Heterogeneity of Sub-retinal Deposits in MacTel Type 2 and Ageing

A Thesis Submitted to University College London for the degree of Doctor of Philosophy

Ashraf Ahmed Gango Omer
MBBS, MSc

Department of Ocular Biology and Therapeutics
Institute of Ophthalmology
University College London
2013
Declaration

I, Ashraf Ahmed Gango Omer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Ashraf Ahmed Gango Omer

Date: 25th of January 2013
Abstract

MacTel type 2 is a slowly progressive neurodegenerative condition primarily affecting the temporal area of the central retina. Subtle generalised increases in foveal autofluorescence are early sign of the disease. During the course of examining autofluorescence imaging at the Reading Centre of Moorfields Eye Hospital, we identified a new hitherto undescribed pattern of autofluorescence in patients with MacTel type 2. This pattern consists of focal hyperautofluorescent deposits (HADs). Cross-sectionally, we studied HADs prevalence and patterns of distribution in MacTel type 2 patients, family members and controls in fundus autofluorescence and fundus colour images. We carried out a detailed phenomenological description of HADs using a multimodal approach consisting of point-to-point correlative analysis of fundus autofluorescence, colour fundus imaging, fluorescein angiography imaging and high-definition OCT. Longitudinally, we examined HADs progression over a 5-year-period and HADs in the clinical progression of MacTel type 2. In parallel, we carried out in vivo investigations to examine our hypothesis that hyperautofluorescent deposits have an autofluorescent signature distinct from that of Bruch’s membrane and the RPE and that this autofluorescent signature could mark specific pathological states. We used the microprobe synchrotron X-ray fluorescence and X-ray diffraction analysis to examine the effect of trace metals and elements on drusen autofluorescence. We demonstrated that HADs are a newly identified pattern of autofluorescence change in patients with MacTel type 2 by showing that HADs prevalence, topographical distribution, progression; all strongly correlate with the disease. We explored the potential of autofluorescence imaging as technique to derive specific information on deposits/drusen and
RPE layer in pathological states. We discovered the presence of calcium is associated with the precipitation of hydroxyapatite, a constituent of drusen that had not been described before. Future studies are required to confirm the potential for the use of HADs as a biomarker in clinical settings.
Acknowledgements

This work would not have been possible without the help and support of so many people in innumerable ways over so many years. The work is a product of the hand of serendipity and the fortuitous encounters with people some of whom were inspirational.

First and foremost, I would like to express my deepest appreciation and gratitude to Professor Philip J Luthert for his inspiring mentorship, patience and kindness throughout the years. Thanks to Drs Lengyel and Peto.

My deepest gratitude to Mr. Robin Shawyer, Executive Director for Windle Trust International, Mr. Mario Konyen Joseph, UK Programme Officer and Anne Lloyd-Williams for their ample support. This PhD would not have been possible without the financial support from Mr. Philip Highlander and the Highland Foundation, for which am deeply grateful.

Special thanks to my dear friends; Christopher Kidner and his parents Virginia and William, Tom Nickalls and his parents; Fiona and Oliver. No words will be enough to express my gratitude and thanks for your help and encouragement in so many different ways. Special thanks to Mr David Wolton, for proof reading this thesis and for generously sharing his positive spirit. I am very fortunate and truly delighted to get to know you and to have you in my life.

Many thanks to Dr. Peter Munro of the imaging department of the Institute of Ophthalmology for his sincere help and encouragement. Special thanks to Robin Howes for guiding my trembling fingers with electron microscopy. Thanks you for sharing your amazing world and experiences of music old planes, motobikes and lots of fascinating life lessons. Thanks to
Hannah Armer. Special thanks to Heidi Barnes for her sincere and indispensable help in the lab and for sharing all her interesting stories about plate tectonics and her volcano adventures. Thanks to Professor Richard Thompson of the University of Maryland School of Medicine for an insightful discussion we once had on autofluorescence and spectral profiling. Thanks to Professor Alan Bird for his scientific advice and many insightful comments.

Thanks are also extended to Professor Jane Flinn of George Mason University (USA) and Sue Wirick of the synchrotron facility at Brookhaven National Laboratory (USA) for their support and guidance with XRD and XRF. Many thanks to Mr. Desta Bokre at the Library of the UCL Institute of Ophthalmology for always being a sympathetic ear and a helping hand. Danny Daniels, the fabulous computer expert whose help made such a difference. Many thanks to my fellow lab mates both past and current for being such a delight to work with; Sabrina Cahyadi, Daniela Florea, Neda Barzegar-Befroei and Valentina Reffatto. Thanks are also extended to Irene Leung and Delna Mariner at the Reading Centre of Moorfields Eye Hospital. Special thanks to Nancy Campbell for proof reading this thesis. Many thanks to Vincent Rocco of the imaging department of Moorfields Eye Hospital.

Cordial thanks to London’s Nuba community for their immense support and encouragement during the period of writing the thesis; Yassir Hamouda, Kamal Kambal, Sallam Tutu, Hussain Koodi and Ezzeldin Rahma, am fortunate to have met you all.

Many thanks to all the wonderful friends and staff members at Goodenough College for creating such a wonderful community.

Last but not least I would like to thank my examiners Professor Dr. Daniel Pauleikhoff and Professor Fred Fitzke for accepting to be my examiners.
Dedication

To my parents; Altuma and Ahmed, for all their sacrifices
To the Nuba Mountains, a land that was filled with thorns, but like a phoenix it shall rise
Contents

I Overview

1 General Introduction

1.1 Overview ..................................................... 29

1.2 Embryonic Development ................................. 31

1.2.1 The Eye ............................................. 31

1.2.2 The Retina & RPE ................................... 32

1.2.3 Bruch’s Membrane ................................. 33

1.3 Functional Anatomy .................................... 34

1.3.1 Retina ............................................... 34

1.3.1.1 Structure ....................................... 34

1.3.1.2 Function ....................................... 35

1.3.2 Retinal Pigment Epithelium ........................ 37

1.3.2.1 Structure ....................................... 37

1.3.2.2 Function ....................................... 37

1.3.3 Bruch’s Membrane ................................. 38

1.3.3.1 Structure ....................................... 38

1.3.3.2 Function ....................................... 39

1.4 Ageing of the Photoreceptor-RPE-Bruch’s Complex .... 39

1.4.1 Introduction ....................................... 39

1.4.2 Photoreceptors ................................... 40
II  Autofluorescence and Metal Distribution in Drusen 59

2  Spectral Profiling of Drusen 60
   2.1  Overview 60
   2.2  Literature Review 62
      2.2.1  Introduction 62
      2.2.2  Photoluminescence 62
2.2.3 Conceptual basis of autofluorescence

2.2.4 Characteristics of fluorescence
   2.2.4.1 The Stokes Shift
   2.2.4.2 Independence of the emission spectrum from the exciting light
   2.2.4.3 Fluorescence Intensity
   2.2.4.4 Quantum yield and lifetime
   2.2.4.5 Quenching
   2.2.4.6 Fluorescence Measurement

2.2.5 Effects of Metals on Autofluorescence
   2.2.5.1 The lightening Effect
   2.2.5.2 Alteration of radiative decay rates

2.2.6 Fluorophores in the RPE-Bruch’s Membrane Complex
   2.2.6.1 Lipofuscin
   2.2.6.2 Bruch’s Membrane
   2.2.6.3 Drusen

2.3 Methods
   2.3.1 Tissue
   2.3.2 Tissue Dissection
   2.3.3 Confocal Microscopy
   2.3.4 Data Analysis & Statistics

2.4 Results
   2.4.1 The Spectral Properties of Drusen
   2.4.2 Spectral Properties of the Spherical Inclusion Bodies in Drusen
   2.4.3 Spectral Properties of RPE, Bruch’s Membrane & Drusen

2.5 Discussion
   2.5.1 Spectral Properties of Drusen
   2.5.2 Spectral Properties of Inclusion Bodies
   2.5.3 Spectral Properties of the RPE-Bruch’s Complex
2.5.4 Conclusions ................................................................. 106

3 Trace Elements in Drusen .............................................. 107

3.1 Overview ................................................................. 107

3.2 Introduction ............................................................. 109

3.3 Metals and Trace Elements Distribution in Ocular Tissue .......... 110

3.3.1 Zinc ................................................................. 110

3.3.1.1 Zinc in the photoreceptor-RPE Complex ......................... 111

3.3.1.2 The role of zinc in the photochemical visual event ........... 112

3.3.1.3 Zinc as a stabiliser of Biomembranes ........................... 113

3.3.1.4 Zinc as a neurotransmitter ....................................... 113

3.3.1.5 Zinc as an antioxidant ........................................... 114

3.3.1.6 Zinc in Bruch’s Membrane & Drusen ........................... 114

3.3.1.7 Zinc and the complement system ................................. 115

3.3.2 Calcium ............................................................. 116

3.3.2.1 Photoreceptor-RPE Complex ..................................... 117

3.3.2.2 RPE ............................................................. 117

3.3.2.3 Bruch’s Membrane ............................................... 117

3.3.3 Iron ................................................................. 118

3.3.3.1 Photoreceptor-RPE Complex ..................................... 118

3.3.3.2 Bruch’s Membrane ............................................... 119

3.3.4 Copper ............................................................. 120

3.3.5 Selenium ........................................................... 120

3.3.6 Heavy Metals ......................................................... 121

3.4 Effects of Metals on the Remodelling of Bruch’s Membrane ....... 122

3.4.1 Remodelling .......................................................... 122

3.4.1.1 Control of Remodelling ......................................... 123

3.4.1.2 Defects of Remodelling ......................................... 123
CONTENTS

3.4.1.3 Changes with Ageing ........................................... 124
3.4.2 Effects of Metals on Remodelling ................................. 124
  3.4.2.1 Zinc ............................................................. 124
  3.4.2.2 Calcium ......................................................... 125
  3.4.2.3 Iron ............................................................. 125

3.5 Methods ................................................................. 126
  3.5.1 Tissue Sources .................................................... 126
  3.5.2 Drusen Dissection ................................................. 126
  3.5.3 Labelling for metals .............................................. 126
    3.5.3.1 Labelling for the bio-available zinc ....................... 126
    3.5.3.2 Labelling for calcium ...................................... 127
  3.5.4 Confocal Microscopy ............................................. 127
  3.5.5 Combined X-ray fluorescence and X-ray diffraction analysis  127
    3.5.5.1 Sample preparation ....................................... 128
    3.5.5.2 Measurements ............................................. 128
  3.5.6 XRF Data Analysis .............................................. 129
    3.5.6.1 Metal distribution topographic maps ....................... 129
    3.5.6.2 Energy dispersive spectra ................................ 129
    3.5.6.3 Metal standards ......................................... 130
  3.5.7 XRD ............................................................... 130
    3.5.7.1 XRD data analysis ...................................... 130
    3.5.7.2 Calibration ............................................. 132
    3.5.7.3 Background subtraction .................................. 132
    3.5.7.4 Integration ............................................. 134
    3.5.7.5 Phase identification .................................... 134
  3.5.8 Transmission electron microscopy imaging ...................... 134
  3.5.9 Drusen autofluorescence ....................................... 136

3.6 Results ............................................................... 137
3.6.1 Trace Metals Concentration in Drusen .......................... 137
3.6.2 Structural Heterogeneity and Staining for Zinc and Calcium .......................... 140
  3.6.2.1 Ultrastructural features .......................... 140
  3.6.2.2 Staining for Zinc and Calcium .......................... 140
3.6.3 Drusen XRD Analysis & Metal Content .......................... 145
  3.6.3.1 Crystalline Structure .......................... 145
  3.6.3.2 Metals & Trace Elements .......................... 146
  3.6.3.3 Phase Identification .......................... 149
3.6.4 Metals and Drusen Autofluorescence .......................... 154
  3.6.4.1 Trace Metal Concentration and Autofluorescence .......................... 154
3.6.5 Drusen Autofluorescence & XRD .......................... 154
3.6.6 Drusen Matrix Vesicles .......................... 156
3.7 Discussion ............................................ 164
  3.7.1 Zinc ............................................ 164
  3.7.2 Calcium ............................................ 168
  3.7.3 Drusen Matrix ............................................ 171
  3.7.4 Drusen Mineral Phase ............................................ 173
  3.7.5 Potential role for hydroxyapatite in drusen biogenesis ............................................ 174
  3.7.6 Speculated Mechanism of drusen formation ............................................ 176
  3.7.7 Conclusions ............................................ 179

III Autofluorescence in MacTel Type 2 ............................................ 180

4 Literature Review ............................................ 181
  4.1 Idiopathic Macular Telangiectasia (MacTel Type 2) ............................................ 181
  4.2 Epidemiology ............................................ 181
  4.3 Genetics ............................................ 182
  4.4 Clinical manifestations ............................................ 184
4.5 Imaging ......................................................... 184
  4.5.1 Colour Fundus Imaging (CF) ................................ 184
  4.5.2 Fluorescein Angiography (FA) .............................. 185
  4.5.3 Optical Coherence Tomography (OCT) ..................... 185
  4.5.4 Confocal reflectance imaging ............................... 187
  4.5.5 Adaptive optics imaging (AO) ............................ 188
  4.5.6 Macular pigment optical density (MOPD) ............... 188
  4.5.7 Fundus autofluorescence (AF) ......................... 190

4.6 Staging ...................................................... 191
  4.6.1 Stage I ................................................ 191
  4.6.2 Stage II ............................................. 192
  4.6.3 Stage III ........................................... 192
  4.6.4 Stage IV ............................................ 192
  4.6.5 Stage V ............................................. 193

4.7 Histopathology ............................................. 194

4.8 Pathophysiology .......................................... 194

4.9 Differential Diagnosis .................................... 196

4.10 Functional Deficits ........................................ 197
  4.10.1 Visual Acuity (VA) .................................. 197
  4.10.2 Fine Matrix Mapping (FMM) ........................... 197
  4.10.3 Vision-related QoL .................................. 199

5 HADs: Prevalence & Distribution ............................ 200
  5.1 Overview .................................................. 200
  5.2 Introduction: ............................................. 202
  5.3 Methods .................................................. 204
    5.3.1 Population ......................................... 204
    5.3.2 Procedure ......................................... 204
CONTENTS

5.3.3 Grading for hyperautofluorescent deposits (HADs): 208

5.4 Results 209

5.4.1 General characteristics and demographics 209
5.4.2 Prevalence of Deposits in CL and AF images 210
5.4.3 Carbohydrates tests & fasting lipid profiles 213
5.4.4 Distribution of deposits in AF and CL images 215
5.4.5 MacTel patients divided on the basis of HADs (present/absent): 221
  5.4.5.1 Demographics and past medical history 221
  5.4.5.2 Test results of carbohydrate metabolism 221
  5.4.5.3 Fasting Lipid Profile 221

5.5 Discussion 226

5.6 Conclusions 231

6 HADs Characterisation 232

6.1 Overview 232

6.2 Introduction 234

6.3 Methods 237

  6.3.1 Human Subjects 237
  6.3.2 Correlative point-to-point AF-CF-FA imaging analysis: 237
  6.3.3 Spectral-domain optical coherence tomography (SD-OCT) 238

6.4 Results 241

  6.4.1 Autofluorescent activity of the deposits 241
  6.4.2 Autofluorescence and induced fluorescence of deposits 241
  6.4.3 Location 241
  6.4.4 The “basket phenomenon: 243
  6.4.5 Cross-comparison of HADs, drusen and sub-retinal drusenoid deposits 243

6.5 Discussion 255

6.6 Conclusions 259
CONTENTS

8.2.2 Fundus Autofluorescence Imaging (AF) . . . . . . . . . . . . . . . . . . . . 291
8.3 Future Directions . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 293

IV Overarching Discussion 296

9 Overarching Discussion 297
List of Tables

2.1 Fluorophores present in extracts of RPE cell lipofuscin .............................. 70
2.2 Summary of drusen’s emission peaks ............................................................ 86

3.1 Summary of metal concentrations in drusen ................................................... 138
3.2 Summary of drusen trace metal XRF concentrations and correlations to auto-
fluorescence emission $\lambda_{\text{exc}}$ at 488nm, 543nm and 633nm ....................... 159
3.3 Summary of drusen’s autofluorescence by deciles and its correlations ............. 160

5.1 Imaging basis for the sub-classification of the category of family members ..... 205
5.2 Basic characteristics, past medical history and HAD prevalence in MacTel pa-
tients, controls and family members ............................................................... 211
5.3 Laboratory test results or participants at enrolment into the MacTel study .... 217
5.4 Combined distribution per subfield (AF) ....................................................... 219
5.5 Combined distribution per subfield (CL) ......................................................... 220
5.6 Demographics and PMHx of MacTel patients divided on the basis of HADs ... 223
5.7 Fasting lipid profile of MacTel patients divided on the basis of HADs .......... 225

6.1 Deposits relative autofluorescence intensity .................................................... 244

7.1 Summary of deposits prevalence in AF and CL images correlated with Gass-
Blodi clinical stages ......................................................................................... 279
List of Figures

1.1 The embryonic development of the eye ............................................ 32
1.2 Light micrographs showing the histology of the retina ...................... 36

2.1 Jabolanski Energy Diagram ............................................................. 64
2.2 Effects of metals on fluorophores .................................................. 68
2.3 Autofluorescence at the RPE-Bruch’s membrane complex and drusen ...... 69
2.4 An autofluorescence optical cross-section of drusen ........................... 76
2.5 Drusen Autofluorescence Heterogeneity .......................................... 77
2.6 A series of XY images of a representative specimen showing emission spectra with $\lambda_{exc}$ at 364nm ................................................................. 78
2.7 Drusen emission with $\lambda_{exc}$ at 364nm ........................................... 79
2.8 Association of emission peaks at $\lambda_{exc}$ with 364nm with mean drusen’s autofluorescence intensity ......................................................... 80
2.9 Drusen emission with $\lambda_{exc}$ at 488nm ........................................... 81
2.10 Association of emission peaks at $\lambda_{exc}$ with 488nm with mean drusen’s autofluorescence intensity ......................................................... 82
2.11 Drusen emission with $\lambda_{exc}$ at 543nm ........................................... 83
2.12 Association of emission peaks at $\lambda_{exc}$ with 543nm with mean drusen’s autofluorescence intensity ......................................................... 84
2.13 Autofluorescence emission peaks of drusen with $\lambda_{exc}$ at 364nm, 488nm and 543nm 85
2.14 A druse exhibiting numerous dark inclusion bodies .......................... 88
LIST OF FIGURES

19

2.15 Optical cross-sections of a druse with inclusion profiles . . . . . . . . . . . . . . 89
2.16 Emission peaks of drusen’s background autofluorescence and spherical inclusions 90
2.17 Emission peaks of drusen’s background autofluorescence and spherical inclusions 91
2.18 Emission peaks of RPE, Bruch’s membrane and drusen with

exc

364nm . . . . 94

2.19 Emission peaks of RPE, Bruch’s membrane and drusen with

exc

488nm . . . . 95

2.20 Emission spectra of RPE, Bruch’s membrane and drusen with

exc

543nm . . . 96

2.21 Emission spectra of RPE, Bruch’s membrane and drusen with

exc

543nm . . . 97

2.22 Autofluoresence emission o the RPE-Bruch’s Complex and drusen with

exc

at

364nm, 488nm and 543nm . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 98
3.1

X26A beamline at Brookhaven National Laboratory . . . . . . . . . . . . . . . 131

3.2

A typical logarithmic multi-channel fluorescence spectra from a druse excited
at 12 keV . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 131

3.3

↵-Al2O3-AgBeh diﬀraction pattern . . . . . . . . . . . . . . . . . . . . . . . . . 133

3.4

Kapton tape diﬀraction pattern . . . . . . . . . . . . . . . . . . . . . . . . . . . 133

3.5

XRD machine . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 135

3.6

Trace Metals in Drusen

3.7

Drusen structural heterogeneity and metal distribution . . . . . . . . . . . . . . 141

3.8

Staining for Ca and Zn in drusen with Alizarin-Red and ZP-1 dyes respectively 142

3.9

Co-staining for Calcium and Zinc . . . . . . . . . . . . . . . . . . . . . . . . . . 143

. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 139

3.10 A confocal XY scan of a druse stained with AR and ZP-1 dyes . . . . . . . . . 144
3.11 simultaneous metal analysis using microprobe synchrotron X-ray fluorescence
(mSXRF) and X-ray diﬀraction analysis (XRD) of a single druse . . . . . . . . . 147
3.12 Metal CPS values at the loci along the horizontal axis in the druse . . . . . . . 148
3.13 A mSXRF of a druse . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 150
3.14 Debye-Scherrer diﬀraction patterns along 20 scan point the horizontal axis of
the druse . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 151


<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.15</td>
<td>X-ray diffraction patterns from the 10 points along the horizontal trajectory of the druse above compared to HAP reference spectrum</td>
</tr>
<tr>
<td>3.16</td>
<td>Representative XRD patterns found in druse compared to a hydroxyapatite standard diffraction</td>
</tr>
<tr>
<td>3.17</td>
<td>Multi-channel X-ray fluorescence metal counts</td>
</tr>
<tr>
<td>3.18</td>
<td>Emission intensities of isolated drusen with $\lambda_{\text{exc}}$ at 488nm, 543nm and 633nm lasers</td>
</tr>
<tr>
<td>3.19</td>
<td>XRF metal distribution maps for Zn, Ca, Cu, Fe and Ni in isolated drusen correlated with autofluorescence with excitation at 488nm</td>
</tr>
<tr>
<td>3.20</td>
<td>Debye-Scherrer patterns of dissected individual drusen</td>
</tr>
<tr>
<td>3.21</td>
<td>Autofluorescence intensity of drusen</td>
</tr>
<tr>
<td>3.22</td>
<td>TEM of a druse</td>
</tr>
<tr>
<td>3.23</td>
<td>Temporally serial X-ray diffraction (XRD) analysis of collagen mineralization process</td>
</tr>
<tr>
<td>5.1</td>
<td>Fundus subfields for grading</td>
</tr>
<tr>
<td>5.2</td>
<td>An AF image of a patient with MacTel type 2</td>
</tr>
<tr>
<td>5.3</td>
<td>Autofluorescence and colour fundus images of an AMD an DM patients</td>
</tr>
<tr>
<td>5.4</td>
<td>HADs distribution in the fundus</td>
</tr>
<tr>
<td>5.5</td>
<td>OGTT baseline results in MacTel patients</td>
</tr>
<tr>
<td>5.6</td>
<td>Carbohydrate test values or MacTel patients divided on the basis of HADs</td>
</tr>
<tr>
<td>6.1</td>
<td>Retinal Architecture in SD-OCT Spectralis</td>
</tr>
<tr>
<td>6.2</td>
<td>Correlative point-to-point AF-CL-FA analysis</td>
</tr>
<tr>
<td>6.3</td>
<td>Characteristics of HADs</td>
</tr>
<tr>
<td>6.4</td>
<td>AF, CL and FA images of a patient with MacTel type 2</td>
</tr>
<tr>
<td>6.5</td>
<td>The “basket phenomenon”</td>
</tr>
<tr>
<td>6.6</td>
<td>Multimodal imaging of a MacTel patient with an HAD deposit</td>
</tr>
<tr>
<td>6.7</td>
<td>SD-OCT scan of a MacTel patient</td>
</tr>
</tbody>
</table>
6.8 Longitudinal light reflective profiles (LRPs) from figure (6.7) .................. 250
6.9 Multimodal Images of drusen in an AMD patient ................................. 251
6.10 SD-OCT scan of an AMD patient .......................................................... 252
6.11 Longitudinal light reflectivity profiles of the druse above ....................... 253
6.12 Different types of sub-retinal deposits .................................................. 254

7.1 Grading for progression ............................................................................ 266
7.2 HADs regression ....................................................................................... 271
7.3 No change in HADs .................................................................................. 272
7.4 Increase in count and size of HADs .......................................................... 273
7.5 High-definition volumetric SD-OCT scan of the HAD deposit .................. 274
7.6 5-year-change in deposits in autofluorescence and colour fundus images ...... 275
7.7 Prevalence of deposits in colour and AF images with the progression of MacTel
type 2 ............................................................................................................. 276
7.8 Prevalence of HADs and MacTel Type 2 progression ............................... 277
7.9 Prevalence of deposits in CL images and progression of MacTel Type 2 .... 278
# Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{em}$</td>
<td>Emission wavelength</td>
</tr>
<tr>
<td>$\lambda_{exc}$</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>$\mu$SXRF</td>
<td>Microproble Synchrotron X-ray Fluorescence</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AF</td>
<td>Fundus Autofluorescence Imaging</td>
</tr>
<tr>
<td>AFF</td>
<td>Affected Family Members</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced Glycation Endproducts</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Scelerosis</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related Macular Degeneration</td>
</tr>
<tr>
<td>AO</td>
<td>Adaptive optics imaging</td>
</tr>
<tr>
<td>AR</td>
<td>Alizarin Red</td>
</tr>
<tr>
<td>BLamD</td>
<td>Basal Laminar Deposits</td>
</tr>
<tr>
<td>BLinD</td>
<td>Basal Linear Deposits</td>
</tr>
<tr>
<td>CBR</td>
<td>Confocal Blue Reflectance</td>
</tr>
<tr>
<td>CF</td>
<td>Colour Fundus Imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CFH</td>
<td>Complement Factor H</td>
</tr>
<tr>
<td>CHOs</td>
<td>Carbohydrates</td>
</tr>
<tr>
<td>CL</td>
<td>Colour Fundus Imaging</td>
</tr>
<tr>
<td>CNV</td>
<td>Choroidal Neovascularisation</td>
</tr>
<tr>
<td>COD</td>
<td>Crystallography Open Database</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CPS</td>
<td>Counts Per Second</td>
</tr>
<tr>
<td>cSLO</td>
<td>Confocal Scanning Laser Ophthalmoscope</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ELM</td>
<td>External Limiting Membrane</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>FA</td>
<td>Fluorescein Angiography</td>
</tr>
<tr>
<td>FAs</td>
<td>Fatty Acids</td>
</tr>
<tr>
<td>FBG</td>
<td>Fasting Blood Glucose</td>
</tr>
<tr>
<td>FI</td>
<td>Factor I</td>
</tr>
<tr>
<td>FMM</td>
<td>Fine Matrix Mapping</td>
</tr>
<tr>
<td>FMs</td>
<td>Family Members</td>
</tr>
<tr>
<td>FoM</td>
<td>Figure of Merit</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full Width at Half Maximum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion Cell Layer</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HADs</td>
<td>Hyperautofluorescent Deposits</td>
</tr>
<tr>
<td>HBA1c</td>
<td>Glycosylated Hemoglobin A1c</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoproteins</td>
</tr>
<tr>
<td>HHDs</td>
<td>Hyperautofluorescent Hyperreflective Deposits</td>
</tr>
<tr>
<td>HTN</td>
<td>Hypertension</td>
</tr>
<tr>
<td>ICZ</td>
<td>Inner Collagenous Zone</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>IJRT</td>
<td>Idiopathic Juxtafoveal Retinal Telangiectasia</td>
</tr>
<tr>
<td>ILM</td>
<td>Internal Limiting Membrane</td>
</tr>
<tr>
<td>INL</td>
<td>Inner Limiting Membrane</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner Plexiform layer</td>
</tr>
<tr>
<td>IS</td>
<td>Photoreceptor Inner Segment</td>
</tr>
<tr>
<td>IS/OS junction</td>
<td>Inner Segment/Outer Segment Junction</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoproteins</td>
</tr>
<tr>
<td>LF</td>
<td>Lipofuscin</td>
</tr>
<tr>
<td>LRP</td>
<td>Light reflectivity Profile</td>
</tr>
<tr>
<td>MacTel Type 2</td>
<td>Idiopathic Macular Telangiectasia Type 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MEHRC</td>
<td>Moorfields Eye Hospital Reading Centre</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Major Histocompatibility Class II</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MP</td>
<td>Macular Pigment</td>
</tr>
<tr>
<td>MPOD</td>
<td>Macular Pigment Optical Density</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MV</td>
<td>Matrix Vesicle</td>
</tr>
<tr>
<td>NCPs</td>
<td>Non-collagenous Proteins</td>
</tr>
<tr>
<td>NFL</td>
<td>Nerve Fiber Layer</td>
</tr>
<tr>
<td>NS</td>
<td>Neurosensory Retina</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical Coherence Tomography</td>
</tr>
<tr>
<td>OCZ</td>
<td>Outer Collagenous Zone</td>
</tr>
<tr>
<td>OD</td>
<td>Ocula Dextra</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
<tr>
<td>OLM</td>
<td>Outer Limiting Membrane</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer Nuclear Layer</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer Photoreceptor Layer</td>
</tr>
<tr>
<td>OS</td>
<td>Ocula Sinistra</td>
</tr>
<tr>
<td>PA</td>
<td>Possibly Affected Family Members</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
</tbody>
</table>
PL Photoluminescence
PLs Phospholipids
PMHx Past Medical History
POS Photoreceptor Outer Segment
ppm Parts Per Million
PUFA Polyunsaturated Fatty Acids
Qm Quantum yield
RBI RPE/Bruch’s Interphase
RI RPE interdigitation
ROI Region of Interest
RPE Retinal Pigment Epithelium
RRA Retino-Retinal Anastomosis
SD-OCT Spectral-Domain Optical Coherence Tomography
SOD Superoxide Dismutase
SRNV Sub-retinal Neovascularisation
TAGs Triacylglycerides
TC Total Cholesterol
TD-OCT Time-Domain Optical Coherence Tomography
TEM Transmission Electron Micrographs/Microscope
TIMPs Tissue Inhibitors of Metalloproteinases
UHR-OCT Ultra-High Resolution Optical Coherence Tomography

UnA Unaffected Family Members

VA Visual Acuity

Vision-specific QoL Vision-specific Quality of life

WHO World Health Organization

XBP Xanthophyll-binding Protein

XRD X-ray Diffraction

XRF X-ray Fluorescence

ZP-1 Zinpyr-1
Part I

Overview
Chapter 1

General Introduction

1.1 Overview

Figures of the global prevalence of blindness are shaped by factors such as socioeconomic conditions and geographical regions. The World Health Organisation (WHO) estimates in 2002 from 55 countries and 17 different WHO epidemiological subregions paint a grim picture of 37 million people afflicted with blindness and a further 124 million people enduring low vision (Foster and Resnikoff, 2005). With a prevalence of 1%, Africa has the highest burden of blindness of those over 50 years of age. Cataract, glaucoma, corneal scars, age-related macular degeneration, diabetic retinopathy and onchocerciasis are the principal causes for global blindness in that order (Foster and Resnikoff, 2005).

The topic of this thesis was the study of a novel phenotype of autofluorescence changes in a neurodegenerative macular disease; idiopathic macular telangiectasia (MacTel type 2). The global impact of the burden caused by MacTel type 2 is currently unknown. However, MacTel type 2 is thought to be a relatively rare disease, whose impact on public health was estimated to be as high as 0.06% in Kenya and Nigeria (Sallo et al., 2012) and 0.1% in USA (Klein et al., 2010). With every development of a diagnostic tool, it becomes more obvious that the impact of MacTel type 2 has been underestimated but what is well recognised is the impact of MacTel type 2 on the quality of life of those afflicted. MacTel type 2 is relentless slowly
progressive disease that causes cumulative profound macular and foveal architectural damage. Normally, the disease is diagnosed in the fifth or sixth decade of life. Functional impairments manifest scotomas, reading difficulties, and metamorphopsia that interfere with the quality of life. From a clinician’s standpoint, there is a need for better diagnostic tools and prognostic indicators that are translatable to earlier diagnosis of the condition allowing room for potential disease-modifying interventions and that help a more accurate prediction of the course of the condition. An antecedent biomarker of disease progression or a surrogate clinical end-point is needed. From a standpoint of a clinical scientist, there is a need for a better understanding of the basic pathophysiologic processes to evaluate potential biomarkers in both human studies and to evaluate animal models and the usefulness of therapeutic endeavours. The non-invasive fundus autofluorescence imaging modality (AF) has recently gained wider acceptance and use in the ophthalmic community. An overall increase in foveal autofluorescence is recognised as an early indicator of developing MacTel type 2 (Wong et al., 2009). Here, I investigate a hitherto undescribed new pattern of focal hyperautofluorescent deposits in patients with MacTel type 2. I also investigate the wider phenomenon of autofluorescence associated with sub-retinal deposits and trace metal content.

The MacTel consortium is a global community of clinicians and biomedical scientists representing 22 different participatory clinical centres located in 7 different countries, all dedicated to the goal of understanding the different aspects of MacTel type 2 in order to develop disease-modifying therapies.

