefore being more prone to changes in perfusion [Chung et al., 1999]. In CAD patients, having a known disturbance of not only the ANS but also endothelial vascular function, changes in ocular perfusion could have led to sub-clinical vision impairment as demonstrated in these results. However, these results should be treated with caution, as all visual field parameters were only assessed once and could be explained by the summation of perimetric learning effect and decreased attention/compliance [Matsuo et al., 2002, Bernardi et al., 2007, Horani et al., 2002].

6.7.4 Conclusion

Visual function in patients suffering CAD is grossly normal. However, when filtering those with greater CV-values which represent a decrease in reliability, some patients appear show decreased visual function which could be artifactual in origin.

6.7.5 Limitations

Both FDT and flicker perimetry, have been shown to be capable of detecting early changes in visual function [Matsumoto et al., 2006]. Furthermore, flicker perimetery offers the advantage of being independent of media opacities and blurred vision [Iester et al., 2000, Pierre-Filho et al., 2006, Ueda et al., 2006, Adams

et al., 1999]. However, both examination techniques are known to exhibit a learning effect [Bernardi et al., 2007]. The subjects had all undergone one trial test to minimize the learning effect; however, all individuals tested struggled to some degree during the test, in particular the flicker perimetry. The increased variability and false positive count present in flicker perimetry in the present study support the theory that besides being within normal limits, flicker perimetry is a much more variable and unreliable test compared to FDT. Flicker perimetry is most difficult to complete because the patient does not press the response button in the same way as they would for FDT and conventional perimetry. In flicker perimetry, the patient presses the response button only if they perceive the presented light as flickering (to assess CFF). Sometimes, the stimulus is presented but does not flicker, in which case the patient should not press the response button. This task requires greater concentration than for other visual field tests and consequently many patients experienced difficulty in completing the test. Furthermore, the judgement of weather the stimuli is flickering or not which gets increasingly more difficult the closer the flicker frequency gets to the CFF [Matsumoto et al., 2006]. This lack of compliance, along with limitation of testing only once after an initial trial examination could have led to the high CV values as measured for flicker perimetry.

Chapter 7

Multiple functional, structural and biochemical vascular endothelial dysfunctions in patients suffering from coronary artery disease: relationships and possible implications

7.1 Abstract

Purpose: The aim of this study was to assess the presence of multiple functional, structural and biochemical vascular endothelial dysfunction in patients suffering from coronary artery disease (CAD).

Methods: the purpose of this study we analysed data obtained from 24 CAD patients and 30 age-matched healthy controls. In order to minimize measurement bias all subjects had to refrain from their usual medication on the study day. Von Willebrandt factor (vWF) was the chosen biochemical marker for endothelial dysfunction. To assess it, all patients and controls underwent fasting venous blood collection on the first study day when they were also fitted with an ambulatory blood pressure (AMBP) and electrocardiogram (ECG) monitor. Visual fields (VF), visual acuity (VA), contrast sensitivity (CS) and colour vision assessments were all performed on a second visit. In addition, various ocular blood flow (BF) measurements using the Retinal Vessel Analyser (RVA), colour Doppler imaging (CDI), Heidelberg Retinal Flowmeter (HRF) as well as peripheral circulation assessments were carried out.

Results: BMI significantly correlated with vWF (p=0.040) in the patient group but not controls (p>0.05). Retinal vascular reactivity showed a significant correlation with peripheral reactivity parameters in controls for all three stimulation cycles (cycle 1: p=0.001, cycle 2: p=0.003, cycle 3: p=0.016); this was only present in the third cycle in the CAD group (cycle 1: p>0.05, cycle 2: p>0.05, cycle 3: p=0.017). In addition, visual field parameters as assessed FDT were strongly related with systemic vascular elasticity (AASI) in controls (FDT superior threshold: p=0.034, FDT inferior threshold: p=0.018) but not CAD patients (all p>0.05).

Conclusion: In CAD patients, functional, structural and biochemical vascular changes are present at multiple levels and have a strong relationship between them.

7.2 Introduction

Arteriosclerosis, the underlying cause of CAD, affects the vascular tree throughout the body. However, even if various vascular impairments occur at multiple levels in this disease [Cheung et al., 2007c, Troseid et al., 2006], to date it is still unclear whether there is a relationship or any pathological cascade that links the different tissue sites. This lack of knowledge is partially due to the fact that the function of vessels of various calibres and locations is governed by different local and systemic regulatory mechanisms. While microcirculation has lots of collaterals, which in case of injury will maintain function, other larger vascular beds do not have this type of backup; therefore any impairment at this level will result in loss of function and structure.

Structural and functional changes of the vascular system can be assessed at various tissue levels by a number of different tests. In addition to various blood flow measurement technologies outlined in the previous chapters of the present thesis, we have also measured vWF which is one of the most sensitive circulatory markers of endothelial function and it is used to predict cardiovascular disease outcome, severity, morbidity and mortality [Troseid et al., 2006, Vischer, 2006, Woodward et al., 2007, Spiel et al., 2008]. In addition to the other structural and functional assessments, this blood test will help us to elucidate if endothelial dysfunction present in multiple vascular beds (including the eye) in patients suffering from CAD have a common factor identifiable at the biochemical level.

It has been already demonstrated that patients suffering from arterial hypertension exhibit defects of the colour vision [Schroder et al., 2002] but not of the VF as measured standard achromatic automatic perimetry [Schroder et al., 2003]. It is possible that wide spread circulatory abnormalities that exist in patients suf-

fering from CVD will result in various subtle abnormalities of the visual function; to test this hypothesis, a relationship between the measured endothelial function markers and parameters of visual function was also assessed in our subjects.

7.3 Hypothesis

There is an identifiable relationship between functional and structural vascular changes at both ocular and systemic level in patients suffering from CAD that could further result in subtle abnormalities of the visual function.

7.4 Aims

To elucidate the level of vascular dysfunction as measured at functional, structural and biochemical level in patients suffering CAD. The effect of the possible perfusion (ocular and systemic) abnormalities on visual function was also explored.

7.5 Subjects and Methods

7.5.1 Patient recruitment

Patients were recruited from the cardiac rehabilitation unit at City Hospital, Birmingham. Age-matched healthy individuals were recruited from patients spouse and other volunteers.

7.5.1.1 Inclusion criteria

Patients were included in the study if they met all the following:

- Established diagnosis of CAD confirmed by angiography showing abnormalities of coronary arteries
- No age limitations applied (patients had to be at least 18yrs of age)

7.5.1.2 Exclusion criteria

Exclusion criteria are outlined below:

- Diabetes mellitus;
- Hypertension; as defined in Section 1.7.1 ; Table 1.3 on page 62;
- Atrial fibrillation;

- History of any ocular disease;
- Patients with a refractive error of more than +/- 3dpt spherical equivalent and more than +/-1 dpt cylindrical equivalent due to the magnification/ minification causing over/ underestimation of retinal diameter measured;
- History of neurological diseases associated with loss of visual function;
- Ocular surgery, such as cataract or LASIK; and
- Abnormal left ventricular (LV) Function, as defined by depressed left ventricular ejection fraction (LVEF). LVEF represents stroke volume/LV end-diastolic volume, whereas the normal LVEF equals approximately 60%, but impaired LVEF is generally defined as less than 45%.

In addition to the exclusion criteria above, healthy controls had to be free form any form of systemic vascular disease, including diabetes mellitus, hypertension or CAD.

7.5.2 Ethical approval

Prior to the study ethical approval was obtained from NHS Sandwell and West Birmingham Ethics Committee and Aston University Ethics Committee. Written informed consent was received from all subjects participating in the study. The study has been designed and conducted in accordance with the Tenets of Declaration of Helsinki.

7.5.3 Experimental protocol

After approaching the prospect patient/ control subject, a full ocular history and examination took place to ensure the patients were free from any systemic and ocular disease as outlined in the exclusion criteria above. After this initial examination the patient was booked in for the research appointment. All subjects were instructed to refrain from consuming caffeinated products, such as coffee or tea, chocolate, drinking alcohol and smoking on the study day. Additionally, patients were asked to abstain from their usual medication 24 hrs prior to the appointment in an attempt to minimize the effect of medication. Subjects were examined based on the protocol stated below:

Day 1:

1. 12 hr overnight fast
2. Venous blood sample: 8ml EDTA and 3.5ml citrated sampling

3. 24hr BP and ECG monitor

Day 2:

1. VA and CS assessment
2. CDI
3. HRF
4. VF and colour vision assessment
5. IOP assessment
6. Nail-fold capillaroscopy
7. Dynamic retinal vessel assessment using RVA
8. Fundus photography using RVA

All ocular measurements were performed in one randomly selected eye of each participant. As the subjects have been fitted with an ABPM on day one of the study, BP measurements were taken at regular intervals during procedures. The BP values were also used to calculate the AASI according to Equation (1.9) as described in Section 1.7.2.2. The BF measurements as well as the visual function assessments methods used in this study have been already described in the previous chapters of the present thesis.

7.5.3.1 Obtaining venous blood samples

All blood samples were collected by a qualified phlebotomist from the non-dominant arm of the patient. After an over-night fast and refraining form systemic medication, eight ml of venous blood was collected in EDTA (ethylenediamide tetra-acetic acid) tubes and 3.5 ml in citrated tubes (citric acid) by venipuncture to the antecubital vein. All bloods were then processed as described in Section 1.8.6.1.

7.5.3.2 Analysis for von Willebrandt factor

Von Willebrandt factor was analysed using the citrated plasma of both CAD patients and controls according to the protocol described in Section 1.8.6.1.

7.5.4 Statistical analysis

All results are given as the mean +/- standard deviation (SD). Spearman's correlation coefficient R was used to explore the relationship between static and functional retinal vascular parameters as well as between parameters of visual function and systemic circulation. Group differences were analysed by either using the Mann-Whitney-U test or an unpaired t-test if normally distributed. In the absence of normal distribution, the data was log transformed in an attempt to normalize it. When computing multiple comparisons, the significance level was set at $p<0.01$ to minimize bias.

7.6 Results

7.6.1 Sample

60 eyes of 11 women and 49 men were assessed during this study (all patients and controls were of Caucasian origin). However, as a result of careful image analysis, 6 subjects whose data had poor image quality were excluded from the final statistical analysis. Fifty-four data sets remained, comprising of 24 eyes (3 women and 21 men) of CAD patients and 30 eyes (7 women and 23 men) of healthy controls.

7.6.2 Relationship between retinal and peripheral (nail-fold capillaries) vascular reactivity

For evaluation of the relationship between the microvascular beds of the periphery (nail-fold) and the eye (retinal), we correlated the rate of diameter increase due to flicker light stimulation as assessed by slope measurements (for more detail please read Section 1.3.3.3) with the RT as measured at the nail-fold level. While controls showed a strong relationship between the arterial slopes of each flicker stimulation cycle with nail-fold capillary RT (cycle1: $p=0.001$, cycle2: $p=0.003$ and cycle3: $p=0.016$; see Table 7.2 on page 166), in patients such relationship was only significant for the last cycle of stimulation (cycle1: $p>0.05$, cycle2: $p>0.05$ and cycle3: $p=0.017$; see Table 7.2 on page 166). BCF was strongly associated with the average ratio of baseline retinal arterial DA and MD as measured by RVA ($p=0.040$; see Table 7.2 on page 166), however, this relationship was absent in controls ($p>0.05$; see Table 7.2 on page 166). While controls' RT at the nail-fold capillary level was independent of the retinal A/V-ratio ($p>0.05$; see Table 7.2 on page 166), patients showed a strong association ($p=0.010$; see Table 7.2 on page 166).