While I hope here the work in this thesis will help elucidate some aspects of the autofluorescent phenomenon in MacTel type 2, it is also hoped that light is shed on the phenomenon of autofluorescence, which is manifested in a large number of retinal diseases and conditions. The increasing use of fundus autofluorescent imaging (AF) for diagnostic purposes commands endeavours to better understand the clinical and morphologic correlates of the manifested phenomenon in order to unlock the full potential autofluorescence could contribute to our understanding of the molecular processes both in health and disease. The study of trace metals in subretinal deposits gives a further insight to the understanding of the pathophysiologic
processes of ageing and age-related macular degeneration.

1.2 Embryonic Development

1.2.1 The Eye

On day 22 of gestation, the eye starts to develop as a shallow depression on the growing neural tube. The optic groove appears on both sides of the forebrain on the cranial end of the developing embryo. As the neural folds close to form the neural tube, the optic vesicles now project from the walls into the adjacent mesenchyme. The cavities of the optic nerve, which initially are freely communicating with the developing forebrain, start to constrict as optic vesicles enlarge distally to form the narrow optic stalk (Schoenwolf and Larsen, 2009). Through the elaboration of an inductive arsenal of chemical signals, the adjacent mesenchyme plays a pivotal role in driving the early development of the eye. The retina and the posterior layer of the iris and the optic nerve are thought to originate from the neuroectoderm of the forebrain while the surface ectoderm of the head gives rise to the lens and corneal epithelium. The fibrous and vascular elements of the eye, which anatomically lie in the middle, are embryologically mesodermal in origin. The ectodermal cells adjacent to the optic vesicles thicken to form the primordial structures; the lens placodes. Lens placodes later invaginate and sink to form lens pits whose edges approach each other while losing connection with the surface ectoderm to form lens vesicles. The optic vesicles subsequently invaginate forming the optic cups with an initial large opening. The opening rim enfolds around the developing rim, which eventually completely arrests the lens as it loses its initial ectodermal connection. The developing lens induces the thicker inner layer of the optic cup to develop into the neuroepithelium while the proximal growth of the ganglion cells in the superficial layer of the retina through the optic stalk into the brain is behind the gradual constriction of the optic stalk and its transformation into the definitive optic nerve. The hyaloid artery, a branch of the trigeminal artery, is important during the development of the inner layers of the optic cup, the lens and the adjacent mesenchyme. It subsequently degenerates distally leaving behind the central
canal in the vitreous while proximally it becomes progressively trapped in the narrowing optic stalk. Definitively, the hyaloid artery ends as the central artery of the retina enclosed within the neurovascular bundle with the optic nerve.

1.2.2 The Retina & RPE

The retina has a dual embryologic origin; the RPE layer is derived from the outer thinner layer of the optic cup while the inner and much thicker wall gives rise to the multi-layered neurosensory retina. A ventricle initially separates the two layers. The posterior 4/5th of the inner layer which borders the intraretinal space that histogenetically separates the two parts of the developing retina, known as the pars optica retinae differentiate to give rise to the photoreceptive rod and cone cells. On the basal aspect, cells are anchored to a basal lamina of extracellular matrix (ECM), which will become the inner limiting membrane (ILM). The outer rim of cells develops into the outer and inner nuclear and ganglion cell layers of the retina. This duality of origin is reflected in the persistently loose fusion of the RPE and the neurosensory retina in adults eyes. The two structures can easily be ripped apart (Galli-Resta et al., 2008). The anterior 1/5th of the inner optic layer known as pars ceca retinae divides into to pars iridica retinae and pars ciliaris retinae which respectively become inner layer of the iris and the ciliary body.
1.2.3 Bruch’s Membrane

Bruch’s membrane also known as the lamina basalis choroideae, lies between the RPE and the choroid and is an acellular extracellular matrix. It is embryologically derived from both the RPE and the choroid. The mesenchymal cells surrounding the optic cup thought to be of neural crest origin under chemical induction from the developing RPE differentiate into an inner vascular and an outer choroid and fibrous elements that are to become the sclera. Greiner and Weidman studied the development of Bruch’s membrane in laboratory mammals with different gestational periods (Greiner and Weidman, 1991). Retinal samples attached to choroids obtained from hamsters (cricetus auratus), voltes (Microtus pennsylvanicus), rats (Ratus ratus) and ferrets (Mustelo furo). Embryos were serially removed from mid-gestation to birth and studied with light and electron microscopy. Despite interspecies differences, the main steps in the development of Bruch’s membrane are sufficiently similar to derive general conclusions. The first evidence for the appearance of Bruch’s membrane came when a continuous dense fibrillar basement membrane is observed parallel to the base of the RPE in all species. The basement membrane itself appeared as an electron-dense layer separated by an electron-lucent zone. An amorphous electron-lucent material with sparse electron-dense granules, microfibrils and mesenchymal cell processes occupies the zone between the basement membrane and the innermost layer of the developing choroid. The choroidal cells are enriched in ribosomes, polyribosomes and rough endoplasmic reticulum, indicative of active protein synthesis. Subsequently, there is a surge in the deposition of electron-dense particles extracellularly and an increase in the deposition of the microfibrils between the RPE layer and choriocapillaris. Collagen fibrils are then laid down and then the choriocapillary basement membrane develops along with its endothelial fenestrae. Subsequently, the amount of collagen increases and the choriocapillaris assumes its definitive contiguous morphology. Finally, elastin appears as widely spaced dense-staining deposits. First as isolated islands within the collagen it is not till the start of the visual function after birth that these islands proliferate and form a continuous layer dividing collagen into the thicker OCZ and the thinner ICL.
1.3 Functional Anatomy

The anatomy of the eye is organised to optimise its functions: refract light and serve as a portal to the nervous system. In this section the anatomy of the eye will be explored tracing the path taken by light starting at the cornea, iris, lens and finally the retina. Similarly, our discussion of embryonic development, structure and function, changes with ageing and the role of metals and trace elements will be anatomically organised.

1.3.1 Retina

The retina is the light-sensitive component of the eye which contains photoreceptors of two types; the cones, responsible for colour vision, and (2) the rods, which outnumber the cones and are mainly responsible for night vision and vision in dim light. Visual information is coded as electrical signals by the photoreceptors and then transmitted across the different layers of the neurosensory retina to optic nerve fibres, optic chiasm, optic tract and radiation and eventually to the visual cortex.

1.3.1.1 Structure

The retina, which is the light-sensitive part of the eye, is the posterior most part of the eye globe and is approximately 0.5mm in thickness. In a radial section the ganglion cell layer (GCL) is the innermost part of the retina and the photosensor rods and cones form the outermost retina and lie in close association with the the retinal pigment epithelium and the choroid. The outer nuclear layer (ONL) contains cell bodies of the rods and cones while the inner nuclear layer (INL) is made of cell bodies of the bipolar, horizontal and amacrine cells and Muller cells. The ganglion cell layer (GCL) contains cell bodies of ganglion cells and displaced Amacrine cells. Dividing these nerve cell layers are two neuropils where synaptic junctions occur. The inner plexiform layer (IPL) is the second neuropil layer of the retina. It acts as a relay station for the bipolar cells to connect to ganglion cells. Amacrine cells with different orientations interact in further networks to influence and integrate the ganglion cell signals. Muller cells...
are the glial cells of the retina. Muller cells partake in the formation of the outer limiting membrane (OLM) as a result of tight junctions between Muller cells and photoreceptor cell inner segments. Similarly, the inner limiting membrane (ILM) of the retina is composed of laterally contacting end feet of Muller cells and components of its basement membrane. The OLM forms a barrier between the subretinal and the neural retina proper and thus insulating the space in which outer segments interact with the RPE from the neurosensory retina proper. Similarly, the neurosensory retina is shielded from the vitreous by the ILM. Vascular networks of capillaries criss-cross most areas of the retina branching off and confluencing towards the major blood vessels. Capillaries are found from the nerve fibre layer to the outer plexiform layer and occasionally in the outer nuclear layer. Photoreceptors derive their nutrients from the posterior retinal vasculature composed of the choriocapillaris located exterior to the retinal pigment epithelium layer.

1.3.1.2 Function

The neural layer processes light and generates electrical impulses that are transmitted via the neuronal cell axons to the visual cortex in the brain. Counter intuitively perhaps, photoreceptors are located posteriorly behind the conductive layers of the retina and visual data is processed as electrical impulses move anteriorly through the five types of neurones arranged in alternating layers in the retina. As a result, there is considerable scattering as light first crosses the conductive layers first en route to photoreceptors (Kröger and Biehlmaier, 2009). Photoreceptors contain a stack of discs containing the photopigment rhodopsin pivotal for the process of transduction. The photoreceptor inner segment is the origin of the new discs, which progressively get pushed towards the peripheral outer segment. The RPE cells on the apical side possess villi that embrace the ageing termini of photoreceptor outer segments. The RPE is responsible for the phagocytosis of the older discs to make room for regeneration of new discs. The implication of this process is the transfer of various substances and trace elements and trace metals from the outer segment to the RPE (Bok, 2002). The fovea is a minuscule area right at the geometrical centre of the retina. It averages 1 square millimetre with a high
Figure 1.2 – Light micrographs showing the histology of the retina

[Photocredit: (http://webvision.med.utah.edu)]
density of photoreceptor cells specialised for high acuity vision. Visual sensory data exits the retina via the axons of the retinal ganglion cells which converge in the optic nerve. From the optic disc at the posterior of the eye, the optic nerve exits the eye.

1.3.2 Retinal Pigment Epithelium

1.3.2.1 Structure

The retinal pigment epithelium (RPE) is a monolayer of hexagonal cells connected with tight junctions. The apical membrane of the RPE faces the photoreceptor outer segments where apical microvilli invaginate the ends of the outer segments. Externally, the basolateral membrane of the RPE layer faces Bruch’s membrane, which separates the RPE from the functionally vital fenestrated endothelium of the choriocapillaris. RPE cells contain melanosomes, a lysosome-related organelle responsible for the production and storage of the melanin pigments, lipofuscin granules, numerous mitochondria, an active protein synthetic machinery and a variety of organelles whose concentration testifies to the various important functions RPE plays.

1.3.2.2 Function

The RPE is a multifunctional component of the eye and the visual process. It absorbs the light energy focused by the lens on the retina. It forms part of the outer blood retinal barrier and performs essential transport functions for nutrients and metabolites and diffusion of gases between the photoreceptors and the choroidal blood vessels. In the holangiotic variety of mammalian retinas, the two regions that have no direct vasculature are the rim at the periphery and the foveal region. This design necessitates the transfer of various substances and trace elements to and from the outer segment at these zones has to be effected through the agency of the RPE (Bok, 2002). RPE is important for photoreceptor renewal as it phagocytoses and digests the outer segment membrane discs. Phagocytosis of the photoreceptor outer segments is a critical RPE functions by which 3-5% of the outer segments are shed per day are engulfed
by RPE cells are digested. This process is essential for the integrity of both the neural retina as well as choriocapillaris (Young and Bok, 1969). The RPE cells on the apical side possess villi that embrace the ageing ends of photoreceptor outer segments. Bok and Young estimated that the each RPE cell engulfs 7500 photoreceptor discs per day (Bok, 1985) amounting to one million per year, this places a tremendous metabolic strain on the RPE cells requiring its highly developed mitochondria, lysosomes and protein synthetic machinery. A consequence of this process is that the chemical constituents of the outer segments pass to the RPE (Bok, 1985). The RPE manufactures the visual pigments required by the photoreceptors. RPE cells elaborate a variety of growth and trophic factors for the maintenance of the structural and functional integrity of choriocapillaris endothelium and photoreceptors (Strauss, 2005). Neural retina is separated from the blood at two levels; an inner level where the retinal vessels separate the retinal tissue from the blood and an outer level where the RPE forms a tight barrier between the choroid and the and the neural retina.

1.3.3 Bruch’s Membrane

1.3.3.1 Structure

Bruch’s membrane is a pentalaminar extracellular matrix (ECM). It is a semi-permeable filtration barrier that modulates the bi-directional transport of nutrients and metabolic waste products between the metabolically active photoreceptors and RPE cells on the one hand and the choriocapillaris on the other (Hageman and Mullins, 1999). Sumita et al. studied the fine structure of Bruch’s membrane in TEM images. He reported that Bruch’s membrane is composed of 5 different layers organised as follows from the side of the RPE cell layer: (1) The basement membrane of the retinal pigment epithelium. (2) Inner collagenous zone (ICZ) which is between 0.1 and 1μm thick. Most collagen fibrils are orientated along the long axis of the membrane with little cross orientation. (3) An elastic fibre layer is the middle layer of Bruch’s membrane. It is made of elastin fibres. (4) An outer collagenous zone (OCZ) is relatively electron-dense, 0.2 to 1μm in thickness. It shows discontinuity from one place to another
where collagen fibrils are present. (5) The basement membrane of the capillary endothelial cells of the choroid. Both the ICL and the OCL are composed of 65nm striated collagen fibrils I, III and V and they have distinct properties that confer certain functional features to Bruch's membrane. Type I collagen confers tensile strength, while type III possesses elastic properties and collagen type V provides anchorage to basement membranes of choriocapillaris but not in the RPE. Type VI collagen has the function of acting as a mechanical anchor for the fenestrated capillaries to the choroid and hence providing mechanical stabilisation for the choriocapillaris (Marshall et al., 1992). Heparan sulphate is found in the basement membrane adjacent to RPE cells while another important component found in abundance is chondroitin sulphate and dermatan sulphate. These two negatively charged glycosaminoglycans act as an electrical barrier limiting down diffusion in the collagenous layer and along the basal lamina of choriocapillaris (Hewitt et al., 1989).

1.3.3.2 Function

Bruch’s membrane performs an important structural role in order to support the RPE and secure adhesion between the choroid and the RPE. It has been suggested that the elastic component of Bruch’s membrane assists in the absorption of pulsatile energy from the circulation of the choroid.

1.4 Ageing of the Photoreceptor-RPE-Bruch’s Complex

1.4.1 Introduction

There is no distinct cut-off point at which ageing is said to start; rather the changes that happen at the tissue and cellular level are one continuous process that starts with the embryonic development until the functional failure of tissue. In other words, there is a progressive impairment of normal tissue and cellular function (Glenn and Stitt, 2009). This impairment inevitably results in diminished organism and tissue capacity to respond to stress and leads to the development of degenerative diseases (Szweda et al., 2003; Yin and Chen, 2005). Ob-
viously this raises conceptual ambiguities as to where should the line be drawn between the so-called “normal ageing” and pathology. However, ageing is considered as those pathological changes that occur continuously in tissues and cells that despite leading to decreased fitness and functional reserves do not have direct pathological consequences (Booij et al., 2009). In the light of the close anatomical and functional association between the RPE cell layer and Bruch’s membrane, it is hardly surprising that changes in one or the other would not have a direct functional bearing on the functioning of the complex as a whole. It was reported that structural changes in Bruch’s membrane precedes cellular changes in the RPE cells by one or two decades (Pauleikhoff et al., 1990; Marshall et al., 1992).

1.4.2 Photoreceptors

Evidence indicates that photoreceptor cell density decrease as a function of age. At an annual rate of cell loss of between 0.2% to 0.4% (Panda-Jonas et al., 1995) and that this is anticipated by loss of Muller cells (DiLoreto Jr. et al., 1995) crucial for the normal functioning of photoreceptors. The rate of photoreceptor cell loss also relates to lipofuscin concentration in the opposing RPE (Dorey et al., 1989). Evidence also point out that the rate of photoreceptor loss is faster in black individuals compared to whites where the rate of photoreceptor loss relates to lipofuscin accumulation in the RPE layer (Dorey et al., 1989). Normal ageing results in functional decline of photoreceptors evident in electroretinographic changes (Li et al., 2001), structural organisation (Lai and Rana, 1986) and reduced responsiveness to melatonin (Baba et al., 2012). Genetic factors appear to influence the rate of retinal degeneration (Dangiger et al., 2007). Loss of photoreceptors and consequently visual decline within a certain limit is an inevitable process associated with normal ageing in the human organism, however, an accelerated rate of loss or patterns different from normal could spell out pathological processes that could be reversed or at least slowed.
1.4.3 RPE cells

These highly differentiated post-mitotic cells are particularly sensitive to the effects of ageing. A number of factors render RPE particularly susceptible to damage with ageing. The high metabolic activity characteristic of the RPE cells attested to by the high density of mitochondria in the RPE cytoplasm (Liang and Godfrey, 2003) accounts for a high-energy consumption required to fuel the various cellular activities. A central function for the RPE is the engulfment and digestion of the photoreceptor outer segments allowing for replacement and recycling. With ageing, there is a reduction in the efficiency of the protein synthetic machinery leading to the slowing down of this process and eventually accumulation of the undigested ROSs with a variety of effects (Feeney-Burns et al., 1984). RPE autofluorescence, being mostly due to their content of lipofuscin, increases with age as the volume of RPE cytoplasm occupied by residual bodies expand (Okubo et al., 1999). Anatomically, RPE cells are located in an oxygen-rich niche (Winkler et al., 1999) sandwiched between the underlying choriocapillaris with its near-arterial oxygen tension and the overlying PUFA-rich photoreceptors. In addition to that, there is a constant bombardment with light quanta. These conditions are ideal for the generation of reactive oxygen species that mediate oxidative damage. Aspects of RPE physiology that renders it vulnerable to exudative damage include their \(\beta\)-oxidation of PUFA which generates lipid aldehyde radicals and other reactive oxygen species (Esterbauer, 1993). Lipofuscin itself is a powerful source for the generation of free radicals (Boulton et al., 1990) and therefore tissue damage. High-energy photons of solar radiation interact with components of the outer retina leading to a photochemical damage exacerbated by the superimposed photodynamic effects of the abundant oxygen in the retina (Young and Bok, 1969; Winkler et al., 1999). Such interaction generates singlet oxygen atoms further exacerbating oxygen damage. RPE cells are equipped with a robust anti-oxidant system, which includes the water-soluble glutathione system and vitamin C both is primarily active in the cytosol (Winkler et al., 1999). Fat-soluble antioxidants vitamin E and carotenoids are found in the membranes where they counteract PUFA oxidation (Winkler et al., 1999). Additional mechanisms include; SOD,
catalase, lutein and zeaxanthine carotenoids (Bone et al., 1988; Mayne, 1996; Khachik et al., 1997; Snow and Seddon, 1999). Ageing is certain to decrease the functional capacity of this elaborate antioxidant mechanism, which results in progressive and cumulative tissue damage contributing to the functional deterioration associated with ageing. Various mechanisms were put forward to explain the role of ageing in the RPE functional decline. It was suggested that iron accumulation in RPE cells impairs the RPE lysosomal and phagocytic functions (Dunaief, 2006; Chen et al., 2009). Non-enzymatic collagen cross-linking by nitrate modification in the ECM is associated with reduced solubility and associated with a decreased RPE phagocytic function and increased generation of reactive nitrogen species was proposed to contribute to this age-related functional reduction (Sell and Monnier, 1989).

### 1.4.4 Lipofuscin

LF is widely accepted as an age index in eukaryotic cells. It starts to accumulate almost immediately after birth and could reach a maximum of 19% of the cytoplasmic volume by 80 years of age (Feeney-Burns et al., 1984). LF arises as a by-product of \( \beta \)-oxidation of unsaturated fatty acids (PUFAs) as part of normal cellular energy processes (Kennedy et al., 1995). It is linked to membrane damage or dysfunction of mitochondria and lysosomes. Lipofuscin is a generic name for at least 10 different lipid-based fractions (Kennedy et al., 1995). Contained within granules inside RPE cells, LF formation might be linked to the accumulation of trace metals; Zn, Al, Fe and Cu (van der Schaft et al., 1992). Each RPE cell which performs a host of functions critical to photoreceptors anatomic and functional integrity (Strauss, 2005). Each RPE cell is in apposition to 30-50 photoreceptor outer segments (POS). A fraction estimated between 10-15% of the outer segment of each photoreceptor cell is phagocytosed by the apposing RPE cell on daily basis (Young and Bok, 1969; Newsome et al., 1987). This continuous processing of the shedding of the POS places enormous metabolic strains on RPE cells. As a function of age, protein synthetic machinery and cellular functions of the RPE is become progressively less efficient resulting incomplete digestion of POS. Due to their lack of a machinery to either degrade or export LF to a significant degree, the accumulation is
progressive and leads to a variety of effects (Lakkaraju et al., 2007). Progressive accumulation of the complex autofluorescent lipid-protein aggregates known generically as lipofuscin (Sparrow, 2007) which is estimated to increase from 1% in the first decade of life to peak at 19% of the RPE cell volume between the ages of 80 and 90 years (Feeney-Burns et al., 1984). Besides volume occupation evidence points out a prominent role of lipofuscin in cellular auto-oxidation mediated damage (Winkler et al., 1999) and interference with the phagolysosomal digestion of phagocytosed ROS fragments, RPE’s main function (Finnemann et al., 2002). Pathological accumulation of lipofuscin is implicated in a large number of neurodegenerative diseases for instance; age-related macular degeneration (AMD) (Sparrow and Boulton, 2005), Stargardt disease (Lois et al., 2004), Alzheimer’s disease (AD) (Giaccone et al., 2011), Parkinson’s disease (PD) (Ulfig et al., 1989), amyotrophic lateral sclerosis (ALS) (McHolm et al., 1984), lysosomal storage diseases (Terman et al., 2008), acromegaly (Khaleeli et al., 1984) and chronic obstructive pulmonary disease (COPD) (Allaire et al., 2002).

1.4.5 Bruch’s membrane

The thickness of Bruch’s membrane exhibits a linear increase as a function of age (Okubo et al., 1999). With progression of age, both the functional capacity and filtration rates drop for Bruch’s membrane not just as a simple consequence of increased thickness but also due to biochemical and histological transformations of its constituent molecules (Booij et al., 2009) Moore et al. described a linear pattern of reduction of Bruch’s membrane permeability to macromolecules with age, by the 9th decade of life the permeability was found to be only 10% of what it used to be during the second decade (Moore et al., 1995). Substances with molecular weights in excess of 200 kDa are selectively barred. Similarly, Karwatowski et al. found that water solubility of Bruch’s membrane drops by 50-60% between the first and the 9th decade of life, this decline was associated with an increase in the non-collagenous protein debris that also potentially interferes with RPE cell functions (Karwatowski et al., 1995). Using a dynamic method of continuous pressure changes to estimate the water flow across 4mm sections of Bruch’s-choroid complex, an exponential decline of permeability with
a half-life of 9.5 years was described (Moore et al., 1995). An experimental refinement by Starita and co-workers found that the exponential loss of water conductivity is much steeper with a half-life of 15 years in both macular and extra-macular regions (Starita et al., 1996). Calcification in Bruch’s membrane is an age-dependent process. It renders the membrane less elastic and brittle and therefore more vulnerable to breakdown and therefore, facilitating the process of neovascularization (Spraul et al., 1999). Numerous lines of reasoning were followed to account for the decline of Bruch’s membrane with the advancement of age. Membrane remodelling, immune response, changes in collagen structure and other constituent molecules; proteoglycans in particular were proposed (Karwatowski et al., 1995; Booij et al., 2009). Histochemical and ultrastructural analysis of eyes ranging in age from 12 to 80 years by Newsome et al (1987) found that there is increased with ageing, there are differential patterns of histochemical change. It was found there a greater rate of increase in the deposition of glycosaminoglycans in the collagen and elastin of Bruch’s membrane in the macula when compared to peripheral retina. In Bruch’s membrane, it was demonstrated that the thickening of OCZ is disproportionate compared to ICZ in which thickening with age appears to be only minimal (Killingsworth, 1987). There is also a deposition of long-spacing collagen fibrils, which tend to associate with RPE basement membrane (Sarks, 1976).

1.4.6 RPE-Bruch’s Membrane Interactions

The nature of the relationship between Bruch’s membrane and RPE in the context of ageing is characterised by a complex two-way functional interaction. Cai et al explored the genetic interaction between the RPE and Bruch’s membrane at the transcription level (Cai and Del Priore, 2006). Using micro-array technique, they reported that age alterations in Bruch’s membrane affect genetic expression in immortalised human ARPE-19 cells. RT-PCR revealed that ageing Bruch’s membrane up regulates 12 genes and that 8 genes are down regulated at the same time. TGF-α, vitronectin and ABCC5 are the major genes that were found to be altered in the RPE as a result. TGF-α is pivotal for the regulation of granulation tissue formation and angiogenesis while since vitronectin is involved in phagocytosis, it might be
linked to disturbances in RPE outer photoreceptor segment phagocytosis and might therefore have a potential role in the biogenesis of deposits/drusen. The ABCC5 gene is involved in the transport of cholesterol, iron and retinoids in photoreceptor cells. It is also essential for cGMP function, which controls the efflux of substances from cells. Cai et al. presume that the age-related down-regulation of ABCC5 in Bruch’s membrane contributes to the decreased clearance of substances from the RPE and in part the accumulation of lipofuscin and unextruded substances in the cytoplasm of the RPE cells.

1.4.7 Bruch’s-Choriocapillaris and RPE-Choriocapillaris Interactions

The choriocapillaris is a bed of highly fenestrated capillaries situated just exterior to Bruch’s membrane. Bruch’s membrane, which interposes the RPE and the choriocapillaris, controls the bi-directional flow of peptide factors and waste products originating in the RPE. Nutrients from the choriocapillaris destined for the RPE and photoreceptors also cross the Bruch’s membrane. The RPE layer produces the angiogenic vascular endothelial growth factor (VEGF) and anti-angiogenic factors that are required to maintain the normal physiology of the choriocapillaris. Functionally, the RPE and the choriocapillaris are coupled; death of one layer or the other will ultimately lead to death or functio laesa of the other layer (McLeod et al., 2009). With ageing, the choriocapillaris shrinks in volume. It is estimated in the 9th decade of life, the diameters of the choriocapillaris are only 30% of what it used to be and that half of the capillary profiles had already been lost these changes take place at the same time as the thickening of Bruch’s membrane (Ramrattan et al., 1994). The consequent hypoxia induces the RPE layer to increase the elaboration of factors that substantially elevate the risk for neovascularization. With advancing age, there is a predominance of angiogenic factors over anti-angiogenic factors leading to over activation of the choroid and choroidal neovascularization. The retinal architectural manifestations are swelling or scarring whose functional consequence is blindness (Hof and Mobbs, 2009).
1.5 Sub-RPE deposits

Sub-RPE deposit is a generic term that is broadly used to denote extracellular deposits external to the RPE layer. The term encompasses focal deposits (drusen) as well as diffuse deposits. Diffuse deposits are commonly sub-categorised into basal lamellar deposit (BLamD) and basal linear deposit (BLinD) (Loeffler and Lee, 1998). Sub-RPE deposits are composed of a wide variety of heterogeneous materials. Further sub-categorisation is based on the anatomical location of the deposits. Basal lamellar deposits (BLamD) are located between the RPE and Bruch's membrane BLamD, while basal linear deposits (BLinD) are a collection of extracellular material that is located external to the basal lamina of RPE in the ICZ of Bruch’s membrane. The correlation of such divisions with clinical observations and their value have been questioned as they have introduced unnecessary confusion in the terminology with no clear demonstrable clinical usefulness (Loeffler and Lee, 1998). Our discussion on the age-related changes in the RPE-Bruch's complex will focus on the third category of sub-RPE deposits; drusen which are located within the beneath the basal lamina or the RPER basement membrane.

1.6 Drusen

1.6.1 Introduction

Drusen are both risk factors and indicators age-related macular degeneration (AMD), the most important blinding condition in the developed world. Drusen are tiny white or yellow-white extracellular deposits that accumulate between the RPE basement membrane and the inner collagenous zone (ICZ) of Bruch’s membrane (Booij et al., 2009). First reported in 1855 by Francis Donders as “Colloidkugeln” or colloid spheres (Donders, 1855), drusen were given their present name by Heinrich Muller in 1856 who associated “geode” to the retinal vasculature (Müller, 1856). The biogenic origins of drusen are still mysterious, however, at least part of its lipid content arises as waste products resulting from the RPE’s digestion of the photoreceptor outer segments (Hageman et al., 2001). Hard drusen <63mm in diameter and smaller are a
common finding in fundus of those aged 20–49 years found in up to 35% (Silvestri et al., 2005). With age drusen’s prevalence increases especially after 40 years of age. Drusen >125mm in diameter are very rare in healthy population of all ages (Silvestri et al., 2005). Sarks et al. studied 353 eyes without histological evidence for early AMD and correlated the findings with clinical manifestations and evidence from transmission electron microscopy studies in a subgroup of these eyes (Sarks et al., 1999). Drusen were histologically evident in 50% of the eyes studies but clinically detectable in only 34%. All the drusen detected were of the small hard distinct variety with no soft indistinct drusen. They also reported that clinically detectable drusen have to exceed 30μm in size. 90% of normal eyes reportedly have small hard drusen of count less than 10 which are a normal finding and do not stipulate any increased risk for the development of AMD (Sarks et al., 1999).

1.6.2 “Life cycle” of a Druse

Currently, the most widely accepted models for drusen biogenesis postulate drusen arise as secretions from the RPE cells or photoreceptors due to an aberration of a physiologic mechanism by which damaged cellular components are normally removed or as a by-product of phagocytic degradation (Ishibashi et al., 1986; Young, 1987). The development of electron microscopy and immunohistochemical methods of investigation lead to the identification of a core and secondary deposit. It is assumed that drusen deposition is a two-stage process. First, the nucleation stage, in which RPE debris and dendritic cellular material is deposited in the sub-RPE space forming the core drusen material constituting a pro-inflammatory event (Anderson et al., 2002). This is followed by a second step, the “maturation” in which further deposition and modification of drusen takes place (Hageman et al., 2001). On examination using the electron microscope, mature drusen appear to be encased in an apical crust of relatively harder amorphous material underneath which, the druse contents appear to be progressively paler from base upwards. This enclosing follicle appears to be important for the maintenance of the mechanical integrity of the druse. After the period of the initial growth and by the time the druse attains 25μm size, already degenerative changes are evident in the overlying RPE.
CHAPTER 1. GENERAL INTRODUCTION

cells. These changes take the form of out pouchings of the drusen’s material could be seen herniating through breaches in this smooth defensive crust and incorporation of the pigment from the RPE cells as the mechanical pressures arising from the contours of the drusen creates “pressure spots” that weaken the RPE’s basement membranes facilitating incorporation of degenerating RPE cells into the underlying drusen. Some drusen might as well display the inclusion of basal laminar deposits. Towards the end of their lives, some drusen regress by dispersal of their material or by turning into coarsely granular structures. By that time the overlying RPE basement membrane, already overstretched and weakened, collapses and in-folds on itself over the underlying Bruch’s membrane.

1.6.3 Classification

Different approached were used to characterise and classify drusen. The morphologic approach relies on features such as size, shape and location and other approaches on chemical composition or other properties.

1.6.3.1 Fundoscopy-based approaches

Solely based on drusen visualisation in CF images, the widely used Wisconsin classification system was designed with a view to give consistency for clinical descriptions of drusen. “Hard” drusen were defined as discrete drusen between 1 and 63mm in diameter and “soft drusen” by definition are larger >125mm in diameter, or if they were between 63-125mm in diameter, with “visible thickness”. Soft drusen were further sub-categorised into either “soft distinct” if they are large with uniform density or “soft indistinct” when large with graded density and fuzzy edges (Bird et al., 1995).

1.6.3.2 Histological Approaches

Rudolf et al. followed a light microscopic semi-quantitative evaluation procedure of 133 drusen from the macula and compared them to 282 drusen obtained from extra-macular regions to determine the prevalence of the hard, soft and mixed drusen across the macula (Rudolf et
al., 2008). Morphologically, they classified drusen into one of three groups: (1) Soft drusen, principally macular in location, can attain 350 µm in diameter. Soft drusen exhibit structural homogeneity with a lack of well-defined substructural features and might be surrounded by small merging drusen at different stages of development and thick basal laminar deposits (BLamD). The RPE layer overlying soft drusen is often intact. (2) Hard drusen, numerically, are the most prevalent type of drusen. The RPE basement membrane overlying areas of hard drusen is often significantly thinned and weakened. Distinct internal regions and shell formations are characteristic features of this type of drusen. (3) Compound drusen: principally are located at the periphery. They are not characterised by distinct shapes and rather appear as loosely knit structures enriched in shells and displaying the highest degree of remodelling. The authors argue that this type represents a distinct and independent class of drusen and not merely an intermediate type between the hard and soft varieties (Rudolf et al., 2008).