Parameter	Controls [n=30]	CAD [n=24]	p-value
	Mean (SD)	*Mean (SD)*	
Age [yrs]	53 (9)	56 (9)	0.162
BMI [kg/m²]	26 (3)	28 (4)	**0.01**
Systemic circulatory parameters (24hr ABPM)			
SBP-24hr [mmHg]	120 (10)	117 (10)	0.469
DBP-24hr [mmHg]	72 (7)	68 (8)	0.114
MAP-24hr [mmHg]	88 (7)	84 (8)	0.192
AASI	0.24 (0.09)	0.35 (0.15)	0.005
Peripheral circulatory parameters (nail-fold capillaries)			
BCF [mm/sec]	0.23 (0.11)	0.18 (0.06)	0.064
Recovery time [sec]	21 (11)	35 (22)	**0.009**
Ocular circulatory parameters			
OA(PSV) [m/s]	0.362 (0.085)	0.401 (0.094)	0.107
arterial Slope1	0.48 (0.25)	0.35 (0.31)	
arterial Slope2	0.46 (0.29)	0.47 (0.29)	
arterial Slope3	0.48 (0.23)	0.48 (0.22)	
A/V-ratio	0.85 (0.09)	0.83 (0.09)	0.923
average APR	2.20 (0.81)	1.94 (0.94)	
Visual function parameters			
CS: 3 CPD	1.56 (0.32)	1.10 (0.44)	**<0.001**
CS: 6 CPD	1.62 (0.31)	1.49 (0.31)	0.164
CS: 12 CPD	1.08 (0.29)	0.98 (0.34)	0.382
CS: 18 CPD	0.57 (0.38)	0.58 (0.36)	0.862
MS (full-field; FDT) [dB]			
MS (inferior field; FDT) [dB]	27 (3)	27 (2)	0.8
TD (inferior field; FDT) [dB]	0.38 (2.65)	0.31 (2.42)	0.933
Octopus superior field CV [%]	10 (2)	16 (16)	**0.007**
Octopus inferior field CV [%]	10 (5)	18 (21)	**0.003**
FDT superior field CV [%]	12 (4)	13 (5)	0.599
FDT inferior field CV [%]	11 (3)	12 (5)	0.456

Table 7.1: Group characteristics; CAD: Coronary artery disease, BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial blood pressure,

Parameter	Controls	p-value	CAD	p-value
	Spearmans R		*Spearmans R*	
Recovery time CapMic [sec] & slopeA1	-0.61	**0.001**	-0.23	0.338
Recovery time CapMic [sec] & slopeA2	-0.58	**0.003**	-0.35	0.143
Recovery time CapMic [sec] & slopeA3	-0.49	**0.016**	-0.56	**0.017**
BCF [mm/s] & average APR	0.26	0.170	0.42	**0.04**
Recovery time CapMic [sec] & A/V-ratio	0.08	0.715	0.7	**0.01**

Table 7.2: Results for correlations between nail-fold capillaroscopy parameters (baseline capillary flow and recovery time) and static and dynamic retinal vessel parameter (arterial slopes of all 3 flicker cycles, average APR and A/V-ratio); average APR: average arterial peak ratio, A/V-ratio: arterio-venous ratio

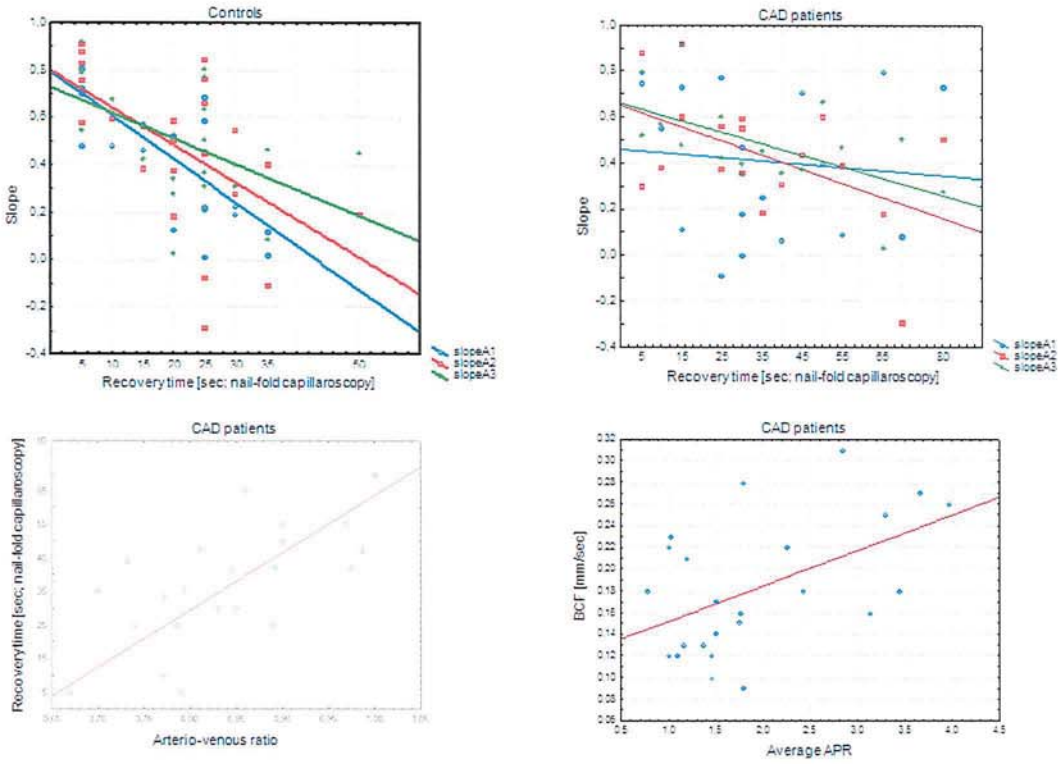

Figure 7.1: Correlations between nail-fold capillaroscopy parameters (baseline capillary flow and recovery time) and static and dynamic retinal vessel parameter (arterial slopes of all 3 flicker cycles, average APR and A/V-ratio); average APR: average arterial peak ratio, A/V-ratio: arterio-venous ratio

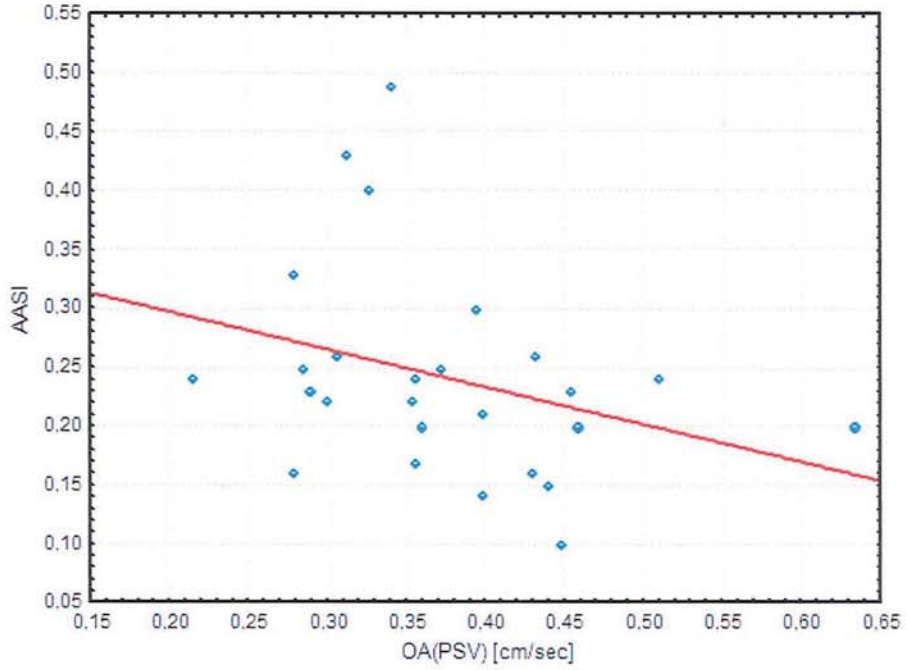

Figure 7.2: Correlaion between the AASI and the PSV of the OA in the control group; AASI: Ambulatory arterial stiffness index, OA(PSV): Peak systolic velocity of the ophthalmic artery

7.6.3 Relationship between systemic blood pressure parameters and ocular circulation

AASI, a measure for large artery stiffness was used to assess its association with the retrobulbar circulation as measured at the level of the OA. This revealed a significant relationship in controls (p=0.044; see Figure 7.2 on page 168) but not in patients (p>0.05). None of the other systemic BP parameters (SBP, DBP, MAP) assessed showed any correlation with the measurements assessed at the ocular circulatory level (all p>0.05).

7.6.4 Relationship between circulatory (ocular and systemic) parameters and visual function

Ocular vascular parameters as assessed by CDI, HRF and RVA did not reveal any correlation with the parameters assessed for visual function (Visual fields, CS and colour vision) in neither CAD patients nor controls, all p>0.05. AASI correlated significantly inverse with the full field threshold and the inferior VF threshold as measured with FDT in controls (p=0.034 and p=0.018 respectively; see Table 7.3 on page 169) but not in the patient group (p>0.05 and p>0.05 respectively; see Table 7.3 on page 169). However, inferior TD as measured by FDT was independent of AASI in both patients and controls (p>0.05; see Table 7.3 on page

Parameter	Controls	p-value	CAD	p-value
	Spearmans R		*Spearmans R*	
AASI & FDT full field threshold [dB]	-0.43	**0.034**	0.05	0.827
AASI & FDT inferior field threshold [dB]	-0.47	**0.018**	0.03	0.863
AASI & FDT inferior TD [dB]	-0.41	0.05	0.01	0.939

Table 7.3: Correlation between the ambulatory arterial stiffness index and visual function (FDT fullfield and inferior threshold and inferior TD)parameters; AASI: ambulatory arterial stiffness index, FDT: frequency doubling technique, TD: total deviation, dB: decibel

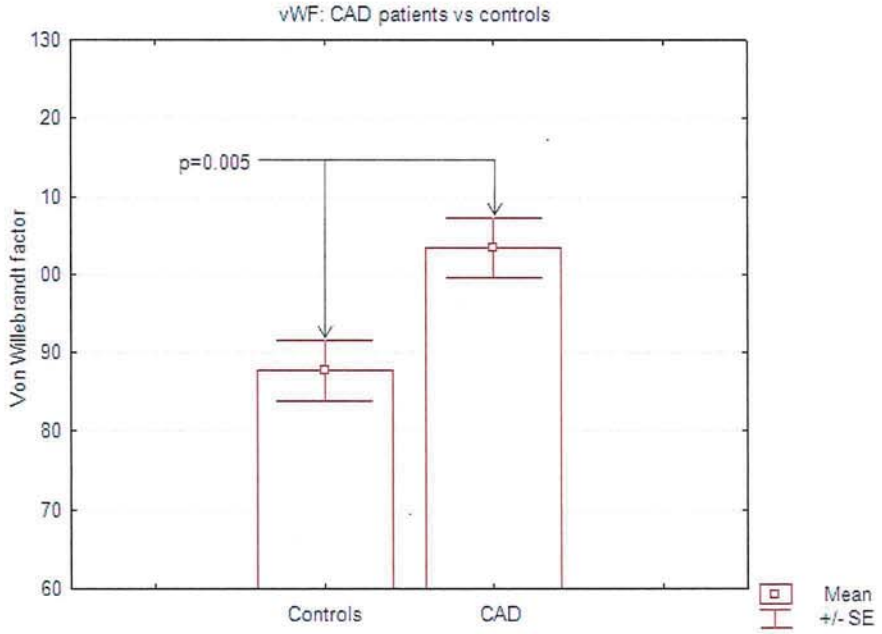

Figure 7.3: Plasma von Willebrandt factor (vWF) levels in CAD patients and controls; vWF: von Willebrandt Factor

169).