1.6.3.3 Clinico-morphologic Approaches

Green devised a dynamic model of drusen classification in the context of histopathologic and clinicopathologic development of AMD (Green, 1999). This staged classification implied a linear clinicopathologic progression of AMD. In stage I, changes are so subtle, that fundoscopically drusen are undetectable and changes are only demonstrable by psychophysical testing. In fluorescein angiograms, there is late fluorescein staining. Morphologically, there are basal laminar deposits (BLamD) and basal linear deposits (BLinD). BLamD are deposits composed wide-spaced collagen between the plasma membrane and the basal lamina of the RPE; on the other hand, BLinD are mainly composed of material with coated and non-coated vesicles located in the inner collagenous zone of Bruch’s membrane (ICZ). With clinical progression, additional ultrastructural changes in the RPE divulge the presence make ophthalmoscope visualisation possible. These changes collectively termed “pigment remodelling” consist of RPE modelling and RPE attenuation with depigmentation indicative of progression into hyperplasia, hypertrophy and ultimately accumulation of cells in the sub-retinal space. The second stage is dominated by the development of soften drusen. Additional morphologic changes res-
ult in the formation of soft drusen, which appear most often due to localised accumulations of basal linear material. Less commonly, soft drusen due to detachment of basal laminar deposits. In the third stage of development, choroidal neovascularisation takes place (CNV). Capillary-like vessels extend from the choroid to invade the space between the BLamD and the RPE and into the sub-retinal space. Later, these incompetent, weak and leaky vessels differentiate into arteries and veins. There is characteristic serous or haemorrhagic exudation and additional RPE and pigment modelling. These leaky vessels give the characteristic hyperfluorescence on FA and staining of the lipid-rich drusen. The last stage of morphologic development of AMD changes is characterised by the presence of disciform scarring with variable histologic patterns. In his classification, Green considered drusen as an external manifestation and a biomarker within the context of the complex and yet not fully understood complex aetiological processes that give rise to AMD. More recent attempts to characterise drusen took advantage larger amount of data pooled from different modalities of investigation interwoven into a coherent and structured theory that aims at not only explaining the variety of morphological presentations of drusen but also placing drusen into the context of an evolving disease process in which immune and non-immune factors stand out in importance.

1.6.3.4 Multi-modal Approaches

Hageman and Mullins described 5 different ultrastructural phenotypes for drusen taken from eyes of donors with age-related macular degeneration (AMD) (Hageman and Mullins, 1999). The first category: drusen with homogenous sub-structure were found to vary in size between 1μm and > 200μm. There are semi-spherical bodies of 20nm in diameter. The authors equated this group to the hard variety. The second is composed of ultra-structurally uniform drusen with electron-lucent inclusion bodies of 80nm in addition to the 20nm granules described above. Some drusen of this group display curvilinear profiles in addition to membrane-bound spherical elements. The third category is highly heterogeneous group with electron-lucent and electron-dense inclusion bodies. Some drusen of this category also contain spherical bodies of varying sizes, fibrin-like profiles, curvilinear and cellular debris in addition some might also
contain large calcium-containing inclusions. The fourth category is composed of electron-lucent membrane-bound vesicles of sizes ranging between 50-400nm in diameter. Drusen of this group tend to occur in clusters and are characterised by their very sloped margins. The fifth category of drusen is made up of numerous membrane-bound vesicles of sizes that range between 80-300nm in diameter; this group displays a characteristic periodicity similar to that of the BLamD and the long-spacing collagen fibrils. Despite the clinical applicability of the morphological approached for drusen classification, a major problem the reliance on phenomenological characterisation (size, shape, borders, vesicles), does not hint on molecular structure. An understanding of the molecular composition of drusen would be vital for the development of testable hypotheses on the origins of drusen that would shed light on the underlying pathophysiological processes that give rise to drusen formation. Sarks et al. followed a multimodal approach in the study of the question of drusen formation as part of an ageing process in the macula (Sarks et al., 1999). 353 eyes with deposits and without clinical evidence of AMD were studied and subset was further examined using a transmission electron microscope. Drusen were histologically evident in 50% but only 34% could be observed on ophthalmoscopy. All the drusen detected were of the small hard distinct variety with no soft indistinct drusen (Sarks et al., 1999).

1.6.3.5 Drusen fluorescence

A distinction could be made between inherent autofluorescence of drusen and induced autofluorescence. In the following discussion, we will use the term inherent autofluorescence to refer to drusen autofluorescence emission as a result of the excitation of its fluorophores with laser light. Induced fluorescence in the following discussion refers to drusen fluorescence emanating from its interaction with the fluorescein dye during the FA procedure. Based on the outcome of drusen’s interaction with the fluorescein dye during FA, drusen could be classified into one of two classes. The first class is drusen that exhibit fluorescence early during the early-phase FA. This fluorescence could lasts till the dye is cleared from the retinal circulation. These drusen tend to be of a larger size suggesting this might reflect an ability to retain more dye.
CHAPTER 1. GENERAL INTRODUCTION

due to their bigger absolute surface area compared to smaller size drusen (Pauleikhoff et al., 1992). The second class is composed of drusen that do not exhibit fluorescence. Pauleikhoff et al. suggested in addition that the former class is chemically distinct from the second in that it is predominantly composed of neutral lipids as compared to the latter that is likely composed of hydrophobic lipids (Pauleikhoff et al., 1992). Drusen autofluorescence has been proposed as a new basis for the classification of drusen. Lengyel and co-workers reported the vast majority 90.2% of these drusen are spatially associated with the honeycomb capillary pillars of the choroidal capillaries (the lateral walls of the choriocapillaris) at the equator of the eyeball (p = 0.028) (Lengyel, 2004). This association might prove important in elucidating the potential links between this type of drusen and AMD. The basis of drusen autofluorescence is not yet clear. Delori et al. argued that drusen autofluorescence is attributed to RPE (Delori et al., 2000). Marmorstein et al. reported in AMD patients with drusen there is a blue shift in fundus autofluorescence (Marmorstein, Marmorstein, et al., 2002). This was attributed to the presence of a novel fluorophores independent of LP. However, no information exists on the nature of this hypothetical fluorophore and if all drusen’s autofluorescence is due to this novel fluorophore (s). The meaning and the implication between the auto fluorescence and non-autofluorescent drusen remains to be elucidated.

1.6.4 Composition of Drusen

1.6.4.1 Lipids

Donders first made the first indication that lipids constitute an important component of drusen in 1854 that described drusen as “fett-reichen” or lipid-rich. Numerous studies amply demonstrated the age-dependent rise in the relative proportion of lipid in Bruch’s membrane (Pauleikhoff et al., 1990; Kopitz et al., 2004). Lipid accumulation in Bruch’s membrane as drusen or laminar deposits compromises Bruch’s membrane central function of transport crucial to the physiology of the photoreceptors. This fat deposition in Bruch’s membrane affects different people differently and varies with age (Pauleikhoff et al., 1990; Bird, 1992). This age-dependent
fat deposition was also found to affect the retina differentially, the central area being affected more than the periphery of the fundus. Holz et al. used the thin-layer chromatography techniques to study the regional distribution of lipid in Bruch’s membrane from macular and peripheral areas in 32 eyes (Holz et al., 1994). Analysis revealed the phospholipids (PLs), triacylglycerides (TAGs), fatty acids (FAs) and free cholesterol with little cholesteryl ester (Holz et al., 1994). The consistently higher level of lipid in the macular area compared to the periphery in addition to the close match in lipid spatial distribution and that of AMD lesions seem to suggest a local cellular origin for the lipid deposits. Lipid qualitative determination experiments by Haimovici et al. using hot stage polarising microscopy (HSPM) identified the presence of cholesterol esters within Bruch’s membrane deposits and drusen (Haimovici et al., 2001). Using biochemical and histochemical techniques, Curcio et al. confirmed these results and demonstrated the presence of both esterified and non-esterified cholesterol in Bruch’s membrane and drusen (Curcio et al., 2001). Similarities in the lipid composition of drusen and atherosclerosis in arterial intima lead the authors to propose a vascular origin for drusen lipids.

It was observed that hyperautofluorescent drusen are associated with with the lateral walls of the choriocapillaris primarily at the equator of the eyeball (Lengyel, 2004). A potential role for lipids in the biogenesis and/or progression and growth of drusen is suspected as annexin II and annexin V were localised in drusen (Rayborn et al., 2006). Annexins are a family of calcium and phospholipid-binding proteins of which annexin II binds to tenascin and tissue plasminogen activator, while annexin V binds to collagen (von der Mark and Mollenhauer, 1997). The proportional lipid content of drusen was also linked to the ability to bind the water-based dye fluorescein during fluorescein angiography. Drusen with a relatively higher proportion of phospholipids were deemed to be hydrophilic and consequently can retain sodium fluorescein and therefore could be visualised as hyperfluorescent on fluorescein angiograms. On the contrary, hydrophobic drusen with very small quantities of fibronectin have the inability to retain the sodium fluorescein dye and therefore appear as hypofluorescent (Pauleikhoff et al., 1992). Age related biophysical changes in Bruch’s membrane are functionally significant in the light of the change in the hydraulic properties and reduced diffusion of Bruch’s membrane that takes
place with age. A better understanding of the compositional changes would undoubtedly help better understand the nature of the processes that reduce the functional capacity of Bruch’s membrane with normal ageing and when these changes become pathologic.

### 1.6.4.2 Proteins

A large number of drusen protein constituents including ubiquitin (Loeffler and Mangini, 1997), integrins (Brem et al., 1994), beta-amyloid (Dentchev et al., 2003; Isas et al., 2009), tissue inhibitor metalloproteinase (Fariss et al., 1997, 1998), advanced glycation end-products (Ishibashi et al., 1998), annexin II and annexin V (Rayborn et al., 2006), fibronectin (Pauleikhoff et al., 1992) and α- and αβ-crystallins (Crabb et al., 2002; Nakata et al., 2005). Hageman et al. noticed there seems to be little relevance between drusen morphological classification systems solely based on drusen size and appearance and molecular composition (Hageman et al., 2001). The assumption that motivated most of drusen composition studies is that molecular composition might provide crucial clues to the understanding of the aetiology of AMD and other drusen diseases that might be valuable in the quest for efficacious treatment. With the advent of proteomics, it became possible to both identify and quantify a considerably wider range of drusen constituents than was possible using immunohistochemical techniques. However despite this, the usefulness of this novel approach of investigation as applied to the study of drusen is hampered by the obvious limitation of its inherent inability to provide information pertinent to the spatial location of the identified protein both within drusen and in the context of Bruch’s membrane. This is further compounded by the practical difficulties of harvesting enough quantities of the drusen protein for analysis and ever-present danger of sample contamination. Crabb et al. developed a technique for the isolation of microgram quantities of drusen and Bruch’s membrane for proteomics (Crabb et al., 2002). Drusen material was collected using blunt forceps to scrap drusen prior to harvesting them by capillary pipettes. By performing proteomic analysis on drusen harvested from 18 normal donors, a total of 129 proteins were identified of which 16% were immunocytochemically localised into drusen. These results seem to agree well with the work by previous investigators using only
immunohistochemical techniques. Tissue metalloproteinase inhibitor-3, clusterin, vitronectin and serum albumin were found to be the most prevalent. While in AMD patient’s crystallins were fairly prevalent proteins. Surprisingly, they reported 65% of the drusen proteins were found in eyes from both patients and controls. Umeda et al. used proteomic analysis to study compositional protein differences between early- and late-onset AMD (Umeda et al., 2005). The study that applied the techniques of fundoscopic and histological examination of drusen in two types of Macaca fascicularis monkeys which have an early-onset variety of macular degeneration at around 2 years against late-onset macular degeneration age-matched controls. Immunohistochemistry and proteome analysis with liquid chromatography tandem mass spectroscopy revealed 60 proteins as constituents of drusen in monkeys with AMD which are also common in human drusen such as annexins, crystallins, immunoglobulins and complement components (Umeda et al., 2005). Drusen in both early and late onset varieties of AMD displayed immunoreactivities to apolipoprotein E, amyloid P component, complement C5, the terminal complement complex C5b-9, vitronectin and membrane cofactor protein. In humans, an increasing body of literature seems to implicate the immune system as a principal player in the events that lead to the genesis of drusen in the first place. Various investigators have reported the presence of antiretinal antibodies (Umeda et al., 2005), amyloid P component, apolipoprotein E, factor X, immunoglobulin lambda, MHC class II antigens and the terminal compliment component C5b-9 complex (Hageman et al., 2001b; Anderson et al., 2002).

1.6.4.3 Carbohydrates

Compared to lipids and proteins, carbohydrate components seem to have attracted much less attention by investigators. Farkas et al. demonstrated the presence of sialomucins which are composed of glycoporoteins associated to oligosaccharides via O-glycosidic linkages (Farkas et al., 1971). Kliffen et al. compared the total quantity of glycosaminoglycans in maculae with and without basal laminar deposits and reported that heparan sulphate was only expressed in maculae with age-related maculopathy and that basal laminar deposits stain positively for chondroitin-4-sulphate, heparin sulphate proteoglycans. The total amount of glycosa-
minoglycans was also significantly higher in maculae with basal laminar deposits (Kliffen et al., 1994, 1996). Mullins et al (1997) reported the detection of glyco-conjugates with termini containing glucose/mannose, N-acetylglucoseamine and sialic acid residues in both hard and soft drusen phenotypes (Mullins and Hageman, 1999). When sections of drusen were treated with the enzyme neuraminidase prior to labelling with Arachis hypogea agglutinin (PNA), distinct drusen sub-domains intensely stained showing cores ranging in size between 5 to 38mm in both soft and hard drusen (Mullins and Hageman, 1999). Only one such core was found per druse raising the possibility that these cores which are found deep into individual drusen but close to RPE might be linked to ontogeny as they might represent early nucleation sites (Hageman et al., 2001). Such a core is presumed to be the oldest part of the druse, it was laid down early in the process of drusen biogenesis. Hageman et al. postulated that formation of this carbohydrate core is an important event in the formation of drusen providing a nidus for the subsequent accumulation of material to follow, in the same way a grain of sand would form the core around which a pearl would grow (Hageman et al., 2001). Carbohydrate composition of drusen is poorly understood and does not seem to have attracted as much attention as other components of drusen. It remains to be seen whether carbohydrates play an important role in drusen biogenesis and indeed if that has any clinical significance.
1.7 Aims of the study

We identified a hitherto undescribed pattern new pattern of autofluorescence during the course of grading autofluorescence images of patients with MacTel type 2 at MEHRC. The patterns consist of focal hyperautofluorescent deposits (HADs) with distinct focal hyperautofluorescence in comparison to their immediate surrounding. We aimed at investigating HADs using two approaches; a specific clinical approach described in part III of the thesis. The clinical part is aimed at uncovering the extent of the phenomenon of HADs and providing a clear description of the nature of the HADs and elucidating the links between HADs and the clinical progression of MacTel type 2. In parallel, we aimed to investigate the general characteristics of autofluorescence of deposits using human cadaveric tissue. We embarked on a series of experiments aimed at defining the spectral characteristics of drusen’s autofluorescence heterogeneity and exploring the effects of zinc and trace metals in sub-RPE deposits in the modification of autofluorescence. We aimed at elucidating the role and effect of the accumulation of zinc and metals in the sub-RPE deposits on autofluorescence. The work described in this thesis is aimed at:

1. First we aimed to study the magnitude of this phenomenon by cross-sectionally examining the prevalence of HADs in MacTel type 2 patients.

2. Then we aimed at examining the patterns of topographical distribution of HADs in patients with MacTel type 2 in comparison with their family members, control subjects and patients with DM and AMD.

3. We aimed at providing a descriptive characterisation for HADs by using multi-modal imaging analysis to construct a clearer picture of HADs’ characteristics.

4. Longitudinally, we examine patterns of change of the HADs in MacTel type 2 patients over a period of 5 years.

5. We aimed at defining the spectral characteristics of drusen’s autofluorescence heterogeneity in comparison to that of the RPE-Bruch’s complex.
6. Finally, we aimed at exploring the effects of zinc and trace metals in sub-RPE deposits in modulating autofluorescence.
Part II

Autofluorescence and Metal Distribution in Drusen
Chapter 2

Spectral Profiling of Drusen

2.1 Overview

Purpose: The main objective of the study is the qualitative examination of the spectral properties of autofluorescence emission of the RPE-Bruch’s complex and drusen deposit with the aim of identifying unique spectral signatures associated with each component. The study also aims at the qualitative characterisation of quantitative differences in drusen’s autofluorescence intensities responsible for drusen’s autofluorescence heterogeneity by examining the properties of the emission spectra of cadaveric drusen with excitations at different wavelengths.

Methods: Unfixed drusen-containing ocular tissue from 7 donors was flat-mounted and examined with a confocal microscope. The tissue was excited at wavelengths of 364nm, 488nm, 543nm and 633nm. Emission spectra were collected with a spectrophotometric detector at 10nm interval windows between 400 and 700nm. Emission spectra were mathematically deconvoluted to their constituent peaks.

Results: Autofluorescence heterogeneity between drusen diminishes in inverse proportion to exciting wavelength; $\lambda_{exc}$ with 364nm laser leads to the excitation of the greatest number of fluorophores as compared with $\lambda_{exc}$ at 488nm and 543nm. The major emission peaks shared between all drusen were observed at 467nm and 551nm. Several drusen display internal autofluorescence heterogeneity evident by spherical inclusion bodies of several sizes exhibiting...
reduced autofluorescence intensity by up to 53% with $\lambda_{exc}$ at 364nm. These spherular profiles display all the emission peaks characteristic of the drusen in addition to peaks unique to spherules indicating presence of additional fluorophores. With $\lambda_{exc}$ at 364nm, the RPE layer has unique emission peaks towards the longer end of the emission spectrum starting at 532nm and longer. Emission spectra of drusen and Bruch’s membrane are similar and show emission peaks at the shorter end of the emission spectrum. However, unique emission peaks associated with Bruch’s membrane were observed at 443nm and 488nm. Drusen display a unique peak at 545nm.

Conclusion: Emission spectra of drusen and Bruch’s membrane show a distinct blue shift in comparison to the autofluorescence emission of the RPE emanating from its LF constituents. The overall relative contribution of Bruch’s membrane and drusen to fundus autofluorescence increases as the exciting wavelength decreases. The use of shorter lasers for excitation gives additional valuable information on drusen and Bruch’s membrane than would otherwise be available from singular excitation with 488nm. Exploration of the potential for such imaging could add an additional tool for the study of Bruch’s Bruch’s membrane and drusen both in vivo and in vitro and in the evaluation of animal models.
2.2 Literature Review

2.2.1 Introduction

The human retina-RPE-Bruch’s membrane interface is a structurally complex zone that consists of heterogeneous tissues with different light scattering properties. It contains chromophores that absorb light, as well as fluorophores, which absorb and re-emit light. Age-related changes as well as changes due to disease processes impact on the optical properties of tissues. When a fluorophore is stimulated by light, it becomes excited and thus light of varying waves is re-emitted and can then be measured. The molecular properties and the environment of the fluorophore determine the emission spectrum (Aslan et al., 2005). Changes in the photo-physical properties of the retinal layers could be employed to characterise the tissue and identify diseases using noninvasive methods. Here a review of the conceptual basis of fluorescence is outlined and a quick review of the spectral properties of the RPE-Bruch’s membrane complex is presented below.

2.2.2 Photoluminescence

Photoluminescence (PL) is the phenomenon when a substance absorbs photons and then re-radiates photons. Upon absorption the substance is excited to a higher energy state for a brief time period, emission occurs upon return to a lower energy state. Depending on the nature of the excited state, the process is categorically divided into fluorescence and phosphorescence.

2.2.3 Conceptual basis of autofluorescence

The conceptual foundations of fluorescence are illustrated in figure (2.1), which represents the energy levels of a fluorophore. These energy levels include the ground electronic state ($S_0$) and higher energy electronic states ($S_1$, $S_2$ etc.). The excited fluorophore vibrates at higher energy upon absorption of light. Each electronic state of a molecule is composed of numerous vibrational and rotational energy levels when a light quantum is absorbed by a fluorophore, the latter is usually excited to $S_1$ or $S_2$ or a higher vibrational state. In most of the cases, a
rapid relaxation process takes place and the molecule reverts to the lowest vibrational state in a non-radiative decay process (internal conversion). The exact vibrational and electronic level reached upon excitation, depends upon the energy content of the absorbed light. Transition from this state to the ground state may be accompanied by the emission of a photon in a process called fluorescence emission. Fluorescence lifetime is the time the molecule persists in this lowest level of the $S_1$ state. The Franck-Condon principle states that the transition between these vibrational states, occurs in 10-15s, and is complete by the time fluorescence emission occurs (Lakowicz, 2006). The nuclear geometry is not altered in during the course of the absorption and emission. In phosphorescence, spin conversion takes place in the molecule by which it spins into the first triplet state ($T_1$) in a process termed intersystem crossing. $T_1$ transition to the ground state is forbidden. (Aslan et al., 2005)

2.2.4 Characteristics of fluorescence

2.2.4.1 The Stokes Shift

When an excited fluorophore decays to the lower vibrational state ($S_0$), the resulting fluorescence occurs at lower energies and higher wavelengths. The difference between the exciting and emitted wavelengths, known, as the Stokes shift is a critical property as it completely prevents the interference of the exciting light with the emitted fluorescence.

2.2.4.2 Independence of the emission spectrum from the exciting light

The same fluorescence emission spectrum is observed independently of the exciting wavelength. When excited to a higher vibrational state, the fluorophore quickly descends to the lowest vibrational level of $S1$ accompanied by a release of the extra energy. This process of relaxation takes $10^{-12}$s and is independent of the exciting wavelength.
With ultraviolet or visible light, typical fluorophores are normally excited to higher the vibrational levels ($S_1$ or $S_2$) of the singlet energy state. In a typical fluorophore, a wide range of allowed transitions populating various vibrational energy levels of the excited states are generated as a result of irradiation with a host of wavelengths; these transitions when combined constitute the absorption spectrum of the fluorophore. Upon absorption of a photon, relaxation to the lowest vibrational energy level of the first excited state ($S_1$) takes place in a process known as internal conversion when accompanied by emission of light or vibrational relaxation in the absence of emitted light. The time frame for these processes is generally within a picosecond.

[Photocredit: (http://www.olympusmicro.com)]
2.2.4.3 Fluorescence Intensity

Fluorescence intensity of a fluorophore is dramatically affected by the biophysical microenvironment in the immediate niche of the fluorophore. Of the various effects on intensity, lifetime of the fluorophores, quantum yield, quenching and presence of metals; are quantitatively, the most important factors to consider (Geddes C.D. et al., 2003; Lakowicz et al., 2008).

2.2.4.4 Quantum yield and lifetime

Quantum yield and lifetime, collectively, determine the autofluorescence intensity of a fluorophore. Quantum yield is a mathematical expression denoting the number of photons emitted by a fluorophore relative to the number absorbed. Quantum yield of a fluorophore is controlled by two factors; the emissive rate of the fluorophore (Γ) and its rate of non-radiative decay (K_{nr}) (Lakowicz, 2006). Since quantum yield is a ratio, the process is governed by rate constants; Γ and Knr, both depopulate the excited state. Therefore, the quantum yield is given by the formula:

\[ Q = \frac{\Gamma}{1 + K_{nr}} \]  

(2.1)

Lifetime of a fluorophore is refers to the time available for the fluorophore to interact with or diffuse to its environment. Lifetime of the excited state is dictated by the average time the molecule spends in the excited state prior to its return to the ground state. Generally, fluorescence lifetime is very short, around 10ns. It is the reverse of the sum of the two rates and is expressed as follows:

\[ \tau = \frac{1}{1 + K_{nr}} \]  

(2.2)

2.2.4.5 Quenching

Is the phenomenon of reduction of the fluorescence intensity of a fluorophore. A number of processes could result in quenching of the fluorescence of a fluorophore upon an encounter
with a quencher. Static quenching occurs when a fluorophore forms a non-fluorescent complex with a deactivating molecule called a quencher at the ground state. Other forms of quenching have been described (Albani, 2008).

### 2.2.4.6 Fluorescence Measurement

Measurement can broadly be categorised into steady state and time-resolved fluorescent measurements. In steady state, the specimen is continuously illuminated with light while intensity is recorded. Time resolved approaches, measure the decay in fluorescence intensity or anisotropy. The principle is the exposure of the fluorophore to a light pulse whose width is shorter than the fluorophore’s decay time. The time frame for the process is typically in nanoseconds. Steady-state fluorescence measurements represent averages of the time-resolved phenomenon over the intensity decay. Steady-state measurements are straightforward, however, molecular information is lost during the averaging process. Time-resolved measurements on the other hand, give much more information; anisotropy, macromolecular shape, conformational state and reveals the nature of quenching. However, much more complex instrumentation is required (Lakowicz, 2006).

### 2.2.5 Effects of Metals on Autofluorescence

By virtue of physical proximity to fluorophores, metals are uniquely suited to modify fluorescence. Physical proximity of fluorophores to metals results in dramatic modifications in the intrinsic photo-physical characteristics of the fluorophores in predictable ways (Aslan et al., 2005).

#### 2.2.5.1 The lightening Effect

Amplification in the rates of excitation of the fluorophore can arise as result of an interaction between the incident light and the freely mobile electrons in the metals, this could result in up to 20,000-fold amplification of the local intensity and rate of excitation (Geddes C.D. et al., 2003).
2.2.5.2 Alteration of radiative decay rates

The interaction between metals and fluorophores is a complex process influenced by the size and shape of the metal and distance of interaction (Geddes C.D. et al., 2003; Aslan et al., 2005). The interaction of plasmon electrons (free electrons in metals) metals with excited-state fluorophores, could result in quenching or amplification of the quantum yield. Quenching occurs as a result of interaction at short distances (0–5 nm), spatial variation of the incident light field (0–15 nm), and changes in the radiative decay rates (0–20 nm) (Aslan et al., 2005). Presence of a nearby metal (m) increases the radiative rate by adding a new rate \( \Gamma m \). The quantum yield (Qm) and the lifetime of the fluorophore (\( \tau m \)) change as predicted by the formulae:

\[
Qm = \frac{\Gamma + \Gamma m}{\Gamma + \Gamma m + Knr}
\]

\[
\tau m = \frac{1}{\Gamma + \Gamma m + Knr}
\]

Metals have dramatic effects on fluorescence; Geddes et al. summarised the effects of metals on fluorescence in figure (2.2). It was estimated that fluorescence could be enhanced by a factor of one million as a result of presence of metals (Geddes C.D. et al., 2003).

2.2.6 Fluorophores in the RPE-Bruch’s Membrane Complex

2.2.6.1 Lipofuscin

Each RPE cell which performs a whole range of functions critical to photoreceptors (Strauss, 2005) is apposed to 30-50 photoreceptor outer segments (POS). 10-15% of the outer segment of each photoreceptor is daily phagocytosed by RPE cell layer (Young and Bok, 1969; Newsome et al., 1987). This continuous processing of the shed (POS) places huge metabolic demands on RPE cells. As a function of age, incomplete digestion of (POS) leads to progressive accumulation of the complex auto-fluorescent lipid-protein aggregates known generically as lipofuscin.
(Sparrow, 2007). Because RPE cells lack the machinery to either degrade or export LF to a significant degree, the accumulation is progressive and leads to a variety of effects (Lakkaraju et al., 2007). Lipofuscin is believed to be the major fluorophore in the fundus that gives rise to the autofluorescence signal. LF is composed of 10 different autofluorescent fragments. All display common absorption peaks between 280nm to 330nm and on the basis of their excitation-emission properties, fall into 4 categories. In table (2.1) Kennedy et al. summarised the excitation and emission properties of the different components of LF (Kennedy et al., 1995). The major fluorophore in LF is believed to be the amphoteric quaternary amine A2E that arises as an Schiff-base reaction product of ethanolamine and retinaldehyde as part of the normal physiologic photochemical visual event.
Figure 2.3 – Autofluorescence at the RPE-Bruch’s membrane complex and drusen

A 0.7µm thick semi-thin section of the RPE-Bruch’s complex embedded in LR White resin imaged with autofluorescence with $\lambda_{exc}$ at 488nm. The collagen component of Bruch’s membrane and the choroid vessels is highly autofluorescent. The lipofuscin in RPE show much less autofluorescent at this laser excitation. Drusen show a range of autofluorescence intensities, some are more autofluorescent than others.
Table 2.1 – Fluorophores present in extracts of RPE cell lipofuscin

<table>
<thead>
<tr>
<th>Fluorophore Emitting Fraction</th>
<th>Excitation peak (s) nm</th>
<th>Emission peak (s) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 green</td>
<td>330</td>
<td>520</td>
</tr>
<tr>
<td>3 yellow/green</td>
<td>280/330</td>
<td>568</td>
</tr>
<tr>
<td>1 golden-yellow</td>
<td>280/330</td>
<td>585</td>
</tr>
<tr>
<td>4 orange/red</td>
<td>285, 335, 420</td>
<td>605, 633, 670</td>
</tr>
</tbody>
</table>

2.2.6.2 Bruch’s Membrane

While it is known the principal source from which most of the clinically-observed fundus autofluorescence emanating as a result of $\lambda_{exc}$ with 488nm is due to lipofuscin, evidence indicates a significant contribution from Bruch’s membrane and drusen deposits (Rückmann et al., 1997; Marmorstein et al., 2002). Collagen type IV is normally thin over the RPE basement membrane but thicker over basement membrane of the choriocapillaris (Marshall et al., 1992) has a distinct autofluorescent activity. Bachmann et al. reviewed fluorophores in human hard and soft tissues, reported that the emission and excitation maxima for collagen cross-links are 335/370 and 390/460 respectively. Cross-linking of elastin to collagen results in a spectral right shift (Bachmann et al., 2006). The excitation and emission maxima for the elastin/collagen cross-links are 420/460 and 500/540 respectively. Precise knowledge of the spectral properties associated with the major fluorophore-containing retinal layers is essential of retinal diseases and could give new insights in the interpretation of AF imaging in the clinical settings.

2.2.6.3 Drusen

Some researchers attributed the spectral signatures of drusen to their content of LF. Gouras et al. postulated that autofluorescent drusen contain LF granules that originated from overlaying RPE cells (Gouras et al., 2008). Delori et al. reported similar results were reported in vivo studies on AMD (Delori et al., 2000). Lengyel and colleagues associated hyper-autofluorescent drusen with the lateral walls of the inter-capillary pillars of the choriocapillaris (Lengyel, 2004). However, there is a lack of clarity on the component fluorophores in the drusen deposits and the RPE-Photoreceptor complex. Stargaadt’s type 1 (STGD1), is a disease characterised by
presence of intensely autofluorescent deposits in the retina manifest on ophthalmoscopy. A study of these drusen-like structures demonstrated their content of large amounts of LF and A2E (Cideciyan, 2004). Marmorstein et al. reported in AMD patients with drusen there is a blue shift in fundus autofluorescence (Marmorstein, et al., 2002). This was attributed to the presence of a novel fluorophore independent of LP. However, no information exists on the nature of this hypothetical fluorophore and if all drusen’s autofluorescence is due to this novel fluorophore (s).
CHAPTER 2. SPECTRAL PROFILING OF DRUSEN

2.3 Methods

2.3.1 Tissue

Seven eyes with drusen from human donors were acquired through the tissue bank of Moorfields eye Hospital. Consent for research was obtained and the tissue was handled in accordance with the tenants of the Declaration of Helsinki. The eyes were enucleated between 4-12 hours post-mortem and kept in a physiologic solution at 4°C until the time of dissection.

2.3.2 Tissue Dissection

Using a surgical skin grafting knife, the globes were circumferentially cut just distal to the ora serrata. Lenses and the iris along with the vitreous were then removed and sent for safe disposal in accordance with the regulations of the department of pathology at UCL Institute of Ophthalmology. The Retina-RPE-Bruch-Choroid’s complex will then be dissected from the sclera using the blunt end of a dissecting forceps. The Retina-RPE-Bruch-Choroid’s complex is then transferred and flattened in a Petri dish containing 70% glycerol solution and left overnight. Using a blunt dissection technique, the neurosensory retina (NS) was removed and safely disposed off as described above. The macular area of the RPE-Bruch’s membrane complex was dissected and flattened in a Superfrost glass slide, overspilled and sealed off using nail polish remover.