7.6.5 Circulating marker for endothelial function

Patients had significantly higher levels of plasma vWf than healthy age matched controls (patients: 103 (16) and controls; 88 (20) p=0.005; see Figure 7.3 on page 169). In addtion, BMI showed a significant positive correlated with the plasma vWf in patients, but not controls (patients: p=0.040 and controls p>0.05; see Figure 7.4 on page 170). However, there was no significant correlation present between plasma vWF levels and any of the ocular and systemic vascular parameter parameters assessed in neither CAD patients nor controls (all p>0.05).

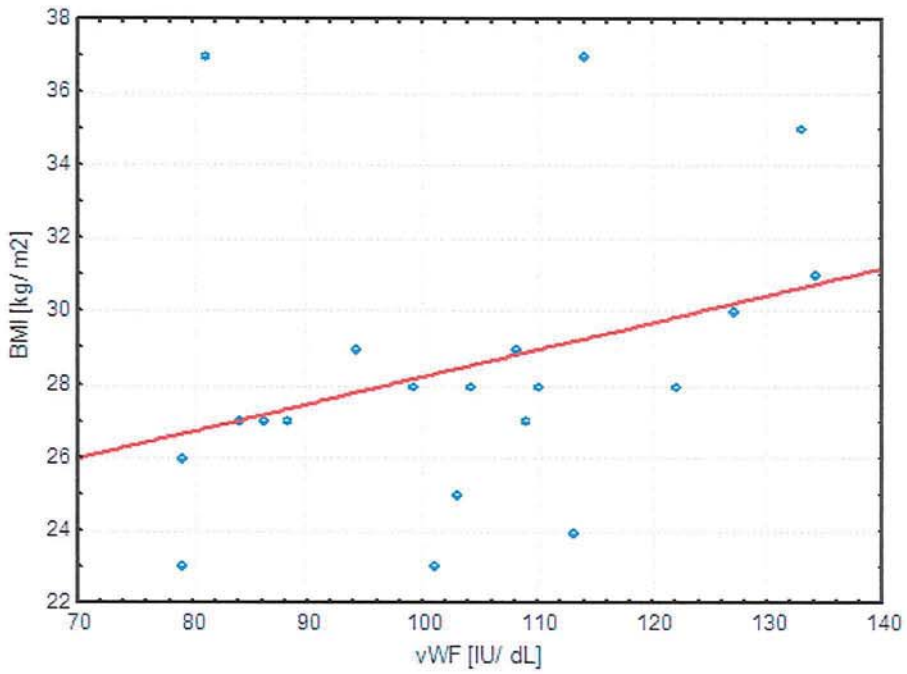

Figure 7.4: Correlation between BMI and vWF in CAD patients; BMI: body mass index, vWF: von Willebrandt factor

7.7 Discussion

7.7.1 Main findings

Besides the fact, that plasma vWF levels were within a "normal range" in both groups, patients had significantly higher levels than the control group. A significant correlation between parameters of vascular reactivity at the retinal and nail-fold capillary level was present in controls but absent in patients. However, within the patient group a functional relationship between parameters assessed at the capillary bed of the nail-fold and variables of retinal elasticity showed significance. Visual function as assessed with FDT was again significantly correlated with AASI, a measure of large artery stiffness, in the control but not the patient group.

7.7.2 Relationship between structurally and functionally different vascular beds

Arteriosclerosis is characterized by numerous structural changes in the vasculature throughout the body, such as retinal vascular alterations [Klein et al., 2002, Liew et al., 2008, Taylor and Lightman, 2003, Werner et al., 2001], changes in circulating endothelial markers [Constans and Conri, 2006, Harris and Matthews, 2004, Vischer, 2006] as well as impaired BF in numerous other vascular beds

[Hamburg et al., 2008, Houben et al., 2003]. Therefore, a normal conclusion would be that there is a common mechanism resulting in all these alterations. Indeed, CAD patients showed a significant correlation between BCF as measured at the nail-fold level and the average APR as measured at the retinal level. However, this relationship was absent in the control group and is most likely the result of similar endothelial dysfunction at level of both vascular beds. Another finding was the correlation between the nail-fold capillary recovery time and the AV-ratio at the retinal level in patients but not in controls. This relationship shows that vascular stiffness associated with CVD is present at both the peripheral (nail-fold capillaries) and retinal level (retinal vessels). In addition, the significant inverse correlation between the three arterial slope values and the capillary recovery time at the nail-fold level found in the control group was present only for the third slope in the CAD group; this result suggests that in a healthy individual: the quicker the recovery at the peripheral vascular level to stimulation, the faster the reaction at the retinal level when stimulated with flickering light. Both measures assess the endothelial function of the microvasculature by using a type of stress (cold and flickering light) therefore; an absence of such relationship in the patient group could suggest a loss of endothelial integrity at the microvascular level.