2.3.3 Confocal Microscopy

An Inverted Zeiss 510 Axiovert motorised microscope equipped with an 8-channel spectrophotometric detector (META UV-vis) was used. Lambda scans were carried out using 364, 488, 543 and 633nm laser lines. The detector exhibited sensitivity in the range between 400-700nm. The emission spectrum was not affected by filter characteristics, as detectors did not depend on the use of filters. The detectors were set to advance in increments of ten from a wavelength longer than the exciting laser until 700nm. A plane lambda-scan was carried out (scaling was X 0.20μm, y 0.20μm) and a Plan-Apochromat 63x/1.4 oil objective with a zoom of 0.7 with
an open pinhole was used. The exciting laser line was powered to T2 16.9%, 9.9%, 74.3% and 86.7% for the 364, 488, 543 and 633nm laser lines respectively. Gains were individually optimised for each scan and were not altered during the course of the scan. Data were obtained in LSM files and was read on Zeiss LSM-5 Image Browser software. Drusen average pixel intensities were plotted off-line using the circle drawing mode of the function “mean of ROI display mode” for the emission associated with each excitation. Data were the exported as Excel files.

2.3.4 Data Analysis & Statistics

Spectral emission curves were deconvoluted into their sharp component peaks using PeakFit software (Sigmaplot Inc). A Fourier deconvolution/ filtering algorithm, which depends on a Gaussian response, was applied for the deconvolution process. Hidden peaks representing local maxima were revealed. The average pixel intensities for drusen autofluorescence were determined using the grey-scale XY image function in the Zeiss LSM-5 Image Browser software. Autofluorescence numerical values were obtained from the image in 8-bit format using Image J software (ImageJ version 1.44p, LIH, USA).
2.4 Results

2.4.1 The Spectral Properties of Drusen

In order to determine whether the quantitative differences in drusen autofluorescence intensities are associated with qualitative differences in spectral emission, we performed a deconvolution on the emission spectrum. The peaks composing the emission spectra of a large number of drusen were identified and compared. Secondly, the percentage contribution of each distinct peak to the overall drusen autofluorescence was determined by the areas under the curve (AUCs) of the peak. Thirdly, the regression of the AUC of the different peaks and drusen’s overall autofluorescence was determined by the $R^2$ ratio to reveal which of these peaks account for the difference in autofluorescence intensities between the different drusen.

Figure (2.4) shows a representative optical cross-section of drusen. The drusen were excited with laser light of 4 different wavelengths; $\lambda_{\text{exc}}$ 364nm, 488nm, 543nm and 633nm. Drusen display a wide range of autofluorescence heterogeneity when excited with lasers of different wavelengths. Different measurements were taken to demonstrate the autofluorescence heterogeneity of drusen at these wavelengths. The measures are summarised in figure (2.5a) and table (2.5b). The highest variation in drusen’s emission autofluorescence was observed with $\lambda_{\text{exc}}$ at 364nm, which showed a coefficient of variation value (CV) amongst drusen of 44%. The second highest drusen autofluorescence variability in relation to the excitation wavelength was observed at $\lambda_{\text{exc}}$ with 543nm for which the (CV) value was 28%. At $\lambda_{\text{exc}}$ with 488nm CV value was 26%. The narrowest variability in autofluorescence emission amongst drusen was noticed with $\lambda_{\text{exc}}$ at 633nm where CV value was 19%.

Figure (2.7) depicts the averaged emission spectra with $\lambda_{\text{exc}}$ at 364nm. The deconvoluted emission spectrum was dominated by the 465nm and 510nm peaks, which represent 35% and 30% of the total emission spectrum’s AUC respectively. There were two other minor contributions from peaks at 582nm (23%) and 547nm (12%).

Figure (2.8) illustrates the correlation coefficient ($R^2$) between the AUCs of the peaks of which drusen’s emission spectrum with $\lambda_{\text{exc}}$ at 364nm is composed. $R^2$ value for the
association between the 510nm emission peak and drusen’s mean autofluorescence intensity was the strongest at 0.8 followed by the 465nm peak ($R^2 = 0.75$). The $R^2$ value for the 582nm and 547nm were 0.6 and 0.05 respectively.

In figure (2.9) at $\lambda_{exc}$ with 488nm, the 554nm peak represented 36% of the AUC of the emission spectrum. Other peaks at 591nm, 525nm and 637nm represented 32%, 21% and 11% of the total AUC for the emission spectrum. In figure (2.10) The $R^2$ value for the association between drusen’s mean autofluorescence intensity and the AUC of the emission peaks at excitation with 488nm was highest for 591nm (0.87) followed by 637nm (0.85), 554nm (0.79) and 525nm (0.11).

Figure (2.10) shows drusen emission with $\lambda_{exc}$ at 543nm. 38% of drusen gross autofluorescence with $\lambda_{exc}$ 543nm is attributed to a peak at 619nm. Secondly, the 599nm peak (29%) followed by 579nm and 649nm peaks contribute the remainder of the drusen autofluorescence at 11% and 21% respectively.

In figure (2.12), With $\lambda_{exc}$ at 543nm, the peaks composing the emission spectrum show similar correlation coefficients with overall autofluorescence. $R^2$ value or the 616nm, 650nm were 0.66 and 0.56 respectively followed by the peak at 578nm (0.46). The smallest correlation was exhibited the peak whose centroid is 599nm (0.06). The autofluorescence’s spectrum emission peaks of drusen with $\lambda_{exc}$ at 364nm, 488nm and 543nm are summarised in figure (2.13). $\lambda_{exc}$ with 364nm laser leads to the excitation of the greatest number of fluorophores as compared with $\lambda_{exc}$ at 488nm and 543nm. It demonstrates that the emission peaks observed at 467nm ± 2, 507nm ± 6, and 551nm ± 4 and 615 ± 4 are the major peaks common between all the drusen scanned. Other emission peaks are present which are not common to all drusen. When excited with 488nm laser light, drusen show distinct fluorescence peaks at 525nm ± 2, 637nm ± 4, 554nm ± 3 and 561nm ± 3. With $\lambda_{exc}$ at 543nm, the peaks at 576nm, 599nm and 619nm show an overlap with peaks observed with $\lambda_{exc}$ at 354nm and 488nm. There was a unique emission peak at 649nm.

Table (2.3) summarises the identified peaks in the drusen emission spectra for the $\lambda_{exc}$ at 364nm, 488nm, 543nm and 633nm. With $\lambda_{exc}$ at 633nm, drusen showed a single emission
Figure 2.4 – An autofluorescence optical cross-section of drusen

A representative autofluorescence optical cross-section showing drusen with (clock-wise rom top right) $\lambda_{exc}$ at 364nm, 488nm, 543nm and 633nm. Drusen display a wide range of autofluorescence heterogeneity when excited with lasers of different wavelengths.

peak with a centroid at 704nm. As the $\lambda_{exc}$ wave becomes longer from 364nm to 633nm, the spectral full width at half maximum (FWHM) of the component peaks become progressively shorter.
Figure 2.5 – Drusen Autofluorescence Heterogeneity

Figures A & B: Illustrate mean drusen autofluorescence intensity with $\lambda_{\text{exc}}$ at 364nm, 488nm, and 543nm and 633nm. The highest variation in drusen’s emission autofluorescence was observed with $\lambda_{\text{exc}}$ at 364nm, which showed a coefficient of variation value (CV) amongst drusen of 44%. The second highest drusen autofluorescence variability in relation to the excitation wavelength was observed at $\lambda_{\text{exc}}$ with 543nm or which the (CV) value was 28%. At $\lambda_{\text{exc}}$ with 488nm CV value was 26%. The narrowest variability in autofluorescence was noticed with $\lambda_{\text{exc}}$ at 633nm where CV value was 19%.
Figure 2.6 – A series of XY images of a representative specimen showing emission spectra with $\lambda_{exc}$ at 364nm
Figure 2.7 – Drusen emission with $\lambda_{exc}$ at 364nm

The figure depicts the averaged emission spectrum with $\lambda_{exc}$ 364nm. The deconvoluted emission spectrum was dominated by the 465nm and 510nm peaks, which represent 35% and 30% of the emission spectrum’s total AUC respectively. There were two other smaller contributions from peaks at 582nm (23%) and 547nm (12%).
Figure 2.8 – Association of emission peaks at $\lambda_{\text{exc}}$ with 364nm with mean drusen’s autofluorescence intensity

The figure shows the correlation coefficient between the peaks AUCs and drusen gross mean autofluorescence intensity with $\lambda_{\text{exc}}$ at 364nm. The correlation coefficient ($R^2$) value for the association between the 510nm emission peak and drusen’s mean autofluorescence intensity was the strongest at 0.8 followed by the 465nm peak ($R^2 = 0.75$). The $R^2$ value for the 582nm and 547nm were 0.6 and 0.05 respectively.
The figure shows drusen emission peaks associated with $\lambda_{exc}$ at 488nm. The 554nm peak represented 36% of the AUC of the emission spectrum. Other peaks at 561nm, 525nm and 637nm represented 32%, 21% and 11% of the total AUC of the emission spectrum.
Figure 2.10 – Association of emission peaks at $\lambda_{exc}$ with 488nm with mean drusen’s autofluorescence intensity

The $R^2$ value for the association between drusen’s mean autofluorescence intensity and the AUC of the emission peaks at excitation with 488nm was highest for 591nm (0.87) followed by 637nm (0.85), 554nm (0.79) and 525nm (0.11).
Figure 2.11 – Drusen emission with $\lambda_{exc}$ at 543nm

The figure shows 38% of drusen gross autofluorescence with $\lambda_{exc}$ 543nm is attributed to a peak at 619nm. Secondly, the 599nm peak (29%) followed by 579nm and 649nm peaks contribute the remainder of the drusen autofluorescence at 11% and 21% respectively.
Figure 2.12 – Association of emission peaks at $\lambda_{exc}$ with 543nm with mean drusen’s autofluorescence intensity

With $\lambda_{exc}$ at 543nm, the peaks composing the emission spectrum show similar correlation coefficients with overall autofluorescence. $R^2$ value or the 616nm, 650nm were 0.66 and 0.56 respectively followed by the peak at 578nm (0.46). The smallest correlation was exhibited the peak whose centroid is 599nm (0.06).
Figure 2.13 – Autofluorescence emission peaks of drusen with λ_{exc} at 364nm, 488nm and 543nm

λ_{exc} with 364nm laser leads to the excitation of the greatest number of fluorophores as compared with λ_{exc} at 488nm and 543nm. It demonstrates that the emission peaks observed at 467nm ± 2, 507nm ± 6, and 551nm ± 4 and 615 ± 4 are the major peaks common between all the drusen scanned. Other emission peaks are present which are not common to all drusen. When excited with 488nm laser light, drusen show distinct autofluorescence peaks at 525nm ± 2, 637nm ± 4, 554nm ± 3 and 561nm ± 3. With λ_{exc} at 543nm, the peaks at 576nm, 599nm and 619nm show an overlap with peaks observed with λ_{exc} at 354nm and 488nm. There was a unique emission peak at 649nm.
Table 2.2 – Summary of drusen’s emission peaks

<table>
<thead>
<tr>
<th>$\lambda_{exc}$</th>
<th>Centroid (nm)</th>
<th>FWHM</th>
<th>Amplitude</th>
<th>AUC</th>
<th>% Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>364</td>
<td>465</td>
<td>53</td>
<td>2045</td>
<td>114850</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>510</td>
<td>46</td>
<td>1994</td>
<td>97018</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>547</td>
<td>39</td>
<td>955</td>
<td>39893</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>582</td>
<td>70</td>
<td>1008</td>
<td>75648</td>
<td>23</td>
</tr>
<tr>
<td>488</td>
<td>525</td>
<td>23</td>
<td>1322</td>
<td>32450</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>554</td>
<td>35</td>
<td>1442</td>
<td>53900</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>591</td>
<td>45</td>
<td>1001</td>
<td>47687</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>637</td>
<td>45</td>
<td>358</td>
<td>17259</td>
<td>11</td>
</tr>
<tr>
<td>543</td>
<td>578</td>
<td>12</td>
<td>235</td>
<td>3065</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>17</td>
<td>443</td>
<td>7882</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>616</td>
<td>20</td>
<td>479</td>
<td>10178</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>650</td>
<td>31</td>
<td>169</td>
<td>5627</td>
<td>21</td>
</tr>
<tr>
<td>633</td>
<td>704</td>
<td>20</td>
<td>35</td>
<td>741</td>
<td>100</td>
</tr>
</tbody>
</table>

The table above summarises the identified peaks in the drusen emission spectra or the $\lambda_{exc}$ at 364nm, 488nm, 543nm and 633nm. With $\lambda_{exc}$ at 633nm, drusen showed a single emission peak with a centroid at 704nm. As the $\lambda_{exc}$ wave becomes longer from 364nm to 633nm, the spectral full width at half maxima (FWHM) of the component peaks become progressively shorter.
2.4.2 Spectral Properties of the Spherical Inclusion Bodies in Drusen

Figure (2.14) shows an optical cross-section of a druse taken with $\lambda_{\text{exc}}$ at 488nm with. The image shows numerous dark spherical profiles in one druse. The spherical profiles exhibit a wide variation in size. In figure (2.15) 3 optical cross-sections of a druse with dark inclusion bodies. The cross-sections A, B and C were taken with $\lambda_{\text{exc}}$ at 364nm, 543nm and 633nm. Autofluorescence emission from one such spherical inclusion was compared with druse’s background autofluorescence (both indicated with arrows in A). The intensity of the autofluorescence of the spherical inclusion profile was 53%, 41% and 45% of that of the indicated area in the drusen with $\lambda_{\text{exc}}$ at 364nm, 543nm and 633nm respectively. In figure (2.15), the emission peaks of the spherical inclusion bodies are compared to drusen’s background autofluorescence. The emission spectrum of the spherical inclusion bodies when compared to drusen’s background shows all the peaks present in the drusen’s background and additional peaks with all excitation wavelengths. This indicates the presence of additional fluorophores in the spherical inclusion bodies. With $\lambda_{\text{exc}}$ at 364nm and 488nm, the spherical inclusion body displays all the background peaks in the drusen in addition to a peak at 491nm. With $\lambda_{\text{exc}}$ at 543nm, 3 unique peaks are displayed by the spherical inclusion body at 607nm, 662nm and 699nm.
Figure 2.14 – A druse exhibiting numerous dark inclusion bodies

An optical-cross section of drusen with excitation at 488nm. Some drusen exhibit numerous spherical inclusion bodies that show markedly decreased autofluorescence intensity as compared to their immediate vicinity.

2.4.3 Spectral Properties of RPE, Bruch’s Membrane & Drusen

Figure (2.17) shows emission peaks of RPE, Bruch’s membrane and drusen with $\lambda_{exc}$ 364nm. The AUC of drusen’s autofluorescence emission is 25% and significantly larger than the AUC of RPE emission spectrum, it is also 19% larger than that of Bruch’s membrane although the difference did not attain statistical significance. RPE layer showed 8 different emission peaks at 506nm, 531nm, 553nm, 574nm, 593nm, 617nm, 640nm and 662nm. There is no clear
Several drusen display autofluorescence heterogeneity. There are spherical inclusion bodies of several sizes, which display reduced autofluorescence intensity compared to the surrounding. Images A, B and C are optical cross-sections of drusen with $\lambda_{\text{exc}}$ at 364nm, 543nm and 633nm. Autofluorescence emission from one such spherical inclusion was compared with druse's background autofluorescence (both indicated with arrows in A). The intensity of the autofluorescence of the spherical inclusion profile was 53%, 41% and 45% of that of the indicated area in the drusen with $\lambda_{\text{exc}}$ at 364nm, 543nm and 633nm respectively.
Figure 2.16 – Emission peaks of drusen’s background autofluorescence and spherical inclusions

The autofluorescence emission of the spherical inclusion bodies when compared to drusen’s background, show additional peaks with all excitation wavelengths. This indicates the presence of additional fluorophores in the spherical inclusion bodies. With $\lambda_{exc}$ at 364nm and 488nm, the spherical inclusion bodies display all the background peaks in the drusen in addition to a peak at 491nm. With lambda excitation at 543nm, 3 unique peaks are displayed by the spherical inclusion body at 607nm, 662nm and 699nm.
The autofluorescence emission of the spherical inclusion bodies when compared to drusen’s background, show additional peaks with all excitation wavelengths. This indicates the presence of additional fluorophores in the spherical inclusion bodies. With $\lambda_{exc}$ at 364nm and 488nm, the spherical inclusion body displays all the background peaks in the drusen in addition to a peak at 491nm. With $\lambda_{exc}$ at 543nm, 3 unique peaks are displayed by the spherical inclusion body at 607nm, 662nm and 699nm.
domination for one particular peak in the spectrum. In drusen, the peak 465nm contributes 35% of the overall autofluorescence emission. The 510 (30%) and the peaks 547nm and 542 account for 12 and 23% of the overall autofluorescence AUC respectively. In Bruch’s membrane, the largest contribution to the overall autofluorescence comes from a peak at 554nm (27%) followed by the 465nm peak (23%). The rest of the autofluorescence is due to peaks at 488nm (18%), 513nm (23%) and 9% from the peak at 443nm. Drusen and Bruch’s membrane autofluorescence emission with 364nm is characteristically blue-shifted and both have the same $\lambda_{max}$ at 364nm. RPE layer’s $\lambda_{max}$ emission is at 573nm.

Figure (2.18), illustrates emission peaks of RPE, Bruch’s membrane and drusen with $\lambda_{exc}$ at 488nm. The AUC of the emission spectrum of the RPE layer is significantly larger than that of Bruch’s membrane. Drusen show emission peaks at 525nm, 554nm, 591nm and 637nm, which account for 36%, 24%, 18% and 6% of the AUC of the emission spectrum. Bruch’s membrane emission spectrum is composed of identifiable peaks at 526nm (26%), 554nm (30%), 634 (6%) and the unique peaks at 585nm (29%) and 616nm (9%). The RPE emission spectrum is shifted towards the red as compared to Bruch’s membrane and drusen. The AUC of the RPE emission spectrum is composed of peaks at: 554nm (36%) and 593nm (24%). In addition to smaller contributions from 617nm (16%), 643nm (18%) and 6% is accounted for by a peak at 764nm. Both drusen and Bruch’s membrane have $\lambda_{max}$ at 554nm while the $\lambda_{max}$ for RPE layer was noticed at 597nm.

Figure (2.19 shows the RPE, Bruch’s membrane and drusen emission peaks at 543nm excitation. The RPE emission dominates the collective emission from the RPE-Bruch’s complex and drusen. RPE emission AUC is 308% and 233% and significantly larger than that of Bruch's membrane and drusen respectively. The RPE emission AUC is dominated by a single fluorophore whose emission peak is 615nm accounting for 70% of the spectrum’s AUC. Smaller contributions are due to peaks at 660nm (14%) and 679nm (16%). Drusen display peaks at 579nm (12%), 599nm (29), 619nm (38%) and 549nm (21%). Bruch’s membrane emission spectrum is dominated by the 599nm and 619nm peaks, which account for 33% and 41% of the total emission spectrum AUC respectively. Minor contributions are due to peaks at 575nm
(6%), 607nm (5%), 637nm (6%) and 653nm (9%). $\lambda_{exc}$ values for drusen, Bruch’s membrane and RPE layer with $\lambda_{exc}$ at 543nm, were at 596nm, 554nm and 618nm respectively.

Figure (2.20) shows the RPE, Bruch’s membrane and drusen emission peaks at 633nm excitation. The RPE emission dominates the collective emission from the RPE-Bruch’s complex and drusen. RPE emission AUC is significantly larger than that of Bruch’s membrane and drusen respectively.

Figure (2.21): Summarises the RPE-Bruch’s complex and drusen emission spectra with $\lambda_{exc}$ at 364nm, 488nm, and 543nm and 633nm. With $\lambda_{exc}$ at 364nm, RPE layer has unique emission peaks towards the longer end of the emission spectrum at; 532nm ± 1, 576nm ± 3, 596nm ± 4, 618nm ± 1, 634nm ± 2 and 661nm. Drusen and Bruch’s membrane show emission peaks at the shorter end of the emission spectrum. Unique emission peaks associated with Bruch’s membrane were observed at 443nm and 488nm ± 1. Drusen display a unique peak at 545nm ± 3. A peak at 510nm ± 3 was common and its AUC is significantly different between RPE, Bruch’s membrane and drusen. With $\lambda_{exc}$ at 488nm, RPE, Bruch’s membrane and drusen share common emission peaks at 554nm and 634nm ± 2. The AUC of the former is significantly different different between RPE and drusen while the AUC of the latter is significantly different between RPE and Bruch’s membrane. Bruch’s membrane displayed a unique peak at 584nm ± 2. At excitation with 543nm laser light, The RPE layer has a unique emission peak at 612nm ± 2 and 682nm ± 2. Bruch’s membrane has a unique emission peak at 646nm ± 5, while drusen displayed a unique peak at 650nm.
The figure shows emission peaks of RPE, Bruch’s membrane and drusen with $\lambda_{exc}$ 364nm. The AUC of drusen’s autofluorescence emission is 19% larger than that of Bruch’s membrane and 25% significantly larger than the AUC of RPE emission spectrum. RPE layer showed 8 different emission peaks at 506nm, 531nm, 553nm, 574nm, 593nm, 617nm, 640nm and 662nm. There is no clear domination of one particular peak in the spectrum. In drusen, the peak 465nm contributes 35% of the overall autofluorescence emission. The 510 (30%) and the peaks 547nm and 542 account for 12 and 23% of the overall autofluorescence AUC respectively. In Bruch’s membrane, the largest contribution to the overall autofluorescence comes from a peak at 554nm (27%) followed by the 465nm peak (23%). The rest of the autofluorescence is due to peaks at 488nm (18%), 513nm (23%) and 9% from the peak at 443nm.
The AUC of the emission spectrum of the RPE layer is significantly larger than that of Bruch’s membrane. Drusen show emission peaks at 525nm, 554nm, 591nm and 637nm, which account for 36%, 24%, 18% and 6% of the AUC of the emission spectrum. Bruch’s membrane emission spectrum is composed of identifiable peaks at 526nm (26%), 554nm (30%), 634nm (6%) and the unique peaks at 585nm (29%) and 616nm (9%). The RPE emission spectrum is shifted to the right as compared to Bruch’s membrane and drusen. The AUC of the RPE emission spectrum is composed of peaks at: 554nm (36%) and 593nm (24%). In addition to smaller contributions from 617nm (16%), 643nm (18%) and 6% is accounted for by a peak at 764nm.
Figure 2.20 – Emission spectra of RPE, Bruch’s membrane and drusen with $\lambda_{exc}$ 543nm

The figure shows the RPE, Bruch’s membrane and drusen emission peaks at $\lambda_{exc}$ 543nm excitation. The RPE emission dominates the collective emission from the RPE-Bruch’s complex and drusen. RPE emission AUC is 308% and 233% and significantly larger than that of Bruch’s membrane and drusen respectively. The RPE emission AUC is dominated by a single fluorophore whose emission peak is 615nm accounting for 70% of the spectrum’s AUC. Smaller contributions are due to peaks at 660nm (14%) and 679nm (16%). Drusen display peaks at 579nm (12%), 599nm (29), 619nm (38%) and 549nm (21%). Bruch’s membrane emission spectrum is dominated by the 599nm and 619nm peaks, which account or 33% and 41% of the total emission spectrum AUC respectively. Minor contributions are due to peaks at 575nm (6%), 607nm (5%), 637nm (6%) and 653nm (9%).
Figure 2.21 – Emission spectra of RPE, Bruch’s membrane and drusen with $\lambda_{exc}$ 543nm

The figure shows the RPE, Bruch’s membrane and drusen emission peaks at At 633nm excitation. The RPE emission dominates the collective emission from the RPE-Bruch’s complex and drusen. RPE emission AUC is significantly larger than that of Bruch’s membrane and drusen respectively.
CHAPTER 2. SPECTRAL PROFILING OF DRUSEN

Figure 2.22 – Autofluorescence emission o the RPE-Bruch’s Complex and drusen with $\lambda_{exc}$ at 364nm, 488nm and 543nm

With $\lambda_{exc}$ at 364nm, RPE layer has unique emission peaks towards the longer end of the emission spectrum at; 532nm ± 1, 576nm ± 3, 596nm ± 4, 618nm ± 1, 634nm ± 2 and 661nm. Drusen and Bruch’s membrane show emission peaks at the shorter end of the emission spectrum. Unique emission peaks associated with Bruch’s membrane were observed at 443nm and 488nm ± 1. Drusen display a unique peak at 545nm ± 3. A peak at 510nm ± 3 was common and its AUC is significantly different between RPE, Bruch’s membrane and drusen.

With $\lambda_{exc}$ at 488nm, RPE, Bruch’s membrane and drusen share common emission peaks at 554nm and 634nm ± 2. The AUC of the former is significantly different between RPE and drusen while the AUC of the latter is significantly different between RPE and Bruch’s membrane. Bruch’s membrane displayed a unique peak at 584nm ± 2. At excitation with 543nm laser light, The RPE layer has a unique emission peak at 612nm ± 2 and 682nm ± 2. Bruch’s membrane has a unique emission peak at 646nm ± 5, while drusen displayed a unique peak at 650nm.
2.5 Discussion

2.5.1 Spectral Properties of Drusen

Fluorescence intensity may change depending on a number of factors including the amount, configuration and the biophysical environment of a fluorophore. Whilst changes in the latter two may affect the intensity of autofluorescence, they might not be reflected in the structure of the spectral emission (Fukushima et al., 1987). The aim of this chapter was:

1. To provide a descriptive characterisation of drusen’s autofluorescence heterogeneity quantitatively by describing the magnitude of differences in autofluorescence manifest with different excitation wavelengths.

2. To identify the common and the unique components of drusen’s emission spectrum and in order to study the relationship of each to the overall drusen’s autofluorescence emission.

With $\lambda_{exc}$ at 364nm, drusen display the highest degree of heterogeneity in autofluorescence emission intensity. This variability in autofluorescence intensity diminishes as a function of the increase in the wavelength of the exciting laser as less and less fluorophores are excited. Autofluorescence heterogeneity is lowest with $\lambda_{exc}$ at 633nm laser. Fluorophore peaks at 465nm and 510nm, which are common between all drusen studied, dominate Drusen’s emission at $\lambda_{exc}$ with 364nm. Quantitative differences in drusen’s content of these two components are responsible for most of the manifest heterogeneity in drusen’s emission at this excitation. The fluorophore whose $\lambda_{em}$ peak is at 510nm accounts for 30% of drusen’s autofluorescence and quantitative differences in drusen’s content of this fluorophore is strongly associated with drusen’s autofluorescence heterogeneity. This peak is common with the RPE and Bruch’s membrane as well. We endeavour to suggest here, that this peak arises from one or both the green-emitting fractions of lipofuscin. The $\lambda_{exc}$ Max and $\lambda_{em}$ Max of the green-emitting fractions of lipofuscin are 330nm and 520nm (Kennedy et al., 1995). This fits very well with our observed data making this the most likely explanation for the 510nm peak. Another peak at 585nm could also be attributed to the golden-yellow emitting fraction of LF (Kennedy et al., 1995). It is very likely that some of the drusen’s fluorophores including LF originated in the
RPE and were transferred to drusen via cytoplasmic conduits (Ishibashi et al., 1986). However, the lack of identifiable lipofuscin granules in TEM images of drusen might be attributed to modification by immune cells (Gouras, Ivert, Mattison, et al., 2008). These results may be relevant to the ongoing debate on the sources of drusen’s material and drusen biogenesis.

The budding hypothesis which postulates that drusen form as a result of tiny cytoplasmic projections budding off from the basal surface of the RPE that act as cytoplasmic conduits by which lipofuscin granules are transferred to the growing druse (Burns and Feeney-Burns, 1980; Ishibashi et al., 1986). Gouras et al. working on rhesus monkeys (Macaca mulatta) demonstrated using autofluorescence and TEM that loss of cytoplasm containing lipofuscin from RPE contributes to drusen formation (Gouras, Ivert, Landauer, et al., 2008). Whilst this study is not designed to answer the question of the origin of materials in drusen, the spectral evidence of a component of lipofuscin adds useful insights into this issue. Our evidence also indicate that 510nm peak is only partially responsible for the drusen’s autofluorescence. A greater contribution to drusen’s autofluorescence emission with $\lambda_{\text{exc}}$ at 364nm is accounted for by peak maxima at 465nm. Quantitative differences in drusen’s content of the fluorophore responsible for this peak are likely to explain drusen’s autofluorescence heterogeneity. Interestingly, the 465nm peak is also found in Bruch’s membrane where it contributes significantly to the overall autofluorescence of Bruch’s membrane. We endeavour to speculate here that with $\lambda_{\text{exc}}$ at 364nm, the 466nm emission peak, which is shared with Bruch’s membrane and crucially absent in RPE, is in fact due to the emission of collagen inside the matrix of the drusen. There is a wide literature consensus that the $\lambda_{\text{exc}}$ max and $\lambda_{\text{em}}$ max of collagen is between 330/370 and 440/460nm (Fukushima et al., 1987; Björkman et al., 1991; Matsumoto et al., 2000; Bachmann et al., 2006b; Shakibaie et al., 2011). The finding that a significant proportion of drusen’s autofluorescence is possibly due to the collagen component of the drusen’s matrix is very important in the light of the ongoing debate on the formation of drusen. A role for age-dependent Bruch’s membrane remodelling defects and expansion of the extracellular matrix had been proposed for the age-dependent thickening of Bruch’s membrane and drusen formation (Kumar et al., 2009; Hussain et al., 2011). It is of interest that cross-linked insoluble
collagen that forms in an age-dependent manner as a result of non-enzymatic glycosylation, displays excitation and emission peaks at 350nm and 460nm respectively (Sell and Monnier, 1989). Emission spectra of some fluorophores show significant structure with multiple peaks with different magnitudes. These peaks correspond to the vibrational energy levels of the ground state and excited states. Spectra of other fluorophores are devoid of such vibrational structures (Lakowicz, 2006). Our approach of mathematical deconvolution of emission peaks would breakdown the emission spectrum of fluorophores with compound emission spectra into its component peaks. However, the advantages of such an approach over gross comparison of emission peaks lie in its ability to identify unique and common peak components when comparing differences in autofluorescence emission and in assigning values to the contribution of different peaks to the overall autofluorescence emission. Acquisition of information on the relative contribution of each peak component to the overall autofluorescence is particularly valuable when considering mixtures of fluorophores without speculation on the exact molecular nature of these fluorophores. Drusen are most likely to contain a mixture of more than one fluorophore and there is certainly a number of fluorophores with different excitation and emission properties in the posterior pole of the eye. Identification of emission markers specific for Bruch’s membrane or drusen would have important clinical implications and could expand the scope of the clinical application of the autofluorescence imaging to giving information on Bruch’s and RPE.