7.7.3 Vascular impairment/ stiffness and visual function

Visual function in CAD patients has been described and assessed in detail in Chapter 6. Patients had significantly lower scores for the larger special frequency grating as assessed by CS as well as showing significantly increased measurement variability as assessed by CV for the Octopus flicker test. Both stimuli, the flickering spot in the Octopus flicker test (Goldman size III) and the grating in the CS assessment, evoke a similar response as the one used to assess retinal vessel dynamics. Therefore the increase in measurement variability of the visual field assessment in particular the Octopus flicker test fits in well with the increased reaction time at the level of retinal arterioles, as measured using RVA, in CAD patients. This increase in reaction time at the retinal level could therefore be the most likely source of the measurements' CV being increased in the patient group compared to the control group. Meaning that in CAD patients, the stimuli used in the visual field assessment was possibly not seen/ or wrongly detected, due to either the delay in retinal arteriole reactivity caused by a disturbed ocular vascular regulatory response or more precisely, photoreceptor impairment caused by vascular dysfunction showing as unreliable visual function. In support of this hypothesis the control group showed a significant correlation between a marker of systemic circulation (AASI) and visual field parameters as assessed using FDT. However, this relationship was absent in the patient group, further undermining the theory that visual function is intact in a healthy vascular environ-

ment and can be compromised due to the loss of vascular function caused by endothelial impairment. Similar findings of decreased visual function have been described in previous studies by Erb and co workers in hypertensive patients [Erb et al., 2000]. Although the visual field and CS tests used in these studies were different from those used in the present study and can therefore not be used for direct comparison, the patient groups assessed also suffered from CVD.

7.7.4 Conclusion

Multiple functional, structural and biochemical vascular endothelial dysfunction in patients suffering from CAD have been found and described in detail in the present and previous chapters. In order to further elucidate the relationship between the parameters of ocular and systemic circulation and visual function with each other, the most relevant variables were selected for the analysis. The correlations computed in this chapter are showing a functional and structural relationship between ocular circulatory and peripheral circulatory parameters as well as between systemic vascular elasticity and visual function. However, more studies using different visual functional stimulation techniques as well as assessing ocular and systemic circulatory parameters are necessary to get more a more detailed insight in the mechanism of functional and structural relationship between these markers in health and disease.

Chapter 8

Summary and Conclusions

8.1 Summary

The importance of endothelial dysfunction, both on the ocular and systemic level, in patients suffering from CVD has been researched previously and relevant scientific literature has been reviewed in Chapter 1 of this thesis. However, it is still unclear how haemodynamic disturbances present at various vascular beds are linked with each other and their impact upon visual function in the presence of CVD. Elucidating these relationships and their underlying mechanisms could open new diagnostic and therapeutic avenues as well as the possibility to utilize these assessments for treatment monitoring purposes. The presented thesis has been concerned with investigating the presence and impact of functional, structural and biochemical vascular endothelial dysfunction in CAD patients and their interaction between each other as well as their impact upon visual function.

In summary, the findings of this work were:

8.1.1 The effect of chronic smoking in healthy individuals

The effect of age upon static retinal vessel parameters, such as the A/V-ratio as well as arterial narrowing and nicking have been researched extensively in previous studies [Wang et al., 2006, Wong et al., 2001a,b, 2002a,b, 2003a,b, 2006a,b]. However, the effect of age on functional retinal vessel parameters and its measurement variability has been rarely assessed. The idea behind this study was to explore if age had a similar effect on functional measures as those described on structural ones. The results discussed and outlined in Chapter 1 did not reveal an effect of age on the dynamic retinal vascular parameters nor on the measurment variability as assessed in non-smoking and smoking individuals.

Due to the lack of studies exploring the cascade and mechanisms of repeated flickering light stimulation on retinal arteries and veins, in the time and diameter domain, an extensive approach using novel analysis examining both was applied.

This included the analysis of the one second mean baseline diameter and its effect on the subsequent initiated vascular dilatory response due to flicker light stimulation in retinal arterioles and veins. The one second mean baseline diameter was found to be predictive for the amount of dilation due to flicker stimulation. These mechanisms and parameters obtained from healthy individuals were aimed to find the most appropriate/ most sensitive parameters for further patient assessment.

Furthermore, the retinal vascular dynamics in smokers compared to non-smokers was assessed, especially as smoking is the number one risk factor for CVD [Hanna, 2006, Lavi et al., 2007, Winniford, 1990] and has a known effect upon ocular vascular parameters as shown in previous studies [Lietz-Partzsch et al., 2001, Morgado et al., 1994, Robinson et al., 1985, Tamaki et al., 1999, 2000, Williamson et al., 1995b, Wimpissinger et al., 2004]. However, to date retinal vascular reactivity in smokers, more precisely its reaction cascade has not been examined. In summary, retinal vessel reactivity to flickering light stimulation was less predictable in smokers compared to non-smokers as described in chapter 1. Retinal arterioles of smokers exhibited a change in reaction pattern when stimulated with flickering light, manifesting as a tendency to constrict with increased stimulation frequency. This vaso-constrictory shift could reflect an abnormal vascular endothelial function at the level of the retina. Furthermore, the one second mean baseline diameter prior to flicker stimulation was not as predictive as in the non-smoking sample which is a further indicator of a loss of autoregulation at the retinal vascular level. However, Wimpissinger and co workers found and increased fundus pulsation amplitude in smokers compared to non-smokers (2005) which could have influenced our measurements due to its vertex distance dependence. In addition, retinal reflectance may have had and additional effect upon the diameter measurement. Due to the measurements dependence on edge detection and frame to frame analysis, an change in retinal reflectance can potentially bias the diameter recording. However, these effects and mechanisms should be separately explored in further studies including both smoking and non-smoking subjects.

8.1.2 The acute and chronic effect of smoking on vascular reactivity

Chronic smoking, the number one risk factor in the development of CVD, is a major contributor to the breakdown of vascular auto-regulation at the systemic [Celermajer et al., 1993, Duprez and Cohn, 2007, Lavi et al., 2007, Rahman and Laher, 2007a] and ocular level [Wimpissinger et al., 2004, 2005, Kaiser et al., 1997, Lietz-Partzsch et al., 2001]. In the group of smokers assessed in chapter 2,

retinal vascular reactivity to flickering light, a measure of retinal vascular endothelial function, was significantly different compared to controls. Vasomotion at both, baseline before flicker stimulation as well as the reactivity to flicker, of retinal arterioles but not veins was changed when compared to a non-smoking age-matched control group; however, the reaction time was not affected by chronic smoking, neither in retinal arterioles nor in veins.