### 2.5.2 Spectral Properties of Inclusion Bodies

In this study, we report a pattern of heterogeneity in drusen autofluorescence with drusen that has not been reported previously. Multiple spherical inclusion bodies with average diameter ranges of (0.3-10µm in diameter) were noticed. Characteristically, these inclusion bodies have markedly reduced autofluorescence intensity when compared to the surrounding area in the druse. The dark spherical inclusion bodies are deceptively black, as appears from the examination of the high contrast autofluorescence images but are between 40-50% less bright than the surrounding area. These inclusion bodies, display all the characteristics of the druse’s
emission spectrum in addition to peaks confined to them. With both $\lambda_{exc}$ at 364nm and 488nm, the spherical inclusions have a peak at 491nm and with $\lambda_{exc}$ at 543nm, 3 unique peaks at 607nm, 662nm and 699nm. The closest report of drusen heterogeneous autofluorescence in the literature was by Delori et al. in in vivo autofluorescence imaging (Delori et al., 2000). The pattern consists of decreased autofluorescence intensity at the centre of the druse, which is surrounded by an annulus of increased intensity whose size matches the outer perimeters of the druse. Delori et al. used an in vivo imaging SLO imaging technique that apparently has resolution sensitivity that is not routinely available in the systems used routinely in clinical practice, but does not have the ability to record in the details of the emission spectrum. Morphologically, this pattern consists of an area of around 17% reduction of autofluorescence intensity at the centre of the druse, which is surrounded by a rim of increased autofluorescence intensity. Delori et al. attribute this pattern to the notion that RPE stretching over discrete drusen would leave a thinner layer of lipofuscin over the middle of the drusen while a much thicker layer of lipofuscin granules would reside over the drusen’s periphery. The problem with this model, it does not explain this pattern of distribution of autofluorescence over hard and soft drusen when their diameters are larger than those of RPE cells. Another explanation by Delori et al is the druse-driven re-distribution of lipofuscin from the centre of the RPE to the periphery. Both of these explanations, stem from the implicit hypothesis that drusen are merely passive transducers of emission spectrum and that they have little or no inherent autofluorescent activity of their own. Drusen are composed of a variety of materials including lipids, over 127 proteins and advanced glycosylation end products (Crabb et al., 2002; Gu et al., 2009) some of which are known to have inherent autofluorescent activity of their own (Handa et al., 1999). Drusen were shown to display distinct emission spectra with a blue shift in comparison to RPE (Marmorstein, Munier, et al., 2002). Lengyel et al. described a class of drusen characterised by high levels of autofluorescence (Lengyel, 2004). For the spectral profiling of the three components of the posterior segment, we used an en face orientation with a confocal laser-scanning microscope with a higher vertical resolution and equipped with the ability to excite at different wavelength and to collect emission data over a wider range. We
observed the presence of multiple spherical inclusion bodies seemingly randomly spread inside the druse with between 40-50% lesser autofluorescence intensity. These bodies are evident with excitations at 364nm, 488nm, 543nm and 633nm wavelengths. A closer inspection of the emission spectrum of the dark spherical bodies reveals that their emission spectra are different from those of the surrounding area of the druse in two important ways. Quantitatively, the emission spectrum’s amplitude is vertically reduced and the comparison of the AUC of the emission spectra shows a reduction in the autofluorescence intensity of the spherical bodies by a factor of 40-50% with slight differences between the exciting wavelengths. Qualitatively, the emission spectrum emanating from the dark spherules displays all the peaks composing the emission spectrum of the drusen and crucially, additional peaks confined to the spherules. Based on this quantitative/qualitative inspection, it is tempting to postulate here, the presence of an additional fluorophore (s) and/or molecule (s) in the dark spherules that exerts a quenching effect on autofluorescence intensity. Morphologically, drusen display a wide range of ultrastructural heterogeneity, it would be plausible to postulate presence of such a quencher. Quenching is the phenomenon of the reduction in the intensity of autofluorescence as a result of a reduction in the quantum yield of the fluorophore (Lakowicz, 2006). Different types of quenching mechanisms have been described. In collisional quenching, the most common example, the reduction of autofluorescence comes by as a result of an encounter of an excited state fluorophore with a deactivating second molecule. However, this model requires an aqueous environment, therefore, it could not be the case in the spherular inclusions of drusen. In static quenching, the model we propose, fluorophores form non-fluorescent complexes with quenchers at the ground state and does not rely on collisional encounters. Metal ions or second chromophores could act as quenchers if they are sufficiently close to the fluorophore (Lakowicz, 2000). Although it is tempting to speculate on the molecular nature for this quenching, a large number of candidate molecules could alone or in combination be responsible for this phenomenon.
2.5.3 Spectral Properties of the RPE-Bruch’s Complex

Drusen display greater emission intensity when compared to Bruch’s membrane at all excitation wavelengths. Gross examination of the emission spectra of drusen and Bruch’s membrane reveals a high degree of overlap and a characteristic blue shift is readily manifested in comparison to the emission spectrum of the RPE layer. These characteristics are evident with all excitation wavelengths. The spectra show a progressive overlap with the increase in the wavelength of the exciting laser. With $\lambda_{exc}$ at 364nm, the RPE layer has unique emission peaks towards the longer end of the emission spectrum while drusen and Bruch’s membrane show emission peaks at the shorter end of the emission spectrum. Our data is in line with earlier reports on the presence of a blue shift in these spectra of fundus areas with drusen At $\lambda_{exc}$ with 488nm, the AUC of the emission spectrum of the RPE layer is significantly larger than that of Bruch’s membrane but not significantly different from the AUC of drusen’s emission. Quantitatively, this indicates that a significant contribution to the overall autofluorescence is made by Bruch’s membrane and drusen. In the clinical settings, $\lambda_{exc}$ with 488nm is the basis for fundus autofluorescence, and is relied upon for functional and morphologic assessment of the RPE layer. RPE’s dominant fluorophore is lipofuscin. The fundus autofluorescence is emitted over a wide spectral band (500nm – 750nm) with the peak emission between 620nm-640nm attributed to lipofuscin. It is hypothesised here, that in the clinical settings, in conditions where there is significant deposition of drusen and sub-retinal deposits, disproportionately larger contributions to the overall fundus autofluorescence come from drusen and disturbed Bruch’s membrane sources. Currently, this possible contribution remains largely unexplored and not employed clinically. Our data illustrate two distinct emission spectra associated with Bruch’s membrane and drusen. The two spectra differ both quantitatively and qualitatively from the emission spectrum of the RPE layer. We found that Bruch’s membrane has a distinct and unique emission peak at 488nm ± 3 thus confirming one earlier report by Marmorstein et al. (Marmorstein et al., 2002). Additionally, we have described unique emission peaks associated with Bruch’s membrane were observed at 443nm and 488nm. Drusen display a unique peak at
545nm ± 3. A peak at 510nm is common between RPE, Bruch’s membrane and drusen. With \( \lambda_{\text{exc}} \) at 488nm, RPE, Bruch’s membrane and drusen share common emission peaks at 554nm and 634nm. At excitation with 543nm laser light, the RPE layer has a unique emission peak at 612nm that dominates its emission spectrum. Bruch’s membrane has a unique emission peak at 646nm, while drusen displayed a unique peak at 650nm. The RPE layer’s propensity for longer wavelength excitation and emission results in the phenomenon at \( \lambda_{\text{exc}} \) with 364nm, the AUC of drusen’s autofluorescence emission is 25% and significantly larger than that of the RPE. An emission spectrum with \( \lambda_{\text{exc}} \) at 364nm would carry more information on the Bruch’s membrane and drusen from the fundus as well as the status of the RPE layer. However, in practice, numerous problems are associated with the use of laser light with shorter wavelength for excitation. Its high proportion of absorption by the cornea and the lens limit the 364nm laser. Despite its potential applications for the study of the RPE-Bruch’s complex and drusen, these problems would limit the practicality of the use of the 364nm laser in clinical practice.
2.5.4 Conclusions

1. Emission spectra of drusen and Bruch’s membrane are predominantly blue shifted in comparison to the emission spectrum of the RPE.

2. Drusen autofluorescence is likely to be polyfluorochromic with features consistent with the green-emitting lipofuscin fraction and collagen and/or hydroxyapatite spectral signatures.

3. At $\lambda_{\text{exc}}$ with 488nm, a significant contribution to the overall fluorescence spectrum is likely due to and bearing the spectral signatures of the Bruch’s membrane and drusen. This contribution augments with $\lambda_{\text{exc}}$ at 364nm and progressively diminishes with $\lambda_{\text{exc}}$ at 543 and 633nm.

4. We have identified multiple dark spherical inclusion bodies. These spherulites exhibit markedly reduced autofluorescence intensity when compared to the surrounding area in the druse. The spherulites display all the characteristics of the druse’s emission spectrum in addition to peaks confined to them.

5. The identified unique spectral signatures of drusen and Bruch’s membrane could be potential non-invasive diagnostic tools and useful for assessing animal models for AMD and other diseases that in which drusen or drusen-like deposits and/or derangements of Bruch’s membrane form part of the disease phenomenology.

6. The role of fluorophore-fluorophore interactions and metals in autofluorescence in the retinal biophysical environment merits close investigation.
Chapter 3

Trace Elements in Drusen

3.1 Overview

Introduction: There is a substantial sequestration of zinc and potentially other metals in the sub-retinal deposits possibly contributing to and/or complicating various disease processes. The role of derangements in the homeostasis of metals and trace elements in ocular tissue is far from clear.

Aims: The following study aims at elucidating the link between metals & trace elements and drusen from three angles:

1. Quantitative and qualitative examination of the metals and trace elements content and distribution in soft and hard drusen.

2. Crystal composition and ultrastructural heterogeneity of drusen.

3. Studying the associations between drusen metal content and autofluorescence heterogeneity.

Methods: Donor eyes were procured from human donors bank of Moorfields eye Hospital. Macula, equator, far periphery and central retina were dissected. The neurosensory retina (NS) was removed. The remaining choroidal microcapillaries/Bruch’s membrane/sub-RPE deposit complex was flat mounted. The tissue demonstrated extensive drusen deposition
of the posterior fundus on postmortem examination. 6 donor eyes were processed for and examined by a scanning electron microscope. Trace metal concentration in specimens from 20 eyes was carried out using the microprobe synchrotron X-ray fluorescence (µSXRF) and X-ray diffraction analysis (XRD) at the X26A beam line at the National Synchrotron Light Source located at Brookhaven National Laboratory (Upton, NY). Staining for Zn and Ca was carried out using Zinpyr-1 and Alizarin red dyes respectively.

Results: XRF analysis of the drusen showed that Zn is invariably found in all drusen examined (864 PPM ± 128). Zn concentration was found to be higher in soft drusen as compared to hard drusen, it averaged 747 PPM ± (111) and 364 PPM ± (60) respectively. Ca and Fe were disparately found in hard drusen with concentrations averages of 273 PPM ± (81) and 6 PPM ± (2). XRF micro-probe analysis also demonstrated Zn, Cu, Ni, Co, Fe are invariably present in drusen unlike Ca, K, Cl, S, P, Mg and Na which are occasionally observed in drusen. TEM showed the drusen deposits to be composed of several regular small spherical structures embedded in a dense matrix. Two types of spherules were noticed, one type is bounded by a double-layered membrane-like structure and some spherules are composed of concentric layers of alternating electron dense and less electron dense materials with dense cores and thin shells. Zinpyr-1 and Alizarin red staining revealed that Zn and Ca are contained within different compartments within the drusen, and that Zn is more widespread in drusen while Ca is contained in spherical structures within the drusen whose diameters vary between 0.5-20\(\mu\)m with an average of 3\(\mu\)m. Drusen 1-D 2theta versus intensity plots exhibited wide variations in peak widths and heights reflecting differences in the degree of mineralization, from the predominantly amorphous to the crystalline. Phase identification revealed the presence of hydroxyapatite crystallites in drusen.

Conclusions: (1) Zinc is ubiquitously present in drusen in high concentration. Calcium is also very commonely present. (2) XRD analysis showed calcium often crystallises to hydroxyapatite. (3) There appears to be a correlation between drusen autofluorescence and metal content. Therefore, we hypothesise that metal content is a likely player in druse formation.
3.2 Introduction

Metals and trace elements are essential for the normal physiology of virtually all tissues in the body. It is estimated that a third of all proteins require specific metal ions in their active sites for correct macromolecular folding and function (Hageman and Mullins, 1999). The role of metals and trace elements in numerous neurodegenerative disorders characterised by the accumulation of intracellular or extracellular protein aggregates is currently a subject of immense interest. Metals such as iron, copper zinc and calcium appear to play pivotal roles in many of the underlying pathophysiological processes in diseases such as Alzheimer’s disease and Parkinson’s disease (Gouras and Beal, 2001; Levenson, 2003).

With advancing age, Bruch’s membrane, which is situated between the retinal pigment epithelium (RPE) and the choroid, becomes progressively thickened. The maintenance of Bruch’s membrane structural and functional integrity depends on the health of the remodelling machinery whose principal enzymes have a functional requirement for Zn and Ca (Birkedal-Hansen et al., 1993; Kumar et al., 2009). Zn and Fe have been demonstrated to disrupt key enzymes responsible for the remodelling process (Kato et al., 2010; Osorio et al., 2011). Bruch’s membrane thickening is associated with the deposition of extracellular material that reportedly contain pathophysiologically high concentrations of Zn (Lengyel, Flinn, Peto, et al., 2007). Zinc is known to be abundant in ocular tissue (Karcioglu, 1982a; Anderson et al., 1987; Grahn et al., 2001), in particular, in the RPE-choroid complex (Newsome et al., 1987) which is the interface where sub-RPE deposits form. Due to the high concentration of zinc and the proteins that bind zinc (beta-amyloid, CFH, serum albumin, crystallines) in drusen (Crabb et al., 2002; Nordgaard et al., 2006), several of which bind zinc under pathological conditions (Nan et al., 2008), it is argued here that zinc and other metals are likely to play important roles in the pathophysiological link of associations that lead to the development of sub-RPE deposit formation.

Despite the ever-increasing body of knowledge on metals and trace elements physiology, there are still important outstanding questions: what cellular structures are responsible for
the storage of metal ions before incorporation in metalloproteins as co-factors? What is the retinal normal tissue composition of metals and trace elements and how does disease and normal ageing effect re-distribution? What are the possible interactions amongst metals and trace elements on one hand and between metals and trace elements and key components of the extracellular matrix and the complement system? What is the fate of ‘recycled’ metals with age-dependent functional decline of the photoreceptor-RPE-Bruch’s complex? An important step for answering these questions, is mapping of the distribution of trace elements and metals in normal tissue and diseased tissue. The aim of this study was to map metals and trace elements in drusen deposits. Synchrotron-induced x-ray fluorescence is an accurate technique that is extensively used for the estimation of trace elements concentration and elemental mapping in a variety of fields (Rao et al., 2012). However, the combination of multi-elemental spectrochemical analysis with XRD analysis with spatial resolution on the micrometer scale is a novel application.

3.3 Metals and Trace Elements Distribution in Ocular Tissue

In the following section, we will be reviewing the salient roles of key metals and trace elements in the physiology and pathology of the retina, the discussion will be anatomically based.

3.3.1 Zinc

Evidence indicates that zinc’s differential effects on cellular physiology depend on the concentration, localisation and/or state (bound or free). With a total quantity in the human body estimated at 2g, zinc is quantitatively the second most important trace element (Grahn et al., 2001). The retina is particularly enriched in zinc. Crucially, Zn exists in two pools in ocular tissue. A larger proportion tightly bound to proteins and a smaller loosely bound and biologically more active pool. The latter has been demonstrated using autometallography (Wu et al., 1993; Ugarte and Osborne, 2001), the dithizone method and a variety of fluorescent zinc sensors (Ugarte and Osborne, 1998; Lengyel and Onwochei, 2007). According to one model,
zinc homeostasis is maintained as a result of concerted actions of multiple players in a complex system. Cytosolic zinc ion-binding proteins such as metallothionein (MT) through their capacity to act as buffers are pivotal in keeping intracellular zinc concentrations at picomolar to nanomolar levels. MT normally possesses about 5 of 7-zinc ion binding sites, which are occupied at the steady-state. A second protein in the system acts as “the store” normally binds a small amount of zinc relative to its capacity in the steady state. A third component in the model is a zinc ion sensor protein unoccupied at steady state but only binds zinc ions when there is an influx of free zinc into the cytosol sufficiently strong to raise free zinc concentrations. Muffling reactions are then activated to direct free zinc ions into the intracellular store for which the transfer process necessitates that zinc ions be first bound to a zinc-binding protein such as MT. As the storage capacity is approached, muffling reactions dampen the zinc ion transient and are responsible for its temporal and spatial characteristics. When cytosolic free zinc ion concentrations are at the limit of the system’s buffering capacity, zinc binds to cytoplasmic sensors. Cytoplasmic sensors mostly, are buffering transcription factors that effect changes in the expression of genes coding for key proteins in zinc homeostasis (Colvin et al., 2010).

3.3.1.1 Zinc in the photoreceptor-RPE Complex

Zn concentration in ocular tissues is unusually high, second only to prostatic tissue (Galin et al., 1962). The descending order of zinc concentration, is retina and choroid, ciliary body, iris, optic nerve, sclera, cornea, and lens (Grahn et al. 2001; Karcioglu 1982). Histochemical zinc mapping studies using dithizone, demonstrated zinc only in rat photoreceptor outer segments, and importantly zinc staining was shown to be reduced in zinc deficiency states (Grahn et al. 2001). The advantage of the histochemical approaches lie in its ability to map out the biologically active free or loosely bound zinc fraction (Frederickson et al., 1987). Zinc deficiency both in the prenatal and postnatal period was shown to be associated with retinal degeneration and that these effects are worsened when there is a concomitant taurine deficiency. Electroretinographic evidence demonstrated a global reduction in the functional capacity of
the retina (Gottschall-Pass et al., 1997, 1998)

3.3.1.2 The role of zinc in the photochemical visual event

Zn deficiency in the retina has been demonstrated to have diverse deleterious functional effects, however, an understanding of the mechanistic basis of these effects remains unclear (Grahn et al. 2001). Studies aimed at elucidating the role of zinc in retinal function established an important link between zinc and vitamin A (Christian and West Jr, 1998a). In zinc deficient humans, dark adaptation is impaired (Warth et al., 1981; McClain and Su, 1983) and thought to result from a decrease in the rhodopsin formation (Dorea and Olson, 1986). Animal studies seem to suggest a reduction in the rate of oxidation of retinol to retinal by a zinc-dependent alcohol dehydrogenase (Christian and West Jr, 1998a, 1998b; Grahn et al., 2001b). Mobilisation of vitamin A from its liver storage sites is also reportedly impaired in Zinc deficiency in humans has also been shown to alter dark adaptation (Warth et al., 1981). In bovine retina it was suggested that metallothionein donates zinc to activate protein kinase C that in turn phosphorylates rhodopsin (Ou and Ebadi, 1992). Thus the effect of zinc deficiency on dark adaptation could thus be explained by the impairment of this role. Importantly, in the dark-adapted retina, chelatable zinc was distributed to the RPE cells and photoreceptor perikarya and some of the perikarya of the inner nuclear layer, both plexiform layers and the ganglion cell layer. Upon light adaptation, only the inner segments of the photoreceptors showed evidence of chelatable zinc. It was suggested that this redistribution of the zinc is due to its translocation from the perikarya into the inner and outer segments and that outer segment zinc might become tightly protein bound rendering it unavailable for chelation (Ugarte and Osborne, 1999). Unlike Mg and Ca, which are found in appreciable concentrations in the ROS, Zn concentrations are markedly elevated when the samples were photobleached (Tam et al., 1976) rendering more evidence for the involvement of zinc in the primary photochemical visual cascade.
3.3.1.3 Zinc as a stabiliser of Biomembranes

Zinc is viewed to act as a stabiliser for biomembranes via direct allosteric-like effects on the conformation of individual proteins and its effect on protein-protein interactions of protein complexes at the tertiary level (Bettger and O’Dell, 1993). In the retina, zinc shows a great deal of subcellular compartmentalisation, large concentrations were found in the rod outer segments and in the photoreceptor synaptosomes (Takahashi et al., 1988). ROS functional and structural integrity is undermined with the loss of zinc from disc proteins leading to a depression in the ERG potential (Grahn et al., 2001).

3.3.1.4 Zinc as a neurotransmitter

Significant amounts of bioavailable zinc have been localised to the photoreceptors’ synaptic termini, where strong evidence suggests a modulatory role for zinc in synaptic transmission (Wu et al., 1993). In terminals of isolated dark-adapted zebra fish photoreceptors, Rendenti et al. detected zinc release that was further enhanced by brief exposures to high K+. In this light, synaptically speaking, released zinc may have an appreciable effect on neural processing in the retina by modulating excitatory and/or inhibitory receptors of the signalling machinery (Hageman and Mullins, 1999). Cyclic GMP-gated ion channels generate the electrical response to light in retinal rods. cGMP holds channels open in darkness; light closes channels and hyperpolarises the cell by activating an enzyme cascade that lowers the concentration of cGMP setting off the photochemical visual act (Stryer, 1991). Zinc ions preferentially occupy progressively open cGMP-activated channels in salamander rods. The cations Ni2+, Cd2+, Zn2+ and Mn2+ potentiate the activity of cGMP by more than threefold in the outer segments (Stryer, 1991). Zn was localised within glutamatergic synaptic vesicles at the base of inner segments of the photoreceptors where Wu et al postulated that it acts as a diffusible switch regulating outer retinal neurotransmitter signalling (Wu et al., 1993).
3.3.1.5 Zinc as an antioxidant

The retina is an area with particular susceptibility to oxidative damage. The Photoreceptors are particularly vulnerable because of their high content of polyunsaturated fatty acids (PUFAs), high metabolic rate and constant assault by light quanta (Grahn et al. 2001). On the other hand, the retina has a relatively low capacity for the scavenging of peroxides and reactive oxygen species as shown by low glutathione concentration and glutathione peroxidase activity (Winkler and Giblin 1983; Grahn et al. 2001). In one in vitro study, zinc along with taurine was shown to have significant protective action against peroxidative damage induced by ferrous sulphate (Pasantes-Morales and Cruz, 1984). In rats, lung tissue, another tissue under constant heavy oxidative stress, zinc-deficient animals, have been shown to have a reduced tolerance to withstand oxidative stress (Taylor and Bray, 1991). Supraphysiological concentrations of Zn have antioxidant-like effects in organelle-based systems and isolated cell-based systems in vitro. It was hypothesised that zinc effects this role by inhibiting transition metals from generating reactive oxygen species (ROS) and protecting the sulfhydryl groups against oxidation (Bray and Bettger, 1990). The high zinc concentration in the retina suggests that zinc might play a role in the antioxidative systems of the retina; however, the details and mechanisms of any such roles remain to be elucidated.

3.3.1.6 Zinc in Bruch’s Membrane & Drusen

Drusen and lipofuscin spots isolated from cynomolgus monkeys with an early-onset macular degeneration were reported to have four-fold less zinc and decreased expression of the metallothionein gene in their RPE cells compared to controls (Nicolas et al., 1996). Olin et al. reported that both zinc and copper concentrations are lower in Macaca mulatta monkeys with >10 drusen per fundus as compared to animals with no drusen. It was reported that 47% of an ageing Rhesus macaques population have macular drusen and that those animals with > 10 drusen per fundus also have low serum zinc levels (Olin et al., 1995). In pigmented rats, zinc deficiency results in the accumulation of lipofuscin in the RPE and deposition of thin
CHAPTER 3. TRACE ELEMENTS IN DRUSEN

morphologically abnormal macrophages at Bruch’s membrane (Julien et al., 2011).

In humans several studies concluded that patients with AMD have lower levels of serum zinc compared to controls (Erie et al., 2009) highlighting potential links between the derangements in zinc homeostasis and the pathogenesis of AMD. Based on this view, it was hypothesised that poor zinc intake in elderly persons might result in zinc deficiency, causing or hastening the development of AMD. Some protective effects for zinc supplementation in AMD patients were observed by the AREDS trial (“A randomised, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss,” 2001). Lengyel and co-workers reported supra-physiological concentrations of zinc in drusen; in excess of 500ppm (lengyel et al., 2007). He also reported the concentration of zinc in Bruch’s membrane is up to 254ppm. However, the origins of this high zinc concentration remains unclear. Lengyel et al. reported that the intercapillary pillars are a favourite site for drusen formation (Lengyel et al., 2004) He hypothesised that the origins of the high zinc concentration is possibly the choroid circulation. Evidence seem to support the hypothesis that zinc is involved in the events leading to drusen formation, however, the details of this role are unclear.

3.3.1.7 Zinc and the complement system

The complement system being a component of the innate and the adaptive immune systems constitutes the body’s first line of defence against bacterial infections. Three distinct enzymatic pathways mediate activation of the complement system. Immune complexes activate the classic pathway (CP). The lectin pathway (LP) responds to repetitive carbohydrate moieties on bacterial surfaces. Foreign or bacterial surfaces activate the alternative pathway (AP), which also shows intrinsic activation. The three pathways for complement activation, converge on the enzyme C3b. Due to the high toxicity of the activated complement enzymes, activation is favoured on foreign surfaces and actively inhibited on self-cells. On self-cells, regulators, such as Factor H, inhibit C3b deposition (Oppermann et al., 2006).

Factor H is composed of linear arrangements of 20 short complement regular domains
(SCRs) of 61 aminoacyl residues with C3b and heparin binding sites (Oppermann et al., 2006). Factor H protein normally assumes a compact tertiary form. When ligand interaction takes place, for instance with surface attached C3b, the protein unfolds and exposing the additional binding sites.

Inhibition of CFH by zinc and Cu (and to a lesser extent by other transitional metals) causes widespread polymerisation and functio laesa (Nan et al., 2008). Given the presence of pathophysiologically high concentrations of bioactive zinc in the RPE-Bruch’s membrane-Choroid complex, it was hypothesised that the uncontrolled CFH zinc-driven oligomerisation is at the heart of drusen formation and AMD pathogenesis (Nan et al., 2011).

Zinc has also been implicated in inhibiting factor I-mediated release of CR-1 bound immune complexes and degeneration of cell-bound factors C3b and C4b (Jepsen et al., 1990). Factor I (FI) is largely responsible for complement inhibition via the proteolytic cleavage of activated C3b and C4b. This crucial reaction can only happen in presence of C-4b binding protein and factor H. (Blom et al., 2004). The cofactor activity of C4b-binding protein towards C4b/C3b and factor H towards C3b depends on zinc concentration.

Having higher affinity for C3b and C4b, this activity is increased at micromolar concentrations of zinc but abolished at 2mM Zn\(^{++}\) concentrations and above (Blom et al., 2003). It could be concluded that zinc has complex effects on the complement system and AMD pathophysiology. At normal physiologic concentrations, it might have protective functions. When zinc homeostasis is deranged and concentrations at the RPE-Bruch’s Membrane-Choroid complex reach high pathophysiologic levels, zinc then becomes a complicating factor by triggering an uncontrolled activation of the immune system.

### 3.3.2 Calcium

About 99\% of the Ca\(^{2+}\) in the body is contained in crystalline form in bone and teeth. The remaining 1\% is widely distributed in soft tissue and in the extracellular fluid. In different parts of the eye, Ca performs different essential functions. In the iris, where calcium exists in two pools, is essential for the electro-mechanical coupling action crucial for pupillary constriction.
3.3.2.1 Photoreceptor-RPE Complex

Calcium has been demonstrated to exist in significant quantities in the discs of the rod outer segment (ROS) in frog cells (Fishman et al., 1977) where light stimulation is thought to trigger its release facilitating the process of phototransduction (Yoshikami and Haginst, 1978). A current model suggests that photoexcitation leads to the release of calcium into the cytoplasm with subsequent effects on the sodium dark current of the plasmalemma. A process ultimately triggers the receptor action potential. In vitro calcium accumulation was demonstrated in isolated ROS discs rendering support to this hypothesis (Fishman et al., 1977).

3.3.2.2 RPE

RPE cells have been demonstrated to contain large amounts of calcium that is released upon photoexcitation facilitating the process of phototransduction (Kaupp et al., 1981). Calcium-rich granules with an internal repeating pattern of 5 nm spherules were described in light-adapted frog RPE cells using X-ray contact microscopy (Hageman and Mullins, 1999). Using electron microscopy, a calcium-rich spherular pattern with similar description and dimensions was described in calcium-rich pigment granules in Aplysia (Henkart, 1975). Given calcium is an essential electrolyte for phototransduction and the location and of these spherules, it is proposed that a possible link exists between pigment granules and sequestered calcium in ocular tissues, it is proposed these granules act as internal depots for calcium (Hageman and Mullins, 1999) for functions essential for phototransduction.

3.3.2.3 Bruch’s Membrane

Using the pyroanimonate precipitation method, calcium antimonate precipitates were detected along Bruch’s membrane indicating the presence of substantial quantities of calcium (Fishman et al., 1977). Bruch’s membrane contains large amounts of calcium, far in excess of that associated with collagen indicating the presence of numerous calcium-binding sites. Since collagen
is not known to be particularly calciphilic, the presence of modifying calciphilic proteins or mucopolysaccharides trapped between the collagen fibrils has been postulated (Leaver et al., 1975). Both quantitatively and qualitatively, the localisation of calcium to Bruch’s membrane may mark important pathophysiologic steps in a variety of clinical conditions. Pathologic calcification in drusen (van der Schaft et al., 1992) and in calcium oxalate retinopathy (Bullock et al., 1974) shows a clear predilection of calcium to deposit in Bruch’s membrane.

3.3.3 Iron

3.3.3.1 Photoreceptor-RPE Complex

Iron is a potent source of highly reactive hydroxyl free radicals generated via the Fenton reaction. An elaborate regulatory system has evolved for tight iron homeostasis to prevent ensuing deleterious oxidative damage. Gradual iron accumulation with ageing is inevitable. RPE cells possess the melanin-containing organelles, the melanosome which are enclosed by a lipid membrane. Melanin is thought to sequester iron and provide protection against oxidative damage (Rozanowski et al., 2008). In RPE and photoreceptors cells, iron is thought to play a role in the generation and maintenance of reactive oxygen species (Dunaief, 2006). Transferrin transports iron in the plasma. By binding iron, it prevents the Fenton reaction that would normally generate free radicals. However, the overall role of iron as an antioxidant is limited by its comparatively tiny concentration in the aqueous and vitreous humours as compared to plasma (McGahan and Fleisher, 1986). Antioxidant effects of ferritin are also illustrated by the increase in free and total iron in response to oxidative challenge (McGahan and Fleisher, 1988). Iron’s antioxidant effects in ocular tissue are also re-enforced by its interaction with ascorbate. Ascorbate has been demonstrated to increase the concentration of ferritin, in cultured lens epithelial cells. This effect is believed to come by through an ascorbate-mediated increase in ferritin synthesis at the translational level (McGahan et al., 1994). It is interesting that individuals with a dominantly inherited defect in ferritin synthesis exhibit a form of early-onset bilateral cataracts (Gorlaska et al., 1997). Photoreceptor inner and outer segments
contain relatively high iron concentrations crucial for a variety of basic functions such as the electron transfer chain and oxidative phosphorylation, membrane biogenesis. Because photoreceptor outer segments are continually lost to phagocytosis by RPE, a recycling mechanism was proposed to exist. Hunt et al proposed that RPE cells take iron back from the choriocapillaris to the photoreceptors. Dysfunction of any step in this iron transport process could lead to iron overload and photoreceptor degeneration (Yefimova et al., 2002). It was demonstrated in the Royal College of Surgeon (RCS) rat model of such dysfunction. Rats have a genetic defect due to a MERKT mutation. The ability of the RCS rat RPE to phagocytose shed OS is compromised. Undigested OS tips build up in the subretinal space, forming an iron-rich heterogeneous debris layer that interferes with the normal RPE–photoreceptor interactions (Yefimova et al., 2002). This accumulation presumably leads to free radicals generation, overwhelming oxidative stress and photoreceptor cell death by apoptosis. Indeed injected iron particles in the retina lead to similar results (Wang et al., 1998).

### 3.3.3.2 Bruch’s Membrane

Iron levels in the retina not only increase with normal ageing (Hahn et al., 2006) but also in numerous pathological conditions (He et al., 2007). Hahn et al. demonstrated that eyes of patients with AMD contain substantially more iron than normal controls (Hahn et al., 2003). RPE and Bruch’s membrane are areas especially targeted by this iron mineralization in AMD. The sources of the abnormally high iron content in this area are not clear. In drusen, iron-containing proteins were identified, for instance haemoglobin beta 2 (Crabb et al., 2002). It could potentially act as a source of iron that could enzymatically be liberated and deposited in Bruch’s membrane or the sub-RPE space. It is speculated that heme oxygenases (1 and 2) mediate the release of iron from heme-containing proteins. The activity of these heme oxygenases is reportedly increased in RPE of AMD afflicted individuals (He et al., 2007). It is not clear whether the supranormal concentration of iron in patients with AMD plays any role in the pathology of the disease, but by drawing an analogy from the brain it is interesting to note that β-amyloid protein, which is hallmark of Alzheimer’s disease, was also identified in
drusen (Johnson et al., 2002). The toxicity of β-amyloid’s protein stems from its bound iron. The high level of iron in the micro-environment where drusen form complicated by the presence of β-amyloid, heme oxygenase and haemoglobin beta 2 could mean that heme oxygenase could help release free iron. Free ion could then through binding to β-amyloid and/or generation of oxidative damage might cause retinal degeneration by increased iron-mediated oxidative damage.

3.3.4 Copper

Copper’s ability to assume two distinct redox states, oxidised (Cu$$^{2+}$$) or reduced (Cu$$^{+}$$) allows it to play pivotal roles in as diverse cellular functions as a catalytic cofactor in the redox chemistry of enzymes, mitochondrial respiration, iron absorption and crucially in ocular physiology free radical scavenging and elastin cross-linking. A delicate balance between uptake and efflux maintains the concentration of cellular copper. The functional demands of the eye, with its intense exposure to light and slow tissue turnover rates make it uniquely vulnerable to oxidative stress. UV light exposure triggers a series of reactions that generate various reactive oxygen species (ROS), including superoxide anion, H$_2$O$_2$ and singlet oxygen (Linetsky and Ortwerth, 1996). The Cu and Zn-containing superoxide dismutase (SOD) is an important component of the antioxidative defence battery in the eye (Rose et al., 1998). Copper/zinc superoxide dismutase (Cu/Zn-SOD) catalyses the important step of converting superoxide anions to peroxides for ultimate disposal by the catalase and glutathione peroxidase systems. Organs were proved to be more vulnerable when the SOD system was blocked (Flood et al., 1999).