The assessment of individuals at risk of developing CVD, in particular that bearing major risk factors such as smoking, is very important for unveiling the mechanisms of the pathogenesis of endothelial function and its progression. Future studies need to further elucidate the impact of endothelial markers of vacular function such as NO and ET-1 at the time of dynamic retinal vessel assessment, as well as their impact in smoking cessation, e.g. if normal levels are reached, does retinal reactivity normalise too?

8.1.3 Ocular Blood Flow and Retinal vessel reactivity in patients suffering CAD compared to healthy controls

Endothelial vascular function in patients suffering from CVD has been extensively reported in the literature [Harris and Matthews, 2004, Taddei et al., 2003, Verdecchia et al., 2006, Constans and Conri, 2006, Felmeden and Lip, 2005, Luscher et al., 1995, Noll and Luscher, 1998, Remme, 1998, Spieker et al., 2006, Vischer, 2006] and large population studies have been convened to explore static retinal vessel changes in this patient group. Ocular BF parameters and retinal vessel reactivity, being functional rather than structural parameters, have rarely been assessed in patients suffering from CAD [Fujioka et al., 2006]. Those suffering from CVD are most likely to be affected by more than one disease entity such as diabetes and hypertension which in turn makes drawing conclusions from such data very difficult. In order to minimise these cumulative effects due to multiple diseases the aim of the present thesis was to assess only those patients suffering from CAD who were also free from any other vascular abnormality (e.g. diabetes mellitus, hypertension) and ocular disease/surgery (e.g. glaucoma, cataract and LASIK). Due to these exclusion criteria, the sample of patients assessed were all of Caucasian origin as the majority of CAD patients of Asian and African-Carribean origin suffer from the-above listed comorbid diseases and therefore were excluded from participating in the present study. The original aim was to assess newly diagnosed CAD patients; however, newly diagnosed patients often suffer from unstable angina which makes the assessment impossible. Those patients in a stable condition are usually post-surgery subjects undergoing cardiac rehab, which is the main reason for having recruited only patients that underwent cardiac rehabilitation.

Cardiac rehabilitation has been shown in previous studies to improve endothelial function [Lee et al., 2006, Erbs et al., 2006, Hambrecht et al., 2000a, 2004, Linke et al., 2006, 2008], which may have lead to the preservation of retrobulbar and retinal BF as assessed in Chapter 4. Despite these findings the presented sample still showed a disturbance at the level of retinal arterioles manifesting in a progressively increasing reaction time when stimulated repeatedly with flickering light. Furthermore, patients exhibited an increase in retinal venular diameter when compared to age-matched healthy controls, a finding which has been reported in previous studies [Wang et al., 2006, Wong et al., 2006a,b]. This finding along with the correlations described between the average APR and velocity parameters of the CRA in patients and controls supports the hypothesis of numerous loss of vascular elasticity in CAD along the vascular tree. In addition, all CAD patients had to abstain from their usual medication; however, the long term benefit of systemic medication improving BF via e.g. NO donation, statins and anticoagulants in combination with cardiac rehabilitation may have helped to restore ocular blood flow to some degree, explaining the finding of similar retinal vascular dilatory response to flicker stimulation. However, despite improved endothelial function, the CAD patient group still exhibited a significantly delayed reaction, reflecting an impaired autoregulatory response (a list of medication taken by CAD patients included for this study can be found in the appendix).

8.1.4 Visual function in CAD

Visual function in patients suffering CAD is grossly normal. Meaning visual acuity and visual field parameters were comparable to those as measured in healthy controls. However, when filtering those with greater CV-values, in particular the visual fields analysis, which represent a decrease in reliability, some patients appear show decreased visual function which could be artifactual in origin.

8.1.5 Multiple functional, structural and biochemical vascular endothelial dysfunctions in patients suffering from CAD: relationships and possible implications

CVD is characterised by a multi level endothelial dysfunction and ANS impairment [Harris and Matthews, 2004]. Multiple functional, structural and biochemical vascular endothelial dysfunction in patients suffering from CAD have been found and described in detail in the previous chapters. In order to further elucidate the relationship between the parameters of ocular and systemic circulation and visual function with each other numerous systemic, ocular vascular as well as parameters of visual function were correlated with each other. These correlations all

show a functional and structural relationship between ocular circulatory and peripheral circulatory parameters as well as between systemic vascular elasticity and visual function. Recent studies (ANS papers on endodys) have introduced a model of the combined effects of ANS impairment and endothelial dysfunction in the pathogenesis of CVD. The CAD patients assessed in this thesis have shown a significant sympathetic overdrive compared to controls during both day and night time; however, none of the analysed parameters of ANS function showed a significant correlation with ocular vascular parameters or visual function.

Cardiac rehabilitation, known to improve ANS function, may well have contributed to the lack in finding a relationship at this level. Furthermore, vWF a strong marker of endothelial function was within "normal range" for both CAD patients and controls but still, patients had significantly increased levels compared to age-matched healthy controls which may also lie in the fact that all CAD patients underwent cardiac rehabilitation at the time of the assessment. Therefore, further studies to elucidate the effect of cardiac rehabilitation not only on the ocular but also on the link between systemic, ocular and ANS function is necessary.