3.3.5 Selenium

Selenium serves as a cofactor for the enzyme, glutathione peroxidase (GPx). GPx is an antioxidant enzyme "family" whose main function functions is the scavengering and inactivation of hydrogen and lipid peroxides (Levander, 1987; McGahan and Grimes, 1991). It forms an important member of the body’s antioxidant arsenal. Very little else is know about selenium. Methodological differences and species differences gave widely different estimates for the
distribution of selenium in ocular tissue. Spectrofluorometric measurements gave very low concentrations in ocular tissues without appreciable differences between the different components of the eye leading to conclusions of limited role of selenium in ocular biology (Christian and Michaelis, 1966). Taussky et al also using fluorometric assays, estimated the concentration of selenium in 5 different species, in descending order of concentration; iris, retina, lens, cornea, sclera and humour (Taussky et al., 1966). McGahan et al. used electrothermal atomic absorption spectroscopy (EAAS) to estimate the amounts of selenium in different human tissues. The kidney and liver were found to have the highest concentration of selenium followed by the lens and the retina (McGahan and Grimes, 1991). The role of selenium is poorly understood, it is known that selenium excess or deficiency has cataractogenic effect (Shearer et al., 1983; Palmquist et al., 1986). Rats fed selenium deficient diet for more than 1.5 years showed reduction in the photoreceptor cells count, but importantly, no increase in RPE cells lipofuscin content.

Selenium seems to be differentially present in ocular tissue in very small quantities. However, major gaps of knowledge exist regarding selenium, partially because of the methodological difficulties. Better and more sensitive methods are needed to study selenium distribution and in order to ascertain its possible significance in ocular biology and physiology.

### 3.3.6 Heavy Metals

Although broadly speaking, metals such as Fe, Zn, Ca and Mg fall under the category of heavy metals, they are metals with special bio-importance in human physiology and within certain limits are beneficial. Exposure to other elements for instance arsenic, cadmium and lead, even at very low concentrations is toxic (Duruibe et al., 2007). The way it is applied in medical sciences, the term “heavy metal poisoning” is a misleading one, it is broadly used to include all toxic metals irrespective of their atomic weight (Duffus, 2002).

Mechanisms of toxicity of heavy metals involve a change in the target proteins structure and function as a result of an interaction with the heavy metal ion. The RPE cell layer is a metal-chelating and protective layer for the retina. The particularly high affinity of the RPE cells for binding essential and toxic metals renders it vulnerable to heavy metal toxicity.
When RPE cells were incubated with a variety of metals, percentages for binding with heavy metals were as follows: strontium, 26%; calcium, 30%; zinc, 37%; iron, 64.5%; nickel, 62%; mercury, 72%; copper, 82%; and lead, 62% (Potts and Au, 1976). Using energy-dispersive x-ray microanalysis, Ulshafer et al. reported the presence of aluminium in Bruch’s membrane and in melanosomes where mercury and selenium were also identified (Ulshafer et al., 1990).

Another proposed mechanism for heavy metal toxicity is the mediation of oxidative damage. Lead and cadmium have been shown to exist in all ocular pigmented tissue (Erie et al., 2005) and are believed to exert oxidative stress by producing reactive oxygen species that overwhelm the tissue’s antioxidative defences and result in lipid peroxidation. In summary, a variety of heavy metals that accumulate in the retinal pigment epithelium and choroid, ciliary body, and the retina of humans are toxic to the retina and the RPE. Oxidative stress and depletion of the antioxidative defences appears to be the primary mode of damage. The interactions of these heavy metals amongst themselves and with the functional systems of the Photoreceptor-RPE-Bruch’s complex and their possible links to retinal degenerative conditions are all questions remain to be elucidated.

### 3.4 Effects of Metals on the Remodelling of Bruch’s Membrane

#### 3.4.1 Remodelling

A continuous process of homeostatic remodelling of the ECM is operational for the maintenance of the functional integrity of Bruch’s membrane (Guo et al. 1999). Since most of the age associated-decline in the diffusional capacity of the Bruch’s membrane occurs without evident gross ultrastructural deformities, defects in the remodelling machinery is postulated to explain both the age-related decline and the deposition of sub-retinal and sub-RPE lipid-rich debris. Mechanistic defects in Bruch’s membrane remodelling form a spectrum at one end of it, is the age-related changes and at the other extreme is the pathological changes associated with AMD.
CHAPTER 3. TRACE ELEMENTS IN DRUSEN

3.4.1.1 Control of Remodelling

Homeostatic ECM turnover is a delicate process finely tuned by biosynthetic and degradative processes. Proteolytic breakdown of the ECM components is effected by matrix metalloproteinases (MMPs) also designated matrixins, which hydrolyse components of the extracellular matrix. A family of Zn$^{2+}$-containing Ca$^{2+}$-dependent proteolytic enzymes (Woessner Jr 1991; Birkedal-Hansen et al. 1993; Kumar et al. 2009) MMP-1, -2, -3, and -9 have been demonstrated in cultured RPE and choroidal endothelial cells and therefore, assumed to be the machinery responsible for Bruch's membrane remodelling (Alexander et al. 1990; Guo et al. 1999). MMPs are released as inactive prozymogens whose functional activation by cleavage of the propeptide, to yield active forms, is a pre-requisite for degradation (Ahir et al. 2002). When activated, MMPs are capable of digesting most components of the ECM. Part of the control mechanism, is the presence of inhibitors of metalloproteinases (TIMPs) whose function is to prevent over activation of MMPs. In Bruch's membrane TIMP-2 and -3 have been confirmed. The degree of ECM breakdown is controlled by the balance between the activities of MMPs and their inhibition by TIMPs. Defects in either the biosynthetic or the degradative processes of the remodelling system could result in structural-functional derangements with consequent effects on photoreceptors.

3.4.1.2 Defects of Remodelling

Sorsby’s fundus dystrophy (SFD) is a rare retinal genetic degenerative disease due to a 22q13–qter defect (Weber et al. 1994). It manifests with rapid loss of central vision followed by progressive loss of peripheral vision and ultimately blindness. Histological features show a great deal of overlap with AMD. It is characterised by abnormal deposition of lipofuscin-like material and thickening of Bruch’s membrane. Eventually, there is subretinal neovascularization RPE atrophy and atrophy of the choriocapillaris. The genetic defect is due to a mutation of the TIMP-3 gene, the abnormal ECM turnover results in Bruch’s membrane thickness up to six times the normal (30µm compared with 2–4 µm in normal) (Fariss et al. 1998). Some
evidence suggests that TIMP-3 activity is raised in drusen rendering them a cold spot for MMP activity (Leu et al. 2002), it was also reported that MMP-2 and MMP-9 concentrations are decreased in AMD patients (Hussain et al. 2011)

3.4.1.3 Changes with Ageing

Evidence indicate, with normal ageing, there is not only an age-dependent increase of the gelatinase component (MMP-2 and -9) of the MMP family but also redistribution of the active form to the peripheral regions leaving the macula practically devoid of activity (Guo et al. 1999). High-molecular-weight gelatinase monomers or heteropolymers MMP2 and MMP9 are the principal species in the ECM of Bruch’s membrane. They undergo aggregation to macromolecular complexes consisting of HMW1, HMW2, MMP9, and some MMP2 (LMMCs). HMW1 and HMW2 and their further polymerisation to LMMCs result in the sequestration of pro-MMP monomers reducing their availability for enzymatic activation (Hussain, Lee, and Marshall 2009). The progressive sequestration of MMPs by binding, trapping, or polymerisation would be the net reduction of the free MMP pool. The functional implications of the increase of the MMPs/TIMPs ratio on the remodelling system is the predominance of the biosynthetic process and expansion of the ECM in the macula.

3.4.2 Effects of Metals on Remodelling

3.4.2.1 Zinc

Local zinc deprivation was shown to inhibit the activity of MMPs. Calprotectin a 36 kDa calcium-binding protein constitutes a significant proportion of the soluble cytosolic proteins in human neutrophil granulocytes (Johne et al. 1997). Calprotectin released from granulocytes within physiologic concentrations has been proved to be sufficient to cause a 50% or more inhibition of MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 (Isaksen and Fagerhol 2001). Interestingly, calprotectin was associated posterior uveits in the connective tissue disease, anti-neutrophil cytoplasmic antibodies (ANCA) (Olson et al. 1996). Zinc excess is a known
inhibitor of collagen degradation by dentin matrix metalloproteinase (Osorio et al. 2011; de Souza, Gerlach, and Line 2000). Given the pathophysiologically high concentrations of bioavailable zinc in Bruch’s membrane and in sub-retinal deposits, it is hypothesised here that MMPs are similarly inhibited and therefore contributing to the expansion of ECM of Bruch’s membrane.

3.4.2.2 Calcium

Metalloproteinases are Ca\(^{2+}\)-dependent endopeptidases (Alexander et al. 1990). Calcium was also found to play an important role at the transcriptional level on ECM remodelling directly by affecting key MMPs and indirectly via its capacity to act as a second messenger. In RPE cells, angiotensin II, by binding to the AT1 receptor on RPE cells, activates G-protein-coupled phospholipase C and inositol-1,4,5-triphosphate, leading to an increased intracellular calcium concentration where calcium acts a second messenger to increase levels of expression of MMP-2 mRNA and protein (Striker et al. 2008). A similar regulatory role for calcium on MMP-2 was described in human cancer cells (Kohn et al. 1994).

3.4.2.3 Iron

It was observed that increased cellular iron levels selectively enhanced the expression of MMP-9 in microglia (Mairuæ, Connor, and Cheepsunthorn 2011). Given the potential of iron for oxidative injury in the brain and the retina, a similar regulatory role for iron accumulation in the RPE-Bruch’s on MMP could be speculated upon. Studies on human cancer cells found a similar up-regulatory effect for iron on MMP-9 (Kaomongkolgit et al. 2008). These results are in contrast with reports of an inhibitory effect of iron on MMP-2 and MMP-9 in dental tissue (Kato et al. 2010). Different roles of iron in different tissues have been extensively studied, however, the role of iron (if any) on the regulation of MMP activity in ocular tissue and Bruch’s membrane is an untouched area.
3.5 Methods

3.5.1 Tissue Sources

Eyes were acquired through the tissue bank of Moorfields Eye Hospital. Consent for research was obtained and the tissue was handled in accordance with the tenants of the Declaration of Helsinki. The eyes were enucleated between 4-24 hours post-mortem and kept in isotonic normal saline at 4°C until the time of dissection.

3.5.2 Drusen Dissection

Using a surgical skin grafting knife, the globes were circumferentially cut just distal to the ora serrata. The lens and the iris along with the vitreous were then removed and sent for safe disposal in line with the protocol applied at the pathology department at UCL Institute of Ophthalmology. The Retina-RPE-Bruch-Choroid complex was dissected from the sclera using the blunt end of a dissecting forceps. The complex was then transferred to a Petri dish and flattened in distilled water. The neurosensory retina (NS) was removed and safely disposed off in line lab protocol as outlined above. Using a fine speculum, RPE cells were scrapped off exposing the underlying drusen under direct stereoscopic observation using a Nikkon ZE 1500 dissecting microscope equipped with a fluorescent lamp. Drusen were then grouped and manually transferred using a fine micropipette to a Mylar film or a Kapton tape.

3.5.3 Labelling for metals

3.5.3.1 Labelling for the bio-availabe zinc

Zinpyr-1 (ZP-1), a fluorescent zinc sensor in HEPES (5 mM) saline was used for the detection of the bio-available zinc in drusen. ZP-1 was applied for 5 min as described before (Lengyel et al., 2007). Specimens were then rinsed in a fresh zinc-free buffer to remove the excess ZP-1. Imaging was performed using a Zeiss LSM 510 Meta Confocal Microscope ($\lambda_{ex}=460$-500nm and $\lambda_{em}=530$-560nm). Negative control specimens were examined to demonstrate ZP-1 specificity for zinc labeling.
3.5.3.2 Labelling for calcium

For labeling calcium we used the fluorescent calcium indicator, Alizarin Red (1, 2-dihydroxyanthraquinone). AR was prepared fresh by adding 2g of the AR powder to 100ml of water. Drusen-containing tissue was incubated for 10 minutes and excess AR was then removed by rinsing in water. Negative control experiments were performed to confirm the pattern of labeling of AR to calcium in drusen. The specimens were viewed and imaged with a Zeiss LSM 510 confocal microscope with a plane-apochromat 40x (Wavelength 488nm T1 2%; 555nm T2 2%).

3.5.4 Confocal Microscopy

An Inverted Zeiss 510 Axiovert motorised microscope equipped with an 8-channel spectrophotometric detector (META UV-vis) was used. Lambda scans were carried out using 364, 488, 543 and 633nm laser lines. The detector exhibited sensitivity in the range between 400-700nm. The emission spectrum was not affected by filter characteristics as detectors did not depend on the use of filters. The detectors were set to advance in increments of 10µm from a wavelength longer than the exciting laser until 700nm. Plane lambda-scan was carried out (scaling was X 0.20µm, y 0.20µm) and a Fluar 20x/0.75 UV objective with a zoom of 0.7 with an open pinhole was used. The exciting laser line was powered to T2 16.9%, 9.9%, 74.3% and 86.7% for the 364, 488, 543 and 633nm laser lines respectively. Gains were individually optimised for each scan and were not altered during the course of the scan. Data were obtained as LSM files and were read on Zeiss LSM-5 Image Browser software. Drusen average pixel intensities were plotted off-line using the circle drawing mode of the function “mean of ROI display mode” for the emission associated with each excitation. Data were the exported as Excel files.

3.5.5 Combined X-ray fluorescence and X-ray diffraction analysis

Trace metal content and structural information were obtained at the National Synchrotron Light Source, Brookhaven National Laboratory, USA. Samples were sent to Professor Jane Flinn, George Mason University (USA) for scanning at the X26A beam line with the help of
3.5.5.1 Sample preparation

Following the isolation of samples with drusen samples were flat mounted and sent for measurements or drusen samples were isolated as described in (3.5.2). Then whole drusen were either transferred onto Mylar films or Kapton tapes or collected in Eppendorf tubes. The latter samples were first centrifuged before removal of phosphate buffer saline. Samples were then embedded on OCT and sectioned at 20µm thickness before mounting on Mylar films.

3.5.5.2 Measurements

A through description of the procedure of measurement at the x26A facility was described before (Sutton et al., 2002). In brief, trace metal concentration in specimens was estimated using the microprobe synchrotron X-ray fluorescence (µSXRF) at the X26A beam line at the National Synchrotron Light Source located at Brookhaven National Laboratory (Upton, NY). A dual silicon monolithic crystals (Si111 & Si311) system was used to fine-tune the X-ray beam in accordance with the requirements for Bragg’s law. The X-ray beam was then collimated to 1/8 of the size at the source from 350µm by 350µm down to 10µm in diameter. A Kirkpatrick-Baez focusing optics system made of two dynamic Rh-coated silica mirrors is used to focus the X-ray beam in both vertical and horizontal planes to 10µm and 14µm full width at half maxima (FWHM) respectively. Specimens were vertically mounted in a motorised table at 45° to the incident beam. In some experiments the XRF scans were coupled to XRD analysis and crystallographic and X-ray fluorescence data was gathered simultaneously. Each pixel was measured for 7 seconds for XRF. A TV monitor was used to observe specimen images created with a light microscope with 5x and 20x objectives. A Bruker SMART 1500 CCD diffractometer was used to detect the transmitted spectra.
3.5.6 XRF Data Analysis

Data analysis programs described below was carried out using specialised software available at BNL website (http://www.bnl.gov). The programs run on the platform of the IDL virtual machine (IDL VM) image analysis software freely available at (http://www.exelisvis.com).

3.5.6.1 Metal distribution topographic maps

2-D data collected using the 9-element or the 13-element array detectors were opened and topographical maps for metal distribution were generated in the plot window "Graphics Panel" of the program by the function "ROI to plot". The X and Y coordinate of a data point per pixel of interest in the Graphics Panel. The 2-D metal topographic maps were opened using the Itools function, which sent the colour-coded metal intensity images to a new window. Drusen were then identified in the 2-D topographical distribution maps. Regions of interest (ROIs) were highlighted manually on overhead transparent sheets of plastic attached to the computer monitor which ensuring the different trace metal contents were determined in the same ROIs when topographical metal maps were changed. Zn, Ca, Cu, Fe and Ni intensity values were created for each druse. The value of the background signal in each topographical map of a metal was estimated. Three background ROIs outside drusen were manually chosen, background metal concentrations were then averaged. The value of the background noise was subtracted from the metal concentration of each druse separately. Data was collected as cps values and exported as a tab delimited ascii file. The data were opened in an Excel file. Metals’ distribution topographical maps were visually correlated with photographic and autofluorescence images of drusen taken with $\lambda_{\text{exc}}$ at 364nm, 488nm and 543nm.

3.5.6.2 Energy dispersive spectra

1-D data from some drusen were collected with the 9-element Ge array detector using the MCA mode. Energy dispersive spectra (EDS) collected using this mode arose from individual drusen one at a time. Raw data are exported in ascii format. Data is opened in the MCA program.
which is available at the BNL website and has similar requirements as described above. Data files were opened the foreground of the MCA program. Peak fitting was performed using "Fit Peaks". The background was peak fitted. Correct peak fitting was confirmed by a visual inspection and once the routine is set for one scan, it is maintained to the end of the data analysis.

Peaks were then automatically identified in the energy dispersive spectra. The peak for each element was then named manually. After insertion of the names of the different metals, the program inserted the emission lines for the energy peaks corresponding to each element from a look-up table. Data is saved in # delimited ascii files exported and opened in Excel spread sheets.

3.5.6.3 Metal standards

To determine the concentration of metals, biological standards were prepared by spiking sheep brain homogenates with different concentrations of externally added metal solutions. 20µm cryosections of the spiked homogenates were mounted as described for drusen. The procedure for the creation of standards was described before (Lengyel et al., 2007). The standards for zinc, calcium, copper, iron and nickel were prepared and x-ray fluorescence spectrums were generated at 100X100 micron areas. Following peak fitting and averaging of all pixels the cps values were plotted against the concentration of spiked metals in ppm. We used equations of linear regression through these point to determine the metal concentration in drusen samples.

3.5.7 XRD

3.5.7.1 XRD data analysis

As described above (3.5.5). Data analysis programs described below was carried out using specialised software available at BNL website (http://www.bnl.gov). The programs run on the platform of the IDL virtual machine (IDL VM) image analysis software freely available at (http://www.exelisvis.com). Our Powder Diffraction XRD data was collected as 2-dimensional
CHAPTER 3. TRACE ELEMENTS IN DRUSEN

Figure 3.1 – X26A beamline at Brookhaven National Laboratory

Figure 3.2 – A typical logarithmic multi-channel fluorescence spectra from a druse excited at 12 keV
data as described by (Sutton et al., 2002) in Bruker’s SMART 1500 CCD format. The program; Fit2D (written by Andy Hamersley) was used to analyse the results of the experiments. Fit2D is freely available at a number of websites (http://staff.chess.cornell.edu). For integrating our 2-D patterns into 1-D plot of 2-theta angles versus intensity, we followed the steps briefly described below.

3.5.7.2 Calibration

The correct reading of the diffraction patterns requires the program to determine the position of the direct beam, the centre point of the diffraction ring and the sample-to-detector distance. The first step is the calibration of the software to the Bruker’s SMART 1500 CCD camera distance and X-ray wavelength and the specifications of the machines used at BNL. For each run, we performed a separate calibration provided by the operators of the machine. In our experiments, we used 2 standards for calibration; α-Al₂O₃ and AgBeh diffraction patterns combined. Using the calibrant option in the graphical user’s interface (GUI), first we entered the text file containing the 2-theta values of the combined α-Al₂O₃-AgBeh diffraction pattern. The inner Debeye-Scherrer ring of the combined 2-theta angles α-Al₂O₃-AgBeh diffraction pattern was manually selected for the calibration. Next the sample-to-detector distance was set to 226.0770mm. Then sample wavelength from the monochromator was set to 0.709300 Å (Mo-Kα1 of 17.479). Finally the size of the horizontal and vertical pixels was set to 76μm (the specifications of the CCD image).

3.5.7.3 Background subtraction

Kapton tape is characterised by its selective absorption pattern of X-ray fluorescence below 6 keV. To minimise this effect, we performed a background subtraction of the Kapton tape XRD pattern. First the sample pattern is opened in a GUI window. Then Kapton tape pattern is opened in a different GUI window. The Kapton tape pattern is then placed at the background using the exchange function. Subtraction is then performed from the MATHS options and the resultant pattern is the drusen pattern free off the Kapton tape interference.
CHAPTER 3. TRACE ELEMENTS IN DRUSEN

Figure 3.3 – α-Al2O3-AgBeh diffraction pattern

Figure 3.4 – Kapton tape diffraction pattern
3.5.7.4 Integration

In order to create a 1-D diffraction pattern of the 2-theta angle versus intensity, we created chi plots. After the transformation of the 2-D plots to 1-D, excessive background at the beginning of the pattern was removed by zooming-in and cropping out. The integrated 2-theta versus intensity data was then saved and exported as ascii files ready for use in “search match” programs.

3.5.7.5 Phase identification

For search-match we used the Match! Software program version 2.0.5 (Crystal Impact, Kreuzherrenstr, Germany), which uses the Crystallography Open Database (COD) database. The COD database had 205,631 entries of XRD patterns (as of 23/08/2012). First we imported the drusen diffraction pattern into Match2! software. The radiation Mo-Kα1 of 0.709300 Å was manually entered and set as a default. Profile fitting was performed next for the determination of peak parameters. A basic unrestricted search-match is carried out to determine the mineralogical composition of the specimen. To test for our approach we determined the 2 theta values for a biologically derived hydroxyapatite powder from human bone. We ran an unrestricted search-match for the standard HA chi plots against the COD database and found that the best match was obtained with HAP. We then compared all our chi plots against the COD database mineralogy database by setting the search to Hydroxyapatite and performed a restricted search-match by setting the search parameters to multiple-phase (value 1), contribution of intensity deviations (value 1) and contribution of 2theta (value 1). These parameters were applied as default option for all the drusen chi plot search match operations.

3.5.8 Transmission electron microscopy imaging

Drusen-containing tissue was cut into small pieces with a razor a blade and then fixed in alcohol for 2-3 hours on ice. Subsequently, the tissue was washed 5 x 3 minutes in cold 0.15M cacodylate buffer containing 2mM calcium chloride.
The tissue was then incubated for an hour in a freshly prepared solution containing 3% potassium ferrocyanide in 0.3M cacodylate buffer with 4mM calcium chloride combined with an equal volume of 2% aqueous osmium tetroxide (EMS).

The tissues were then washed 5 x 3 minutes with ddH$_2$O at room temperature. Tissues were then placed in the 0.22 micron Millipore filtered TCH solution for 20 minutes, at room temperature. Thiocarbohydrazide (TCH) solution was prepared by adding 0.1gm thiocarbohydrazide (Ted Pella) to 10 ml ddH$_2$O and placed in a 60° C oven for 1 hour during which, solution was continuously agitated by swirling gently every 10 minutes to facilitate dissolving. It was then filtered through a 0.22 um Millipore syringe filter right before use.

Tissues were then rinsed again 5 x 3 minutes in ddH$_2$O at room temperature and placed in 2% osmium tetroxide in ddH$_2$O for 30 minutes, at room temperature. Tissues were washed again for 5 x 3 minutes at room temperature in ddH$_2$O then placed in 1% uranyl acetate and left in a refrigerator (~4°C) overnight.

The following day, the tissue was washed 5 x 3 minutes in ddH$_2$O at room temperature and then placed in the en bloc Walton’s lead aspartate solution and placed in the oven for 30
minutes. Washing was in ddH$_2$O was repeated again before a process of gradual dehydration
of tissue was carried out. The tissue was passed in an ascending alcohol series 30%, 50%, 70%,
90%, 100%, 100% for 20 minutes each, then placed in 100% propylene oxide and left at room
temperature for 10 minutes. Tissues were placed in a 50:50 mixture of 100% propylene oxide:
100% resin (TAAB Hard Plus) on a rotator and left overnight. The following day the samples
were placed in 100% resin for 2 hours prior to fresh resin for a period of 4 hours on a rotator.
Samples were then embedded in a thin layer of fresh resin and placed in a 60°C oven and left
for 48 hours.

Tissue blocks were sectioned into semi-thin sections were cut using a Reichert (Ultracut-R)
microtome. Semi-thin sections were stained at 90-100°C with toluidine blue dye and checked
for the presence of drusen. Ultrathin sections were then prepared from the same block phases
and viewed with a transmission electron microscope (JEOL 1010 TEM) equipped with a Gatan
Orinus digital camera. Files were transported and studied in tiff format.

3.5.9 Drusen autofluorescence

Curves for the spectral emission data were deconvoluted into their sharp component peaks us-
ing PeakFit software (version 4.12 by SeaSolve Software Inc, 235 Walnut St. S7, Framingham,
MA 01702, USA). A Fourier deconvolution/ filtering algorithm which depends on a Gaussian
response was applied for the deconvolution process. Hidden peaks representing local maxima
were revealed. The average pixel intensities for drusen autofluorescence were determined us-
ing the grey-scale XY image function in the Zeiss LSM-5 Image Browser software (version
4.2.0.121 by Carl Zeiss MicroImaging GmbH, Germany). Autofluorescence numerical values
were obtained from the image in 8-bit format using Image J software (ImageJ version 1.44p,
LIH, USA).
3.6 Results

3.6.1 Trace Metals Concentration in Drusen

The results of metal concentration maps by X-ray fluorescence scanning for representative images of hard and soft drusen are shown in figures (3.6a) and (3.6b). Zn is the most abundantly found trace element and is invariably present in all drusen examined. However, average Zn concentrations vary between individual drusen and most importantly vary based on the morphologic type of the druse (hard versus soft). While soft drusen contain significantly more quantities of Zn, hard drusen have more Ca. Ca, Cu, Fe, K and Cl, are also found in drusen at varying quantities. Summary of metal concentration results from 20 donor eyes is given in table (3.1). Mean zinc concentration in drusen, was found to be 848 ppm ± (127). Soft drusen have significantly more Zn than hard drusen; 953ppm ± (173) and 472ppm ± (84) respectively. Calcium was detected in 54% of the drusen studied, hard drusen contained on average 171 ppm ± (52) compared to soft drusen 63ppm ± (17). Copper, iron, and nickel were also detected in 64%, 33% and 41% of the drusen examined. The average concentrations of Copper, iron, and nickel were 45ppm ± 7, 17ppm ± 10 and 26ppm ± 9 respectively.
Table 3.1 – Summary of metal concentrations in drusen

<table>
<thead>
<tr>
<th></th>
<th>Zn</th>
<th>Ca</th>
<th>Cu</th>
<th>Fe</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>% drusen with</td>
<td>100</td>
<td>54</td>
<td>64</td>
<td>33</td>
<td>42</td>
</tr>
<tr>
<td>All (ppm)</td>
<td>848 ± (127)</td>
<td>92 ± (20)</td>
<td>45 ± (7)</td>
<td>17 ± (10)</td>
<td>26 ± (9)</td>
</tr>
<tr>
<td>Hard</td>
<td>472 ± (84)</td>
<td>171 ± (52)</td>
<td>8 ± (4)</td>
<td>4 ± (1)</td>
<td>12 ± (6)</td>
</tr>
<tr>
<td>Soft</td>
<td>953 ± (173)</td>
<td>63 ± (17)</td>
<td>58 ± (10)</td>
<td>22 ± (14)</td>
<td>31 ± (13)</td>
</tr>
</tbody>
</table>

Zinc is universally found in all the drusen examined (100%). Its concentration averaged 848ppm ± (127). Soft drusen have significantly more Zn than hard drusen; 953ppm ± (173) and 472ppm ± (84) respectively. Calcium was detected in 54% of the drusen studied, hard drusen contained on average 171 ppm ± (52) compared to soft drusen 63ppm ± (17). Copper, iron, and nickel were also detected in 64%, 33% and 41% of the drusen examined. The average concentrations of Copper, iron, and nickel were 45pm ± 7, 17pm ± 10 and 26pm ± 9 respectively (n = 20).
Zinc is quantitatively the most abundantly found trace element and is invariably found in all drusen examined. However, average Zn concentrations vary between individual drusen and most importantly vary based on the morphologic type of the druse (hard versus soft). While soft drusen contain significantly more quantities of Zn, hard drusen have more Ca. Ca, Cu, Fe, K and Cl are also found in drusen at varying quantities but inconsistently as some drusen do not contain some of these elements. Drusen were isolated, frozen in optimal cutting temperature solution (OCT) and sectioned to a thickness of 20µm prior to scanning with µSXRF.
3.6.2 Structural Heterogeneity and Staining for Zinc and Calcium

3.6.2.1 Ultrastructural features

Figure (3.7) shows a large druse resting on Bruch’s membrane. The deposit contains small several regularly shaped spherical structures embedded in a dense granular extracellular matrix. The spherules, which are also located in the outer collagenous layer of Bruch’s membrane opposite the deposit, are bounded by a double-layered membrane-like structure (inset A, B and C). Some of the spherules contain tiny inner electron-dense particles (B); some are composed of concentric layers of alternating electron dense and less electron dense materials including a dense core and a thin shell (C and D).

3.6.2.2 Staining for Zinc and Calcium

In figure (3.8a), staining for 10 minutes with the dye Alizarin-Red (AR) reveals a well-localised staining pattern. Drusen are composed of regular spherules embedded in amorphous ground substance. Morphologically, the spherules are composed of a dense outer shell that strongly stains for Ca and a seemingly hollow inner core that does not stain for Ca. In figure (3.8b) ZP-1 displays considerable heterogeneity in the distribution of free bio-available zinc with globular structures that do not stain for Zn and others that stain more strongly for Zn. Unlike with AR; there is a general background Zn staining. Figure (3.9) confirms the same pattern of staining for zinc and calcium in the druse. Co-staining for calcium and zinc using AR and ZP-1 in figure (3.9a) reveals areas of greater staining and areas of lack of staining against a background of Zn. In (3.9b) staining with AR reveals a pattern of spherules with thick shells that strongly stain for Ca and cores with lack of staining for Ca. The merged image (3.9c) shows some cases of overlap in staining (the orange colour) but in the majority of areas, AR and ZP-1 stain distinctly different regions of the same druse. Figure (3.10): A confocal XY scan (with XZ and YZ projections) centred on a spherule stained by AR shows that the spherule has a thick shell that strongly stains for AR with a seemingly empty core.
Figure 3.7 – Drusen structural heterogeneity and metal distribution

A large deposit resting on Bruch’s membrane. The deposit is composed of small several regular spherical structures embedded in a dense matrix. The spherules, which are also located in the outer collagenous layer of Bruch’s membrane opposite the deposit, are bounded by a double-layered membrane-like structure (inset A, B and C). Some of the spherules contain tiny inner electron-dense particles (B), some are composed of concentric layers of alternating electron dense and less electron dense materials including a dense core and a thin shell (C and D). In image (E), Alizarin Red stains the outer shells spherules of various sizes embedded within the matrix of a druse. In image (F) ZP-1 in comparison stains the matrix around but not the spherules embedded within a druse.
Figure 3.8 – Staining for Ca and Zn in drusen with Alizarin-Red and ZP-1 dyes respectively

In figure (A) staining for 10 minutes with the dye Alizarin-Red (AR) reveals a well-localised staining pattern. Drusen are composed of regular spherules embedded in amorphous ground substance. Morphologically, the spherules are composed of a dense outer shell that strongly stains for Ca and a seemingly hollow inner core that does not stain for Ca. In image (B) staining for Zn with ZP-1 reveals considerable heterogeneity in the distribution of Zinc with globular structures that do not stain for Zn and others that stain more strongly for Zn. Unlike with AR; there is a general background Zn staining.
Figure 3.9 – Co-staining for Calcium and Zinc

The figure shows XY images of a druse co-stained for calcium and zinc using AR and ZP-1. (A) reveals areas of greater staining and areas of lack of staining against a background of Zn. In (B), staining with AR reveals a pattern of spherules with thick shells that strongly stain for Ca and cores with lack of staining for Ca. The merged image (C) shows some cases of overlap in staining (the orange colour) but in the majority of areas, AR and ZP-1 stain distinctly different regions of the same druse.
Figure 3.10 – A confocal XY scan of a druse stained with AR and ZP-1 dyes

The image is centred about such a spherule, it demonstrates the spherule has a shell that stains for calcium and a seemingly empty core. Calcium and zinc are located at distinct areas of the druse with little or no overlap.
3.6.3 Drusen XRD Analysis & Metal Content

3.6.3.1 Crystalline Structure

Simultaneous metal analysis using microprobe synchrotron X-ray fluorescence (µSXRF) and X-ray diffraction analysis (XRD) of a single druse results are shown in figure (3.11). The indicated druse come from a sample of isolated drusen placed on a Kapton tape and was simultaneously scanned for metal analysis using microprobe synchrotron X-ray fluorescence (µSXRF) and X-ray diffraction analysis (XRD). Simultaneous multi-channel µSXRF analysis and XRD was performed at 40 different loci within the druse. 20 loci were on a horizontal trajectory and the other 20 loci were on vertical trajectory at 90 degrees. The two trajectories intersect at the middle of the druse in a cruciate form. MCA counts per second (CPS) results of the vertical and the horizontal scans (a and b, respectively) indicate that Zn is found in all scanned areas of the druse, while Ca shows a wide degree of variability in its presence across the druse. Simultaneous Debeye-Scherrer diffraction patterns indicate the disparate presence of a crystalline phase in some areas of the druse along the horizontal and vertical trajectories. Two distinct Debye-Scherrer patterns were noticed in the scans, one type illustrated in scan 5 (horizontal) and 13 (vertical) and another type in scans 10 and 15 (horizontal) and 9 (vertical) trajectories. The Debye-Scherrer diffraction patterns along 20 scan point the horizontal axis of the druse are shown in figure (3.14). Two patterns of diffraction were evident, an identical single-crystal zone pattern in 6 scan areas and and a polycrystalline pattern in 5 other scanned spots. Areas towards the periphery of the druse did not show discernible diffraction patterns.
3.6.3.2 Metals & Trace Elements

In figure (3.12), MCA metal CPS values at the loci co-scanned for XRD along the horizontal axis in the druse. Various metals were observed in the horizontal trajectory. The highest quantities for zinc were observed in the middle loci of the trajectory. Se, Zn, Cu, Ni, Co and Fe were detected in all loci. Ca, K, Cl, S, P, Mg and Na were found in smaller quantities in some loci but not others. The ratio Ca:P was higher in the middle loci 10, 11, 13, 14 and 15 were 1.93:1, 1.17:1, 1.05:1, 1.25 and 1:1 respectively. A micro-XRF metal distribution map for the whole druse is shown in figure (3.13) for Zn, Ca, Cu, Fe, and Ni. The scanned druse area totalled 1967 pixels. At each pixel, counts per second were measured for Zn, Ca, Cu, Fe, and Ni. The highest mean CPS values and widest variability for a metal in the druse were recorded for Zn (48 ± 20) followed by Fe (19 ± 8) then Ni (8 ± 5), Cu (5 ± 3) and the lowest CPS were detected for Ca (1 ± 0.1). A high degree of heterogeneity in the distribution of metals was evident in the metal distribution map, particularly for Zn, Ni and Cu. In figure (3.17), multi-channel X-ray fluorescence (XRF) metal counts results from 60 drusen from 20 patients are summarised. XRF micro-probe analysis using multi-channel analysis of metals demonstrated that Zn is invariably present in all drusen (100%) with the highest counts, while Ca (54%), Cu (65%), Fe (33%) and Ni (30%). Other elements, K, Cl, S, P, Mg and Na are found in smaller quantities in some but not all drusen.
Figure 3.11 – simultaneous metal analysis using microprobe synchrotron X-ray fluorescence (µSXRF) and X-ray diffraction analysis (XRD) of a single druse

The figure above shows a sample of isolated drusen on a Maylar film. The druse in the inset image was scanned for metal analysis using microprobe synchrotron X-ray fluorescence (µSXRF) and X-ray diffraction analysis (XRD). Simultaneous multi-channel µSXRF analysis and XRD was performed at 20 horizontal and 20 vertical points along the cruciform trajectories shown with the intersecting red lines in the inset image. MCA counts per second (CPS) results of the vertical and the horizontal scans (a and b, respectively) indicate that Zn is found in all scanned areas of the druse, while Ca shows a wide degree of variability in its presence across the druse. Simultaneous Debye-Scherrer diffraction patterns indicate the disparate presence of a crystalline substance in some areas of the druse along the horizontal and vertical trajectories. Two distinct of Debye-Scherrer patterns were noticed in the scans, one type illustrated in scan 5 (horizontal) and 13 (vertical) and another type in scans 10 and 15 (horizontal) and 9 (vertical) trajectories. No clear relationship between the MCA metal counts and XRD patterns is evident.
Various metals were observed in the horizontal trajectory. The highest quantities for zinc were observed in the middle loci of the trajectory. Se, Zn, Cu, Ni, Co and Fe were detected in all loci. Ca, K, Cl, S, P, Mg and Na were found in smaller quantities some loci but not others. The ratio Ca:P was higher in the middle loci 10, 11, 13, 14 and 15 were 1.93:1, 1.17:1, 1.05:1, 1.25 and 1:1 respectively.
3.6.3.3 Phase Identification

X-ray diffraction patterns from 10 points along the horizontal trajectory of the druse that showed discernible Debye-Scherrer diffraction patterns are shown in figure (3.15). The XRD patterns are compared against a commercial hydroxyapatite (HA) standard showing the typical diffraction planes present in a randomly oriented powder pattern of HA. The sharp peaks indicate a high degree of crystallinity. A single crystal pattern was also noticed in certain areas of the druse. In figure (3.16), representative XRD patterns found in druse compared to a hydroxyapatite standard diffraction. In (A) is the characteristic Debye-Scherrer pattern of a known sample of commercial hydroxyapatite (HA) powder used as a standard in the experiment. (B) And (C) represent patterns most often encountered in drusen. (D) Single crystal pattern encountered in some drusen.
CHAPTER 3. TRACE ELEMENTS IN DRUSEN

The scanned druse area totalled 1967 pixels. At each pixel, counts per second were measured for Zn, Ca, Cu, Fe, and Ni. The highest mean CPS values and widest variability for a metal in the druse were recorded for Zn (48 ± 20) followed by Fe (19 ± 8) then Ni (8 ± 5), Cu (5 ± 3) and the lowest CPS were detected for Ca (1 ± 0.1). A high degree of heterogeneity in the distribution of metals was evident in the metal distribution map, particularly for Zn, Ni and Cu.
Debye-Scherrer diffraction patterns and its integrated polycrystalline powder XRD patterns collected at 20 different points along the horizontal trajectory of the druse shown in figure (8.6). Two patterns of diffraction were evident, an identical single crystal zone pattern in 6 scan areas and a different pattern consistent with HAD most scanned spots. Areas towards the periphery of the druse did not show discernible diffraction patterns.
Figure 3.15 – X-ray diffraction patterns from the 10 points along the horizontal trajectory of the druse above compared to HAP reference spectrum

XRD of various locations in drusen compared against a commercial hydroxyapatite (HA) standard showing the typical diffraction planes present in a randomly oriented powder pattern of AH. The sharp peaks indicate a high degree of crystallinity. A single crystal pattern was also noticed in certain areas of the druse.
In (A) is the characteristic Debye-Scherrer pattern of a known sample of commercial hydroxyapatite (HA) powder used as a standard in the experiment. The Figure of merit (FoM) which indicates the degree of agreement of the specimen with the database was 0.999. (B) and (C) represent patterns most often encountered in drusen whose FoM values were 0.976 and 0.973. (D) Single crystal pattern encountered in some drusen.
3.6.4 Metals and Drusen Autofluorescence

3.6.4.1 Trace Metal Concentration and Autofluorescence

Emission intensities of isolated drusen are shown in figure (3.18): With $\lambda_{\text{exc}}$ at 488nm, 543nm and 633nm lasers, individual drusen exhibit a considerable degree of variability in autofluorescence intensity that substantially varies in emission intensity. Figure (3.19): XRF metal distribution maps for Zn, Ca, Cu, Fe and Ni in isolated drusen correlated with autofluorescence with $\lambda_{\text{exc}}$ at 488nm. The most abundantly found trace metal was Zn followed by Fe. However, the correlation coefficient ($R^2$) between the Zn counts and the intensity of autofluorescence emission with $\lambda_{\text{exc}}$ at 488nm was not significant. Autofluorescence emission intensity with $\lambda_{\text{exc}}$ at 488nm showed a high correlation with Ca concentration ($R^2 = 0.71$) and moderately strong correlation with drusen Fe concentration ($R^2 = 0.52$). Cu concentration showed quite a small correlation with drusen autofluorescence intensity, while Ni concentrations did not show any correlation with drusen’s autofluorescence emission with $\lambda_{\text{exc}}$ at 488nm ($R^2 = 0.06$).

The correlation results between trace metal XRF concentrations and drusen’s autofluorescence emission with $\lambda_{\text{exc}}$ at 488nm in addition to 543nm and 633nm are summarised in table (3.2). The highest correlation values were observed with $\lambda_{\text{exc}}$ at 488nm followed by $\lambda_{\text{exc}}$ at 543nm and 633nm. With $\lambda_{\text{exc}}$ at 488nm, Ca, Fe and Cu showed strong ($R^2 = 0.71$), moderately strong ($R^2 = 0.52$), and low correlations ($R^2 = 0.25$), with drusen autofluorescence respectively. With $\lambda_{\text{exc}}$ at 543nm, only Ca ($R^2 = 0.38$) and Fe ($R^2 = 0.47$) showed significant correlations with autofluorescence. Still at $\lambda_{\text{exc}}$ at 633nm, Ca and Fe concentrations displayed significant albeit small correlation values to autofluorescence ($R^2$ values were 0.25 and 0.22 for Ca and Fe respectively).

3.6.5 Drusen Autofluorescence & XRD

Figure (3.20) shows an emission autofluorescence image of dissected drusen on a Mylar film along with X-ray diffraction patterns. The autofluorescence image was taken with a $\lambda_{\text{exc}}$ at 488nm. Drusen display a wide range of variation in emission intensity. The marked drusen,
XRF micro-probe analysis using multi-channel analysis of metals demonstrated that Zn is invariably present in all drusen (100%) with the highest counts, while Ca (54%), Cu (65%), Fe (33%) and Ni (30%). Other elements, K, Cl, S, P, Mg and Na are found in smaller quantities in some but not all drusen (n= 20).

Figure 3.17 – Multi-channel X-ray fluorescence metal counts
shown along with the accompanying XRD Debye-Scherrer patterns, represent different autofluorescence intensity grades. They range from the brightest druse (A) at decile X to the least bright; drusen (F, G) at decile I. The intensity of the Debye-Scherrer rings diminishes with decreasing autofluorescence intensity of the druse. This indicates an increase in drusen’s autofluorescence is associated with the degree of crystallinity. Figure (3.21) shows a strong correlation exists between the AUC of the XRD patterns and drusen’s autofluorescence optical density ($R^2 = 0.88$). Ca counts also displayed a strong correlation with drusen autofluorescence ($R^2 = 0.82$), while drusen Zn concentration displayed a moderate relationship with the autofluorescence intensity ($R^2 = 0.6$). There was no relationship between drusen size as measured in pixels and their autofluoresence intensity ($R^2 = 0.08$). Data is summarised in table (3.3).

### 3.6.6 Drusen Matrix Vesicles

Figure (3.22), shows a TEM of a druse with numerous vesicles, electron-dense and electron lucent structures embedded in its matrix. Long collagen fibrils stretch in parallel to the elastin layer of Bruch’s membrane within the drusen matrix. Calcifying nodules appear as dark osmophilic particles and numerous matrix vesicles dispersed in the drusen matrix. The calcifying nodules and MVs exhibit a close association with the collagen fibrils.
Individual drusen exhibit a considerable degree of autofluorescence activity and that substantially varies with respect to autofluorescence emission intensity when excited with laser lights of different wavelengths.
The figure shows XRF trace metal distribution map of the same group of isolated drusen. The most abundantly found trace metal was Zn followed by Fe. However, the correlation coefficient ($R^2$) between the Zn counts and the intensity of autofluorescence emission with $\lambda_{exc}$ at 488nm was not significant. Autofluorescence emission intensity with $\lambda_{exc}$ at 488nm showed a high correlation with Ca concentration ($R^2 = 0.71$) and moderately strong correlation with drusen Fe concentration ($R^2 = 0.52$). Cu concentration showed small correlation with drusen autofluorescence intensity, while Ni concentrations did not show any correlation with drusen’s autofluorescence emission with $\lambda_{exc}$ at 488nm ($R^2 = 0.06$).
Table 3.2 – Summary of drusen trace metal XRF concentrations and correlations to autofluorescence emission $\lambda_{\text{exc}}$ at 488nm, 543nm and 633nm

<table>
<thead>
<tr>
<th></th>
<th>488nm</th>
<th>543nm</th>
<th>633nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>0.01</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Ca</td>
<td>0.71*</td>
<td>0.38*</td>
<td>0.25*</td>
</tr>
<tr>
<td>Cu</td>
<td>0.25</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>Fe</td>
<td>0.52*</td>
<td>0.47*</td>
<td>0.22*</td>
</tr>
<tr>
<td>Ni</td>
<td>0.06</td>
<td>0.00</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The table above summarises the correlation coefficient ($R^2$) values for drusen’s emission autofluorescence when excited with $\lambda_{\text{exc}}$ at 488nm, 543nm and 633nm. The highest correlation values between autofluorescence and trace metal concentrations were observed when drusen’s $\lambda_{\text{exc}}$ at 488nm followed by $\lambda_{\text{exc}}$ at 543nm and 633nm. With $\lambda_{\text{exc}}$ at 488nm, Ca, Fe and Cu showed strong ($R^2 = 0.71$), moderately strong ($R^2 = 0.52$), and low correlations ($R^2 = 0.25$), with drusen autofluorescence respectively. With $\lambda_{\text{exc}}$ at 543nm, only Ca ($R^2 = 0.38$) and Fe ($R^2 = 0.47$) showed significant correlations with autofluorescence. Still at $\lambda_{\text{exc}}$ at 633nm, Ca and Fe concentrations displayed significant albeit small correlation values to autofluorescence ($R^2$ values were 0.25 and 0.22 for Ca and Fe respectively).
Table 3.3 – Summary of drusen’s autofluorescence by deciles and its correlations

<table>
<thead>
<tr>
<th>Drusen</th>
<th>AF</th>
<th>Decile</th>
<th>Pixels</th>
<th>AUC</th>
<th>CPS values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>A</td>
<td>212</td>
<td>X</td>
<td>70</td>
<td>4875</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>151</td>
<td>IX</td>
<td>20</td>
<td>4572</td>
<td>4.4</td>
</tr>
<tr>
<td>C</td>
<td>117</td>
<td>VIII</td>
<td>14</td>
<td>2525</td>
<td>1.2</td>
</tr>
<tr>
<td>D</td>
<td>113</td>
<td>VII</td>
<td>16</td>
<td>2350</td>
<td>5.4</td>
</tr>
<tr>
<td>E</td>
<td>87</td>
<td>IV</td>
<td>84</td>
<td>2152</td>
<td>1.7</td>
</tr>
<tr>
<td>F</td>
<td>66</td>
<td>I</td>
<td>19</td>
<td>1583</td>
<td>3.8</td>
</tr>
<tr>
<td>G</td>
<td>63</td>
<td>I</td>
<td>30</td>
<td>367</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Drusen featured in the image and the table above have autofluorescence emission intensities that represent decile ranks from the least bright (I) and through to the brightest druse on decile (X). Both Ca and Zn CPM values show proportionate increases with drusen’s mean emission autofluorescence intensity with λ<sub>exc</sub> at 488nm.
Figure 3.20 – Debeye-Scherrer patterns of dissected individual drusen

The figure above shows isolated an emission autofluorescence image of isolated drusen with a drusen on a lambda excitation at 488nm. The drusen display a wide range of emission intensity. The marked drusen shown along with the accompanying XRD Debeye-Scherrer patterns represent a intensity grades from the 10th decile (A) to the 1st decile (F, G). The intensity of the Debeye-Scherrer rings diminishes with decreasing autofluorescence intensity of the druse indicating an increase in autofluorescence is associated with the degree of crystallinity of the druse.
There is a strong correlation between the AUC of the XRD patterns intensity and drusen autofluorescence optical density (O.D) ($R^2 = 0.88$). CPS values for Ca also displayed a strong correlation with drusen autofluorescence ($R^2 = 0.82$), while drusen Zn CPS concentration displayed a moderate relationship with the autofluorescence intensity ($R^2 = 0.6$). There was no relationship between drusen size as measured in pixels and their autofluorescence intensity ($R^2 = 0.08$).
Figure 3.22 – TEM of a druse

Figure (A): There are long collagen fibrils stretching in the drusen matrix (yellow arrow). Calcifying nodules appear as dark osmophilic particles (red arrows) and numerous matrix vesicles (blue arrows) dispersed in the drusen matrix. Figure (B): A close association between matrix vesicles (MV) along collagen fibrils.
3.7 Discussion

3.7.1 Zinc

Here we report for the first time the presence of zinc in a magnitude of several hundreds of ppm concentrations in the sub-RPE deposits that may play a role in the development and/or progression of AMD. Mechanistic details of zinc involvement in pathophysiological events underlying the development of AMD are still shrouded in mystery. The sub-RPE area contains not only supraphysiological concentrations of zinc, but also significant quantities of Ca, Fe, Cu and other metals and trace elements. It is likely that the pathophysiological mechanisms of zinc are complex and involves interaction with other metals and trace elements in the sub-RPE deposits. Below we speculate on the likely mechanistic explanations for the role of zinc and metals in the events that end with creation of sub-RPE deposits.

Origins of the supraphysiologic enrichment of zinc in drusen and sub-RPE deposits are enigmatic. However, it is conceivable that zinc could have relocated to the sub-RPE deposits as a result of the activities or pathology of the RPE layer. Ocular tissue is particularly enriched in zinc (Karcıoglu, 1982; Grahn et al., 2001). Zinc dithizone histochemical mapping studies demonstrated significant zinc concentrations in photoreceptor outer segments (Grahn et al., 2001). New discs are constantly produced at the photoreceptor inner segment and then progressively get displaced outwards towards the outer segment as part of the normal renewal physiology of the photoreceptors. RPE cells located on the apical side possess villi that embrace the ageing termini of photoreceptor outer segments, where older discs get phagocytosed to make room for regeneration of new discs. The implication of this process is the transfer of various substances and trace elements and trace metals from the outer segment to the RPE (Bok, 2002). It is estimated that each RPE cell engulfs 7500 photoreceptor discs per day amounting to one million per year (Young and Bok, 1969). However, the most likely source of the high zinc concentration in the sub-RPE deposits is the RPE-choroid complex. This complex contains large quantities of zinc stored as component of melanin (Ulshafer et al., 1990; Samuelson et al., 1993). As a co-factor zinc, is involved in key proteins such as
metallothioneins (Tate et al., 1995; Maret, 2000) and numerous cellular functions. Presumably, there is a recycling and recovery mechanism by which zinc either gets returned to the photoreceptor cells or passed over to the choroidal circulation thus preventing a build up of abnormal concentrations in the sub-RPE space. Understanding of zinc homeostasis and its derangements in the photoreceptor-RPE-choroid complex is likely to provide some clues on the pathophysiology of zinc in deposit formation. The supraphysiological level of zinc we report in the sub-RPE deposits suggests that zinc is involved in pathophysiological interactions that might relate to deposits formation either causally or consequentially. Some general clues on the potential role of zinc in deposit formation could be deduced by drawing parallels with Alzheimer’s disease, which is characterised by the deposition of β-amyloid protein. It was found that zinc binding to β-amyloid protein induces the latter to amyloid aggregation and deposition (Bush et al., 1994; Esler et al., 1996) a hallmark of Alzheimer’s disease. Both in transgenic mouse models of AD and in humans (Cherny et al., 2001; Rosenberg RN, 2003). These findings established the rationale for the consideration of zinc and metal chelation as a potential therapy for AD (Hoogenraad, 2011). β-amyloid spherular aggregates have been described in drusen (Anderson et al., 2004). β-amyloids have wide-ranging toxic effects in a variety of neurodegenerative conditions. In drusen β-amyloid deposition correlates with retinal pigment epithelium degeneration and photoreceptor cell death (Dentchev et al., 2003). Our data demonstrated that drusen exhibit a general pattern of staining for active bio-available zinc. Some spherular profiles demonstrated strong ZP-1 labeling. Given the staggeringly high levels of zinc we found in the drusen, it is tempting to speculate a similar role for zinc in the aggregation and deposition of β-amyloid in drusen. Questions remain to be answered on the importance of β-amyloid deposition for the process of drusen biogenesis and whether indeed all drusen contain β-amyloid proteins. An attractive hypothesis for the explanation of the possible role of zinc in the formation of sub-RPE deposits in based on its role in the modulation of the immune responses mediated through the complement system. Zinc has an inhibiting effect on factor I-mediated release of CR-1 bound immune complexes and degradation of the cell-bound factors C3b and C4b (Jepsen et al., 1990). Complement factor I (FI) is largely
responsible for complement inhibition via the proteolytic cleavage of activated C3b and C4b. This crucial reaction can only happen in presence of C-4b binding protein and factor H (Blom et al., 2003, 2004). The cofactor activity of C4b-binding protein towards C4b/C3b and factor H towards C3b depends on zinc, physiologic concentrations of zinc enhances this activity but at micromolar concentrations, but this activity is completely abolished at zinc concentrations > 2mM (Blom et al., 2003). Given the supraphysiologic zinc concentrations in the sub-RPE deposits, it is not inconceivable that immunologic effects of zinc are important contributors to the development of drusen. However, whether zinc is the trigger or a consequence of the potential pathologic processes or immune responses is an outstanding question needs to be answered. The potential role of zinc in the modulation of the immune responses through complement factor-H will be discussed in the following sections. In line of the above discussion, it is counter-intuitive that it was reported that drusen and lipofuscin spots isolated from cynomolgus monkeys with an early-onset macular degeneration were found to have four-fold less zinc and a decreased level of expression of the metallothionein gene compared to controls (Nicolas et al., 1996). Macaca mulatta monkeys with >10 drusen per fundus reportedly had lower zinc and copper concentrations when compared to animals with no drusen (Olin et al., 1995).

It was reported that 47% of an ageing Rhesus macaques population have macular drusen and that those animals with > 10 drusen per fundus also have low serum zinc levels (Olin et al., 1995). In pigmented rats, zinc deficiency resulted in the accumulation of lipofuscin in the RPE and deposition of thin morphologically abnormal macrophages at Bruch's membrane (Julien et al., 2011). In humans several studies concluded that patients with AMD have lower levels of zinc compared to controls (Erie et al., 2009) highlighting potential links of derangements in zinc homeostasis to the pathogenesis of AMD. Based on this view, it was hypothesised that poor zinc intake in elderly persons might result in zinc deficiency, causing or hastening the development of AMD. Some protective effects for zinc supplementation in AMD patients were observed by the AREDS trial (Bartlett and Eperjesi, 2003). This assumption might only be true for the early stages of the disease, as examination of early AMD tissue did not reveal any significant amounts of zinc (van der Schaft et al., 1992). However, as the disease progresses
zinc concentration might increase. This could explain our findings of the supraphysiologic concentrations of zinc in the sub-RPE deposits. Another plausible explanation for the beneficial results documented by the AREDS trial, is that oral zinc supplementation by raising the plasma zinc concentration may trigger increased zinc uptake and replenishment in the RPE-choroid complex. This restoration of zinc concentration would then restore the normal function of the RPE-choroid complex. The exact details of the zinc uptake mechanism are not known. Given the plethora of physiological and possible pathological roles zinc plays, it is quite possible that elevation of the zinc availability would have multiple effects. Increased plasma zinc level may also boost the immune system and provide better protection against inflammation.

To summarise, zinc is known to be abundant in ocular tissue (Karcioglu, 1982; Grahn et al., 2001; Anderson et al., 2002), in particular, in the RPE-choroid complex (Newsome et al., 1987) which is the interface where sub-RPE deposits form. The formation of lipid protein rich sub-RPE deposits found in both central and peripheral retinae of patients is likely to be important in the development of AMD (Mullins et al., 2000; Nozaki et al., 2006). Due to the high concentration of zinc and the proteins that bind zinc (beta-amyloid, CFH, serum albumin, crystallines) in drusen (Crabb et al., 2002; Nordgaard et al., 2006), several of which bind zinc under pathological conditions (Nan et al., 2008), it is argued here that zinc and other metals are likely to play important roles in pathophysiologic links of associations that lead to the development of sub-RPE deposits. The finding that a sizeable proportion of the total is in an ionic or loosely bound form suggests metal chelation as potential therapeutic strategy for AMD. However, it is not clear if zinc and metal disturbances are a primary triggering effect for drusen deposition or just another link in the pathophysiological chain of associations that is responsible for the development of age-related macular degeneration.
Calcium’s concentration and exact localisation in Bruch’s membrane are postulated to be important determinant factors for the role switch from physiologic to pathologic. A large quantity of calcium was demonstrated to exist along the RPE side of Bruch’s membrane in light-adapted Rana pipiens and Rana catesbeiana frogs (Fishman et al., 1977). The location of most calcium close to the RPE junctional complexes potentiates a role for a calciphilic protein secreted by the RPE cells. While calcium deposition is a generalised age-related phenomenon, the propensity of calcium to deposit in Bruch’s membrane is well documented in a variety of clinical conditions. In calcium oxalate retinopathy there is a significant deposition of calcium oxalate crystals in RPE cells and Bruch’s membrane (Bullock et al., 1974). In AMD, pathologic calcification in Bruch’s membrane and of drusen is a recognised phenomenon (van der Schaft et al., 1992). However, here for the first time, we report that not only is calcium found in significant quantities in drusen, but also that soft drusen contain significantly less quantities of calcium that hard drusen. The demonstration of the presence of enormous quantities of calcium associated with Bruch’s membrane and the quantitatively differential presence of calcium between soft & hard drusen and macular and extra-macular drusen may be of importance for the switch from normal age-related calcium deposition to AMD. The propensity of calcification to occur at Bruch’s membrane and in drusen could be related to raised calcium concentrations in Bruch’s ECM, whether caused by calcium bound to collagen or a modifying calciphilic protein in the ECM.

Fishman et al. hypothesised that the large calcium content of Bruch’s membrane, far in excess of that associated with collagen in other tissues, indicates the presence of numerous calcium-binding sites (Fishman et al., 1977). Since collagen is not known to be particularly calciphilic, the presence of modifying calciphilic proteins or mucopolysaccharides trapped between the collagen fibrils has been postulated as the mechanism by which mineralization of collagen occurs in dentin and bone tissues (Leaver et al., 1975). Several protein components of the extracellular matrix have demonstrable ability to bind calcium to varying degrees of affinity.
(Kohfeldt et al., 1997). Although there is no clear understanding regarding their role in bone formation, non-collagenous proteins (NCPs) are assumed to play an important role in bone formation. NCPs are believed to regulate and direct crystal growth and orientation (Olszta et al., 2007). The process of osteogenesis proceeds in two stages. It starts from a bone precursor, the epiphyseal cartilage. The epiphyseal cartilage is composed of small (10-20 nm in diameter), very loose type-I collagen fibrils embedded in an amorphous ground substance. Also embedded in the ground substance, are matrix vesicles containing crystals and minerals required for the mineralization process. Mineralization is a rapid and unorganised process that results in the formation of a "woven" bone microstructure (Olszta et al., 2007). By the end of the primary ossification process, type-I collagen, which is not particularly calciphilic, does not assume the lamellar architectural organisation characteristic of bone, but rather loose and wavy and collagen remains unassociated with the hydroxyapatite crystals (Olszta et al., 2007). The proteoglycan matrix is the site of formation of the hydroxyapatite spherulites. Collagen does not appear to play an appreciable role in directing the mineralization process. Certain parallels could be drawn between the environment where primary bone ossification takes place and Bruch’s membrane. In this study, we report the presence of regular spherical structures embedded in a dense matrix. The spherules are also located in the outer collagenous layer of Bruch’s membrane opposite the drusen. The spherules are bounded by double-layered membrane-like structures. Some of the spherules contain tiny inner electron-dense particles. Some spherules are composed of concentric layers of alternating electron dense and less electron dense materials with a dense crystalline core and a thin shell. Ulshafer et al. carried out an X-ray energy spectra (Kevex) study on morphologically similar spherules with the same range of size (Ulshafer et al., 1987) and reported the spherules are primarily composed of phosphorus and calcium. While Ulshafer et al, reported calcium and phosphate, no mention of hydroxyapatite was made and no indication of the state of the development of AMD was made in the study report. In a study on the calcification of drusen and Bruch’s membrane in tissue from donors with early stage AMD, Van der Schaft et al confirmed the presence of calcium and phosphate with a ratio of 1.88:1 but reported that no hydroxyapatite was ob-
served (van der Schaft et al., 1992). Our X-ray data show that drusen display a great deal of heterogeneity in the degrees of crystallinity. Some are predominantly amorphous while others are very crystalline. Hydroxyapatite is the main component of the crystalline phase. Interestingly, even within single drusen, sub-regions differ considerably in their calcium content and hydroxyapatite content. In neoplastic bone disease presence of osmophilic solitary hydroxyapatite crystals within vesicles is a precursor to the accumulation of hydroxyapatite and disappearance of the vesicular membrane and its transformation into calcopherites constituting the calcification front (Hageman and Mullins, 1999). In the ageing environment Bruch’s membrane, a similar set of conditions is present. The spherules and vesicles of the same range of size, a supraphysiologic concentration of calcium, an extracellular matrix and an abundant extracellular matrix containing collagen. However, important differences exist between the process of primary ossification on one hand and osteosarcoma and drusen formation on the other hand.
3.7.3 Drusen Matrix

Our TEM data show that drusen are quite heterogeneous structures. Some drusen contain uniform electron-dense and electron-lucent spherular structures of varying sizes embedded in an amorphous or granular extracellular matrix. Also embedded in the matrix are cellular debris, curvilinear profiles, membrane-bound structures, vesicular elements and strands with periodic spacing similar to that of collagen fibrils. The ICZ and the OCZ of Bruch’s membrane contain collagen type IV, which has also been located in drusen. Collagen type I, is selectively found in drusen from patients with AMD and not drusen associated with normal ageing. Type I collagen exhibits a characteristic period banding due to its unique tertiary structure. According to the Petruska and Hodge model, the collagen supercoil is made up of three left-handed helices predominantly composed of the amino acids proline-hydroxyproline-glycine series. The three small helices intertwine to form the right-handed superhelix, the tropocollagen molecule. Tropocollagen superhelix is composed of three left-twisting smaller helices, each containing is 1000 amino acyl residues. The collagen triple helix has an axial ratio of about 200 giving it its fibrillar shape. Three left-twisting helices intertwine right-handed manner forming the collagen triple helix. This opposing orientation renders the collagen triple helix its great tensile strength (Murray, 2000). Self-organisation of into fibrillar collagen comes about as a result of secondary bonding and stabilisation between the tropocollagen molecules. The Hodge and Petruska’s quarter-stagger model postulates that this organisation leads to hole and overlap regions that give the characteristic period banding pattern on TEM micrographs. Glimcher et al. suggested that hole zones of the collagen superstructure serve as nucleation sites where mineralization takes places via the formation of hydroxylapatite (HA) crystallites (Glimcher, 2006) this process requires the participation of non-collagenous proteins (NCPs) with a high affinity for calcium in the matrix (Boskey, 1989).

In drusen, we speculate the presence of collagen is an important step in the biogenesis of drusen in two ways. It confers the mechanical strength by providing the nascent druse with a “skeleton” which allows growth and enlargement by the accumulation of proteins and lipids,
this structure allows the druse to counter the spatial constraints in this narrow sub-RPE area that would otherwise flatten out or prevent drusen from growing. Secondly, we postulate that collagen fibrils and matrix proteins in drusen play a role similar to that in the bone by creating conditions conducive to the growth of HA crystallite in drusen.
3.7.4 Drusen Mineral Phase

Our XRF data demonstrated the cluster pattern of calcium inside drusen. Unlike Zn, Ca is characterised by focal rather than generalised distribution inside drusen. Staining with a variety of calcium-specific dyes (for instance Alisarin-red) has confirmed our XRF findings and additionally demonstrated that calcium exists in regular spherules of 0.3-20\(\mu\)m in diameter. Outside these spherules, no staining for calcium was noticed. In our structural TEM data, we identified spherular patterns similar to those described as matrix vesicles found during primary bone mineralization, during embryonic development and mineralization of neoplastic bone tissue (Sela and Bab, 1979). Matrix vesicles are known to deliver calcium and minerals to the calcifying front in collagen fibres during the primary mineralization process of different tissues, under both physiologic and pathological conditions (Bab et al., 1979).