8.2 Conclusions

The aim of the presented work was:

8.2.1 To investigate the novel analysis techniques for the assessment of retinal vascular dynamics and the impact of age upon the analysed parameters

The findings of this work were:

- Age did not affect any of the dynamic retinal vascular parameters assessed
- Age did not affect measurement variability
- The one second mean baseline diameter prior to flicker stimulation is a strong predictor of the dilatory response to flickering light induced vaso dilation in both, retinal arteries and veins

8.2.2 To assess the effect of smoking (chronic and acute) upon retinal vascular reactivity in healthy individuals

The findings of this work were:

- The one second mean baseline diameter prior to flicker stimulation was a less strong predictor for the vascular dilatory response to flicker stimulation in smokers compared to non-smokers
- Smoking subjects showed and increase in the arterial baseline pulsation amplitude as well as in the vaso dilatory response to flickering light stimulation

8.2.3 To assess systemic and ocular vascular function in patients suffering from CAD

The findings of this work were:

- While retrobulbar BF velocities were comparable in CAD patients and controls, systolic spectral wave shapes of the OA and CRA were significantly different in patients than controls
- Peripapillary BF volume as measured with the HRF was significantly different in the 4 quadrants assessed in patients but not controls
- Retinal vascular reactivity to flickering light stimulation was significantly different in patients than controls

8.2.4 To assess visual function in CAD

The findings of this work were:

- CS was significantly decreased at 3 cpd in patients compared to controls
- Colour vision was comparable in CAD patients and controls
- Measurement variability of the flicker perimetry but not FDT was significantly increased in CAD patients compared to controls

8.2.5 To assess the relationship of functional, structural and biochemical vascular endothelial dysfunction in patients suffering from CAD

The findings of this work were:

- BMI significantly correlated with VWF (a marker of endothelial function) in the patient group but not controls

- Retinal vascular reactivity showed a significant correlation with peripheral reactivity parameters in controls which lacked in the CAD group
- Visual field parameters as assessed FDT were strongly related with systemic vascular elasticity (AASI) in controls but not CAD patients

8.3 Implications for CAD patients arising from these results

Patients suffering from CAD have exhibited endothelial dysfunction at the ocular, systemic and peripheral vascular level. This change in vascular function is putting the patient at higher risk of development of ocular vascular pathologies. Therefore, extensive systemic as well as ocular vascular assessment of this patient group is recommended not only at the time of diagnosis but also as a monitoring tool in the progression of CVD.

8.4 Future areas of research arising from this work

Of the new questions arising from the presented results, 4 particular avenues of future research are worth highlighting.

8.4.1 The acute and chronic effect of fasting and dehydration upon retinal vessel reactivity and ocular blood flow

Previous studies investigating the effect of fasting and dehydration upon the ocular circulation were able to demonstrate a significant effect upon IOP [Dadeya et al., 2002], retrobulbar BF [Inan et al., 2002] in otherwise healthy subjects. Furthermore, a change in circulating markers of endothelial function has been demonstrated by Ziaee et al 2006. However, to date no study has investigated the effect upon retinal vascular reactivity and BF and its combined effect upon visual function. Moreover, the effect of chronic fasting would enable an insight into the bodies possibility to adapt to such functional metabolic changes at the level of endothelial vascular regulation.

8.4.1.1 Publications

Publication listed in the appendix gave rise to the above proposed research.

8.4.2 The effect of fundus pulsation and retinal reflectivity on the assessment of retinal vessel reactivity

Retinal vessel reactivity based continuous diameter recordings using RVA technology is highly dependent on constant vertex distance. Since the patient can be put into a stationary position with the head against the head rest and the chin on the chinrest as well as applying movement tracking, the measurement of retinal vascular diameters can still vary due to a change in vertex distance caused by fundus pulsation an retinal reflectivity. Fundus pulsation has a main effect upon vertex distance and could theoretically be corrected for if measured simultaneously. Retinal reflectivity however, has a potential major effect upon edge detection software used to define retinal vascular walls as basis for the diameter assessment. As both, reflectivity and fundus pulsation amplitude have been found to influence the assessment with flicker [Wimpissinger et al., 2003, Riva et al., 2005], this is of particular interest due to the fact that most patient groups assessed could bear such structural and functional changes leading to measurement inaccuracy and potential failure in detection of differences when compared to controls.

8.4.3 Analysis of retinal vascular pulsation in health and cardiovascular disease

Vascular pulsation has been shown to be a useful parameter in the assessment of auto-regulatory breakdown [Gugleta et al., 2006a, Wimpissinger et al., 2004]. However, to date, pulsation variables are mostly derived from averaged measurement values and therefore bear bias in both directions: under and over estimation of assessed differences. This problem could be minimised by applying fast Fourier transform (FFT) analysis in the assessment of retinal diameter matrices. FFT could be used in assessing vaso-motion at the retinal level along the vessel segment observed instead of using averaged diameter values. The RVA measurement offers and ideal matrix to apply FFT analysis due to the nature of the matrix incorporating both diameter and time.

8.4.4 Cardiovascular Rehabilitation and its effect on ocular vascular parameters

The patient group assessed in the present study was recruited from a cardiovascular rehabilitation unit at City Hospital, Birmingham. CV rehabilitation has been shown in previous studies to improve not only biochemical markers of vascular health but also ANS function in patients suffering CVD. The extent of this improved endothelial function at the ocular level however is unknown. In order

to assess this effect, patients suffering CVD should be examined before CV rehabilitation as well as during and after this intervention to elucidate the extent of improvement as well as the impact of intensity of such program upon endothelial function.

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Appendix

Medication list: Medication taken by participants suffering from heart disease

Case Report: The effect of voluntary fasting and dehydration on flicker induced retinal vascular dilation in a healthy individual: a case report

List of medications taken by CAD patients included in the present thesis

Statins	***anti-platelet agents***	***Diuretics***	***selective β1-blocker***	***ACE- inhibitor***
Lipitor	Plavix	Bendoflumethiazide	Metoprolol	Ramipril
Simvastatin	Clopidogrel		Cardicor	Perindopril
Atorvastin			Bisoprolol	Trandolapril
Ezetimibe			Atenolol	
Dietary supplements	***Ca antagonist***	***H2 receptor antagonist***	***Anticoagulant***	***Others***
Adacal D3	Amlodipin	Ranitil	Warfarin	Nicorendil
Omeprazole		Ranitinide		paroxetine
		Lansopreqole		Fasamax
		Allopurinol		Candesarta