In our data, hydroxyapatite (HA) Debye-Scherrer patterns from different drusen demonstrated a wide degree of variation and ranged from wide shallow peaks to narrow high peaks. These findings are similar to those reported by Olsza et al. in a time series of experiments on collagen tissue with progressive mineralization (Olszta et al., 2007). They reported the initially broad peaks sharpen time as a result of growth of hydroxyapatite crystallites and increasing ordering of the HA molecules with time (Olszta et al., 2007). The predominantly amorphous drusen gave a Debye-Scherrer pattern with broad peaks and a low degree of autofluorescence at 488nm. The more strongly autofluorescent drusen, gave a Debye-Scherrer pattern with more pronounced peaks that are taller with a narrow FWHM. It is argued here that the difference in the Debye-Scherrer pattern between the two extremes of a continuum of patterns drusen is due to differences in the degree of mineralization of drusen and size of the HA crystallites.

Tiny crystallites in predominantly amorphous poorly crystallised and presumably “nascent” drusen would give rise to broad X-ray diffraction relative to the standard inorganic (HA) peaks. Aged drusen are arguably more crystalline and harden as they contain more calcium and would therefore give XRD patterns with stronger patterns. This assumption is corroborated by our XRF data which show that drusen are calcium-deficient and their Ca:P ratio in drusen is
Olszta et al. demonstrated a change in XRD patterns with narrowing and increasing width of peaks with increasing mineralisation. Patterns 3-5, show progressive mineralization process of artificial collagen at days 1, 2 and 6 respectively. Debeye-Scherrer patterns are compared to equine bone XRD (top) and to a commercial HA powder diffraction pattern. Photocredit: (Olszta et al., 2007)

below the stoichiometric value for inorganic hydroxyapatite (1.67). Similarly to bone tissue, this gives room for calcium deposition and mineralization. It is argued here that not only is calcium important for the structure of drusen, but also as a component that interacts with proteins in the drusen matrix and collagen to form hydroxyl apatite crystallites. The quantity of calcium present in a druse and its association with collagen forms the structural basis on behind the “hard” phenotype exhibited by some drusen. The degree of crystallinity of drusen partially reflects drusen age and availability of calcium for deposition and formation of HA crystallites.

3.7.5 Potential role for hydroxyapatite in drusen biogenesis

Hydroxyapatite (HA), is a crystalline form of calcium apatite whose chemical composition is commonly expressed as Ca10(PO4)6(OH)2 (Doonan, 2004) stressing the composition of its unit cell crystal of two units. In bone tissue and dental tissue (Pankaew et al., 2012), the
Hydroxyl apatite is modified by the replacement of OH- ion with fluoride, chloride or carbonate, creating fluoroapatite or chlorapatite. It crystallises in the hexagonal crystal system, it is the main component of the mineral phase of bone making up to up to 65% of bone by weight (Olszta et al., 2007). In dental tissue carbonated calcium-deficient hydroxylapatite is the main mineral of which dental enamel and dentin are comprised (Pankaew et al., 2012). Deutscher et al. summarised patterns of protein interaction with hydroxyapatite. HA crystals have both positively and negatively charged areas due to their constituent calcium ions, and phosphate groups respectively (Deutscher, 1990). Electrostatic binding takes place between the positively charged amino groups of proteins and the negative charges on the hydroxyapatite molecule while negative carboxyl groups bind by complexing with the positively charged calcium ions in the hydroxyapatite. Binding of acidic proteins can be increased by the presence of groups such as CaCl, and MgCl. Negative ions such as F, Cl, HPO selectively elute basic proteins (Deutscher, 1990).

It is argued here, by drawing parallels to drusen, hydroxyapatite crystallites located in the sub-RPE space, would act as skeletons to which proteins get adsorbed creating nidus points for drusen growth or accelerate the growth of drusen already formed. We postulate here the presence of hydroxyapatite crystallites in close proximity to collagen fibrils necessitates the action of modifying proteins and abundance of calcium supply in the area. Minerals and calcium delivery mechanism is required for the continued to supply the necessary calcium for the process of mineralisation. During primary ossification in bone tissue and during the calcification of osteosarcoma matrix vesicles play this role. In drusen, we have identified morphologically similar bodies in close association with collagen fibrils embedded in the matrix of drusen. We postulate that a similar mechanism exists for the delivery of calcium in drusen tissue.

The local physico-chemical environment of HA crystallites plays a modifying role on the properties of hydroxyapatite and its ability to act as a selective adsorption agent to certain proteins. The pH degree enhances hydroxyapatite ability to adsorb heavy metals Cd, Pb and Cu (Chen et al., 2010). Fuiji et al. demonstrated that replacement of the HO- with Zn...
to create ZnHA enhances the ability modified the physical dimensions of the hydroxyapatite crystals in a way that enhances its selectivity for adsorption to β2-Microglobulin (Fujii et al., 2006).

It is possible that the properties of hydroxyapatite crystallites in the sub-RPE deposits are modified by the supraphysiological concentrations of zinc and other metals and trace elements in such a that enhances adsorption of key drusen components in the area. The role of metals and hydroxyapatite merits further investigation.

### 3.7.6 Speculated Mechanism of drusen formation

Drawing parallels from the histological similarities between drusen and bone tissue could give some elucidative insights into mechanisms of mineralization and druse biogenesis. Bone is a biocomposite tissue in which minerals make up 65% of weight (Olszta et al., 2007). Bone’s mineral phase is composed of carbonated and non-stoichiometric HA with substitutions of Na⁺, K⁺, Mg²⁺, Sr²⁻, Cl⁻, F⁻, HPO₄²⁻ ions embedded in the extracellular matrix (Lima et al., 2006). These circumstances and metal composition are similar to what we found in drusen. The structural basis for mineralization is the presence of collagen type I which direct the process of primary osteogenesis. Osteoblasts release collagen type I molecules which self-organise into collagen fibrils assuming the architectural organisation described in the Petruska and Hodge Model (Petruska and Hodge, 1964). Calcium phosphate nanocrystals are delivered to the mineralization front via matrix vesicle synthesised by the osteoblasts well Hydroxyapatite nanocrystals epitaxially grow on collagen molecules (Kikuchi et al., 2011). The process of bone formation and mineralization is not fully understood. However, the similarities with drusen are numerous. Bruch’s membrane normally contain collagen type IV (Marshall et al., 1992), but drusen from AMD patients contain collagen type I (Newsome et al., 1987).

Here, we propose a sequence of events, which could explain our key findings and observations regarding the role of elements trace metals in drusen biogenesis or growth. We suggest that the supraphysiological concentrations of zinc in the sub-RPE space would have an effect on the normal turn over of the extracellular matrix of Bruch’s membrane. ECM homeostasis
is a delicate involving a balance between the biosynthetic and degradative processes. ECM breakdown is effectuated matrix metalloproteinases (MMPs) which have a functional requirement for Zn and Ca (Woessner Jr, 1991; Birkedal-Hansen et al., 1993; Kumar et al., 2009). The activity of the MMPs system is counterbalanced by inhibitors of metalloproteinases (TIMPs). The degree of ECM breakdown is controlled by the balance between the activities of MMPs and their inhibition by TIMPs. Zinc is known inhibitor of collagen degradation by dentin matrix metalloproteinase (de Souza et al., 2000; Osorio et al., 2011). Defects in the degradative processes of the remodelling system would result in the expansion of the extracellular matrix and drusen formation. Studies have documented a high concentration of TIMP-3 within drusen speculated to inhibit MMPs rendering drusen “cold spots” for proteolysis (Leu et al., 2002). Given the pathophysiologically high concentrations of bio-available zinc in Bruch’s membrane and in sub-retinal deposits, it is assumed here that MMPs are similarly inhibited and therefore contributing to the expansion of ECM of Bruch’s membrane.

Another mechanism by which zinc could conceivably play a role in the biogenesis and/or progression of drusen in the sub-RPE space is through its modulation of the adsorption properties of hydroxyapatite (discussed above). Indeed replacement of calcium for zinc in hydroxyapatite to form zinc apatite (ZnHA), was found to affects α- and c-axis unit cell parameters of HA creating a markedly reduced crystallites size with proportionately larger surface areas (Lima et al., 2006). Conceivably, this change alters the rates surface reactions (Fujii et al., 2006). As a consequence there is a reduction of crystallinity of HA (Lima et al., 2006). Animal studies have demonstrated that such a replacement has an acceleratory effect osteogenesis and lead to an increase in the area of formed bone in relation to HA (Calasans-Maia et al., 2008). The high concentration of zinc in the sub-RPE deposits could potentially modulate the physical characteristics of the HA crystal lattice to enhance its protein adsorption function and thereby facilitating attachment of proteins to HA crystallites in addition to the enhancement of the activity of matrix vesicles and that in turn would increase calcium and minerals availability to the mineralization front. The third mechanism by which we propose zinc might be involved in the formation and/or complication of already existing drusen is via
the induction of the immune system. Factor H normally inhibits the potentially toxic but key regulatory component of the complement system, C3b (Oppermann et al., 2006). Inhibition of CFH by zinc causes widespread polymerisation and over-activation of the complement system (Ruodan Nan et al. 2008). Given the presence of pathophysiologically high concentrations of bioactive zinc in drusen and the sub-RPE deposits, it is argued here that CFH zinc-driven oligomerisation although does not explain drusen formation but is part of the plethora of effects attributed to zinc in the sub-RPE deposits. It is possible that no single mechanism would be able to explain the wide array of pathological phenomenon seen in the sub-RPE deposits and drusen in which metals are involved. This is in part due to the wide range of biological activities in which metals and trace elements are engaged and also because of the nature of the complexity of the phenomenon itself. However, we maintain here, that this understudied area could provide important clues on the pathophysiology of metals and the role hydroxyapatite plays in the formation of drusen and sub-RPE deposits. A better understanding of this role could help direct future research and the quest for therapies in a whole new direction.
3.7.7 Conclusions

1. Zinc is abundantly found in all drusen in supraphysiologic concentrations where it could result from or complicate the pathophysiologic processes that are responsible for drusen biogenesis.

2. Soft drusen which constitute a risk for AMD contain significantly more zinc than hard drusen.

3. XRF demonstrated that hard drusen contain significantly more calcium than soft drusen.

4. XRD analysis demonstrated that calcium often crystallises to hydroxyapatite in drusen. Differences in the degree of mineralization of hydroxyapatite strongly correlate with druse’s autofluorescence.

5. A large proportion of the difference in autofluorescence intensity between drusen that underlie their autofluorescence heterogeneity could be explained by the quantitative differences in their metal content and calcium in particular.

6. Drusen also contain Cu, Ni, Co, Fe, K, Cl, S, P, Mg and Na at varying quantities the implications of which need to be elucidated.
Part III

Autofluorescence in MacTel Type 2
Chapter 4

Literature Review

4.1 Idiopathic Macular Telangiectasia (MacTel Type 2)

4.2 Epidemiology

Four large population-based studies have attempted to elucidate the question of the prevalence of MacTel type 2. Two of which were carried out on populations largely of Caucasian extract; the Beaver Dam study in the US and the Australian study. The Beaver Dam study reported a 0.1% prevalence (Klein et al., 2010), much higher than that reported in the Australian study (5 to 23 cases per 100,000 of individuals with stages II and III or higher who are 47 years of age or older) (Aung et al., 2010). The disconcordance in the results was attributed to the protocol followed in the Australian study in which no mydriatic agents were used which could have lead to under diagnosis of cases. Two studies conducted on black population from Kenya and Nigeria estimated the combined prevalence of the disease in those two countries to be 0.06% (Sallo et al., 2012). These figures are in general agreement to those drawn from largely white cohorts. All data published so far are based on the analysis of colour fundus images only and are thus likely to underestimate the true prevalence of this disease. However, it is known now the earliest evidence of an ensuing MacTel type 2, are subtle and currently only manifestable in fundus autofluorescence images (Wong et al., 2009). The reliance of the four
studies on colour fundus imaging for the diagnosis of MacTel type 2, could have lead to an underestimation of the prevalence of the disease. This data proves what was once thought of as rare condition is has in fact a much higher burden on the quality of life of many people. This information is of direct relevance to clinicians and health policy makers alike.

4.3 Genetics

Certain clinical features in MacTel type 2 suggest a genetic causation. The symmetry and the bilateral nature of the lesions of the disease in addition to the higher occurrence in monozygotic twins (Menchini et al., 2000; Siddiqui and Fekrat, 2005) and siblings and families (Hutton et al., 1978; Gillies et al., 2009) and families lead to a believe that genetic causation might have a role to play. Gillies et al. suggested the different clinical disease stages of the disease in a pair of identical twins could entail incomplete or variable penetrance of the disease (Gillies et al., 2009). Szental et al. examined two polymorphic sites of the GSTP1 gene in DNA samples from 39 MacTel type 2 with disturbed macular pigment. They identified the GSTP1 gene as a candidate causative gene in MacTel type 2 (Szental et al., 2010). Uptake of xanthophyll pigment into the macula is a process thought to be effectuated by the xanthophyll-binding protein (XBP). The GSPTP1, which is located at chromosome 11q13, is an isoform of glutathione S-transferase with a particularly high affinity for XBP binding. Parmalee et al. carried out a large candidate-gene study, which excluded the previously suspected missense mutation Pro33Ser at 4 (FZD4) as a causative gene. It concluded that the above-mentioned mutation is a benign polymorphism for familial exudative vitreoretinopathy. The study did not identify genes that could be linked to disease causation (Parmalee et al., 2010). Failure to identify causative genes for MacTel type 2 added to the potential links to systemic conditions and evidence of familial clustering of cases could hint at a complex multifactorial causation. Twins and family members do not just share genes, but also share similar microenvironments, which could prove important for the disease development. Incomplete penetrance and links to systemic diseases like diabetes mellitus and hypertension could mean that MacTel type 2 is multi-factorial.
in causation. Indeed it could share similarities with AMD and diabetes mellitus. Whether or not an individual develops MacTel type2 phenotype could be determined by the outcome of a complex interplay between environmental susceptibility and genetic predisposition as a result of or gene-gene-interactions and/or epigenetic influences. Identification of a candidate gene would boosts the development of animal models to help a better understanding of the disease.
CHAPTER 4. LITERATURE REVIEW

4.4 Clinical manifestations

4.5 Imaging

The original Gass-Blodi classification system was solely based on the imaging technology available at the time; fundus colour images (CF) and fluorescein angiography (FA). Development of several new imaging modalities provided unprecedented elucidative insights into tissue architectural derangements that helped construct a better image of the nature of the underlying possible pathophysiologic processes. The uncovering of new features of the disease could necessitate creation of a new staging system.

4.5.1 Colour Fundus Imaging (CF)

Is a widely used imaging modality in ophthalmology where the retina is illuminated with white light. Colour fundus imaging (CF) uses reflected light to create 2-D representations of the 3-D retinal tissues where image intensities represent the amount of a reflected light as recorded by the spectral sensitivity of the detector (Abràmoff et al., 2010). The earliest evidence of pathology in CF images in patients with MacTel type 2 consists of subtle temporal parafoveal retinal greying at or before the clinical diagnosis with MacTel type 2 stages II and I. Changes become more pronounced as the disease progresses clinically; capillary dilations and right-angled venules draining are seen draining the telangiectatic capillary networks. Characteristic RPE pigments proliferation and migration that takes the form of dark/black clumps around the dilated right-angled venules characterises the latter stages of the disease. At stage V, sub-retinal exudation and haemorrhage in addition to various types of anastomosis are evident on colour fundus images (Nowilaty et al., 2010). Small intensely shining superficial crystalline deposits are a common finding in colour images at all stages of patients with MacTel type 2 (Sallo et al., 2011). Colour Fundus imaging is a widely-used imaging modality in the clinical settings, however, images are dominated by the red component of the signal while most of the critical detail is green (Hubbard, 2009). It is common to enhance the contrast when
analysing the images by digitally removing the red component to create a red-free image (Abràmoff et al., 2010). In modern ophthalmic practice, the trend is to combine CF imaging with other modalities that give information on different aspects of health and disease of the fundus. Adoption of such imaging approaches in MacTel type 2 greatly widened the scope of the recognised pathological spectrum and phenomenology of the disease.

4.5.2 Fluorescein Angiography (FA)

A subtle increase in intraretinal staining on fluorescein angiography (FA) represents an early finding in the course of MacTel type 2. Stereoscopic examination of FA images shows a classical pattern of early rapid staining of morphologically thickened walls in the outer capillary network, predominantly in the temporal parafoveal area, followed by a late diffuse hyperfluorescence of the middle and outer retinal layers. Yannuzzi et al. described a disruption in the superficial retinal circulations with segmental dilation superimposed on dilated deep retinal telangiectatic vessels. (Yannuzzi et al., 2006). Right-angled retinal vessels and subretinal pigmented plaques are among the commonest findings on FA in MacTel type 2 patients. Dilated and incompetent veins and arteries also characterise the disease. Possibly in response to ischaemia, three types of anastomosis were described in patients with MacTel type 2; retino-retinal anastomosis (RRA) (Yannuzzi et al., 2006b), retinal-subretinal anastomosis (RSR) and retino-choroidal anastomosis (Nowilaty et al., 2010). Final stages of structural and functional deterioration in MacTel type 2 are characterised by the development of sub-retinal neovascularization (SRNV). Development of SRNV marks a rapid deterioration of visual function. On FA, SRNV is evident in the temporal parafoveal area and is followed by the development of exudation and subretinal haemorrhages and fibrovascular proliferation (Nowilaty et al., 2010).

4.5.3 Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) is a robust, non-contact, non-invasive ophthalmologic diagnostic imaging technology that provides in vivo images of retinal sections. High-resolution volumetric SD-OCT scans allow the visualisation of the microscopic details of the retinal ar-
Architecture. Modern SD-OCT could attain a resolution of 3-4 micrometers. The application of OCT imaging in MacTel patient has revealed a wealth of information on tissue architectural derangements at all clinical stages of the disease. To summarise OCT findings in macular telangiectasia type 2: Overall retinal thickness varies significantly between different MacTel type 2 patients and does not bear any clinically important relationship to observed hyperfluorescence visible on FA (Hageman and, 1999; Bottoni et al., 2010). It is more informative to investigate the individual retinal structures. Loss of the normal pit in the fovea has been described in all stages of the disease, in more severe cases a complete flattening has been observed (Yannuzzi et al., 2006a; Sanchez et al., 2007, 2007). Thinning and disruption of inner segment/outer segment photoreceptor junction (IS/OS) more readily visualised by OCT and outer retinal atrophy is a common and consistent finding (Gaudric et al., 2006; Paunescu et al., 2006; Cohen et al., 2007). The IS/OS disruption correlates with visual acuity and accelerates with the clinical progression of the disease (Paunescu et al., 2006; Cohen et al., 2007; Sanchez et al., 2007) In advanced cases substantial tissue loss is strongly evident by the presence of cyst-like structures at the foveola and inner retinal layers in 50-100% Stage III or higher (Gaudric et al., 2006; Paunescu et al., 2006; Yannuzzi et al., 2006). There is a consensus position that the evolution of the intraretinal cysts superimposed on an atrophic neurosensory retina is considered an important step in the clinical progression of the disease (Nowilaty et al., 2010). Although rare, it was reported these cysts could span the entire width of the retina (Koizumi et al., 2007). Visual acuity (VA) in eyes with cysts vision is typically between (20/40 to 20/70). Disease progression and an accelerated rate of visual loss had been linked to enlargement of macular cysts (Yannuzzi et al., 2006a). Investigators took advantage of the new insights provided by OCT to devise new OCT-based staging systems for MacTel type 2 (Cohen et al., 2007; Sanchez et al., 2007). In a 5-stage-classification system, Sanchez et al. proposed a stage 1 in which 62.5% of the patients had inner retinal highly reflective dots corresponding to microvessels evident on FA (Sanchez et al., 2007). In stage 2 is characterised by the presence of intraretinal cysts (81.8%) and highly reflective dots (90.9%). In stage 3, there thickening and disruption of RPE/choriocapillaris complex in 81.2% of the pa-
tients. Highly reflective areas caused by RPE proliferation, nasally or temporally to the fovea characterise stage 4. In stage 5, neovascularisation is evident in the scans as thickening and duplication of RPE/choriocapillaris complex highly reflective bands. The application of OCT in a longitudinal investigation could provide valuable time-sequenced information on the nature of retinal architectural changes that would help understand the natural history and progression of MacTel type 2. Such an application would not only help in understanding disease progression but could also prove valuable for monitoring of disease processes and effects of interventional strategies.

4.5.4 Confocal reflectance imaging

Confocal reflectance imaging is an imaging modality that utilises a blue short-wave laser with $\lambda_{exc}$ at 488nm or a longer wave-length infra-red laser source with $\lambda_{exc}$ at 820nm to reveal localised areas of altered reflectance in retinæ. The strength and applicability of the confocal blue reflectance ($\lambda_{exc}$ with 488nm) lies with its ability to detect clinically silent and otherwise difficult to detect early cases of MacTel type 2 (Charbel Issa, Finger, et al., 2008). Changes evident on CBR take the form of a well-demarcated oval-shaped area of increased reflectance in the temporal parafoveal area corresponding to and slightly bigger than its corresponding area of hyperfluorescence visualised in the late-phase fluorescein angiography (FA). The highly reflective inner segment-outer segment border of photoreceptor layer is an area for significant tissue structural derangements in MacTel type 2 is particularly well visualised using this technique (Charbel Issa, Finger, et al., 2008). On the other hand, confocal infrared reflectance (with $\lambda_{exc}$ at 820nm), is more suited for monitoring the disease progress. In early cases, it shows evidence of increased reflectance in affected areas as the disease advances areas of pigment clumping and choroidal neovascularization appear as hyporeflective areas (Charbel Issa, Finger, et al., 2008). The use of newer CBR imaging in future epidemiological studies on the prevalence of MacTel type will significantly improve the detection of early cases and should provide a more accurate picture of the impact of MacTel type 2, currently thought to be underestimated in the four major epidemiological studies conducted so-far.
4.5.5 Adaptive optics imaging (AO)

Adaptive optics (AO) is a novel imaging technology used to improve the performance of optical systems by reducing the effect of wavefront distortions. It was adapted from use in astronomical telescopes (Beckers, 1993) into retinal imaging systems (Liang et al., 1997) to reduce optical aberrations and provide photoreceptor-level optical resolution. Wavefront distortions are measured and errors are compensated for via the use of a deformable mirror. High-definition OCT Spectralis established disruptions in at IS/OS junction in patients with MacTel type 2; however, its resolution is not sufficient enough to resolve photoreceptor-level changes. AO is better suited for studying these miniscule changes. Ooto et al. using a prototype of adaptive optics coupled to a confocal scanning laser ophthalmoscope (AO-SLO) identified dark regions in the cone mosaic in eyes with MacTel type 2 (Ooto et al., 2011). These dark regions corresponded to areas of disruption in the IS/OS on SD-OCT images representing photoreceptor abnormalities to the areas of hyperfluorescence on fluorescein angiography (Ooto et al., 2011). No study on the effects of MacTel type 2 on rod photoreceptors has been conducted so-far using AO. Despite the fact that cones are outnumbered by rods by a factor of 20:1, the relatively smaller size of rods and their reduced waveguide capabilities (Alpern et al., 1983) meant it was not possible to adapt this technology for the imaging of rod photoreceptors. Recently Dubra et al. reported their ability to obtain high resolution rod mosaic images with either 680nm and 775nm light in agreement with histological studies (Dubra et al., 2011) Introduction of adaptive optics in both longitudinal and cross-sectional studies on MacTel type 2 patients, would give the best evidence (save in vitro investigation) on the structure-function derangements of MacTel type 2. Such information will be vital to construct a more lucid picture of the nature of the pathological processes operative at a photoreceptor level.

4.5.6 Macular pigment optical density (MOPD)

The macular pigment (MP) of the human retina is perceptible visually as the yellow spot at the centre of the macula (macula lutea). It has been charged with attenuating chromatic aberra-
tion of light through its function of absorbing blue light (Landrum and Bone, 2001). Macular pigment which is a combination of stereoisomers of lutein (L) and zeaxanthin (Z) (Bone et al., 2007), is highly enriched along the axons of the cone photoreceptors in the central retina. In addition to filtration of light and, functions of macular pigment as an anti-oxidative function of neutralising reactive oxygen species (Subczynski et al., 2010), an important function in the oxygen-rich lipid-rich neurosensory retina. The pattern of distribution of macular pigment has been reported to show a great deal of uniformity across the population with the largest concentration in at the macula lutea. The concentration rapidly drops with eccentricity to be lowest at eccentricities of 8 degrees. Using a subtraction technique of fundus autofluorescence images obtained with two different excitation wavelengths ($\lambda_{\text{exc}}$ at 488 and 514nm), a consistently abnormal pattern of distribution of MP in MacTel type 2 patients has been described (Charbel Issa et al., 2009). There is an oval-shaped depletion of MP at the central retina that is consistent with the hyperfluorescence area in the late-phase fluorescein angiography (FA). This area is encircled by a an area of spared MPOD at about 6° eccentricity (Issa and others, 2008; Al-Shamsi et al., 2010; Bottoni et al., 2010). Chabrel Issa et al. noted the disproportionate nature of the diminution of zeaxanthin concentration would suggest impaired trafficking and/or storage is likely involved in MacTel type 2. In vitro examination of one eye with MacTel type 2 corroborated conclusions drawn from in vivo imaging techniques (Powner et al., 2010). Evidence that oral supplementation of Higher animals including human lack the capability of synthesising carotenoids and therefore, rely on the diet for supply. Serum concentration of leutein and zeaxanthin displayed a dose-dependent response to supplementation (Bone and Landrum, 2010). Dietary supplementation of carotenoids has been explored as a way of boosting MP concentration in the treatment of age-related macular degeneration (AMD).

In MacTel type 2, daily oral supplementation of 12mg of lutein and 0.6mg zeaxanthin for the duration of 9 months lead to accumulation of macular pigment only to the baseline level, indicating that restoration of depleted MP at baseline might not be possible (Zeimer et al., 2010).
4.5.7 Fundus autofluorescence (AF)

Abnormalities in the autofluorescence in MacTel type 2 became evident soon after the introduction of the technique in clinical ophthalmology. A number of patterns of deviation from normal have been recognised in fundus autofluorescence images of MacTel type 2. Below is a summary: The earliest evidence of a supervening MacTel type 2 is subtle increases in central AF signal. These subtle increases are observed long before the more overt angiographic, MP changes or changes on OCT are evident (Wong et al., 2009). The subtle changes are attributable to early anatomic changes preceding vascular abnormalities, these changes could be due to the loss of macular pigment (Helb et al., 2008) and consequently its unmasking effect on autofluorescence and/or quantitative and qualitative changes in fluorophore (s) content of the affected areas (Wong et al., 2009). Gass and Blodi described a pattern of focal yellow foveal spots on funduscopic examination that does not follow the general pattern of temporal location in 4% of patients with MacTel type 2 (Gass and Blodi, 1993). Chabrel Issa et al postulated that the pathophysiologic correlate could be a focal deposition of fluorophores or shed photoreceptor outer segments persisting in the sub-neurosensory space (Charbel Issa et al., 2012). Using short-wave laser excitation, unbleached photoreceptor pigment has a mild attenuating effect on fundus autofluorescence by absorbing the exciting light and therefore reducing light quanta available for the excitation of LF granules (Theelen et al., 2008). Consequently, areas of photoreceptor atrophy exhibit increased autofluorescence intensity relative to areas of intact photoreceptors (Theelen et al., 2008).
4.6 Staging

Idiopathic macular telangiectasia type 2 (MacTel type 2) is the most prevalent of currently acceptable three subtypes of idiopathic macular telangiectasia. It tends to affect people between 38-82 years with mean ages of 55 to 59 (Gass and Blodi, 1993; Yannuzzi et al., 2006). No gender or racial predilections have been reported in the disease, which is bilateral but might be asymmetric. However, MacTel type 2 might start off as a unilateral condition and visual loss might also be disparate between the two eyes (Gass and Blodi, 1993). In their original work, Gass and Blodi devised their classification system on the basis of five interlocking stages of phenomenological derangements of MacTel type 2 that ultimately end with neovascularization and blindness. In 2006, Yannuzzi made use of new clinical, angiographic and optical coherence tomography (OCT) imaging observations. He suggested two distinct types: Type 1 "aneurysmal telangiectasia" to replace idiopathic juxtafoveal retinal telangiectasia (IJFT) group I (both A and B), and type 2 "perifoveal telangiectasia" equivalent to IJFT group IIA. (Yannuzzi et al., 2006). Yannuzzi also proposed a further simplification for the five stages of clinical progression into a mere two distinct stages. Nonproliferative and proliferative stage equivalent to the first four stages and the fifth stage of the Gass-Blodi classification respectively.

4.6.1 Stage I

Is largely asymptomatic and generally, ophthalmoscopically inconspicuous. Capillary dysfunction in the area might be detected in the late-phase of fluorescein angiography (FA) evidenced as staining (Yannuzzi et al., 2006) from which the fovea is normally excluded. Wong et al. reported that a tiny increase in foveal autofluorescence on fundus autofluorescence imaging (AF) is detectable in phase I where normally no functional or anatomical disruptions are evident (Wong et al., 2009).
4.6.2 Stage II

Marks the transition to the symptomatic stage for some patients; others might remain symptom-free or have minor changes in vision throughout stage II. Changes in the central vision include; blurred vision, metamorphopsia or paracentral positive scotoma. Anatomic changes constitute retinal parafoveal greying that might be partially or completely surround the fovea. If there is telangiectasia at this stage, it is often slight and no capillary thickening (Yannuzzi et al., 2006). Two distinct patterns are manifested on fluorescein angiography (FA), there is an initial phase of rapid staining of the walls of the thickened capillary vessels in the temporal parafoveal area followed by diffuse staining during the late-phase of FA mainly in the middle and outer retina. Crystalline deposits are commonly seen.

4.6.3 Stage III

Is characterised by a slow and insidious deterioration of vision. Anatomically, slightly dilated right-angled telangiectatic vessels are evident on biomicroscopy. These vessels now thought to be of both arteriolar and venular origin are thought to develop temporally forming part of a proliferating capillary network in the deep layers of the retina. On FA, beneath the right-angled capillaries there is an abnormal capillary permeability change due to dilation beneath the right-angled capillaries. No fluid collections or cystic changes in the plexiform layer are evident in this stage (Al-Shamsi et al., 2010).

4.6.4 Stage IV

Loci of clumps of retinal pigmented epithelial could be seen around the parafoveal right-angled vessels. There is hyperplasia and migration of the RPE cells along the abnormal vessels. Thus the stellate plaques are evident on colour or AF imaging. Collectively, the first four stages make the non-proliferative stage according to the simplified classification proposed by Yannuzzi et al. (Yannuzzi et al., 2006a).
4.6.5 Stage V

Is the equivalent of the the proliferative stage in Yannuzzi’s proposed classification (Yannuzzi et al., 2006). This stage is characterised by sub-retinal neovascularization (SRNV). There is retinal capillary remodelling alongside proliferation and invasion of the outer retina which is already weakened by atrophy, this combination of events culminates in sub-retinal neovascularization. There are three types of anastomosis that could be seen, retino-retinal (RRA), retinal-sub-retinal and retino-choroidal could be seen. SRNV starts temporally and is associated with an accelerated visual decline brought about by a combination of factors seen on FA. Exudation, neurosensory elevation, intra and sub-retinal haemorrhages, and fibrovascular proliferation are all seen on FA angiograms. Further gross morphologic distortion of the tissue occurs as a result of dragging of neighbouring vessels.

Tiny superficial golden crystals with characteristic hyper-reflective on OCT may be seen in NFL layer at any stage of the disease. Often located anterior to the retinal vessels overlying the telangiectatic area. These crystals were attributed to crystals resulting from Muller cells degeneration. These crystals are an inconsistent finding in MacTel type type 2.
4.7 Histopathology

The main histopathological features of MacTel type 2 have first been described by Green et al (Green et al., 1980). The major features are: (1) Narrowing of the lumen of retinal capillaries in patients with MacTel type 2 brought about by a combination of proliferation of the basement membranes and thickening of capillary walls. (2) Global degeneration of pericytes, sporadically of endothelial cells that extends well beyond temporal parafoveal area. (3) Cellular debris from degenerated endothelial cells and pericytes, lipid particles were entangled between layers of capillary basement membrane. (4) Focal defects in the anatomical integrity of endothelial cells mainly in the temporal parafoveal area were described. (5) Intracellular and extracellular oedema more marked in the inner retinal layers. (6) Proliferation of the retinal capillaries and invasion of the outer retina that might extend as far as photoreceptors.

These findings were substantiated in a histopathological case report study performed on a MacTel type 2 patient with SRNV, Eliassi-Rad and Green noticed two additional features (1) Intraretinal migration of the RPE along the course of the telangiectatic vessels (1) Proliferation and dilatation of the retinal capillaries which extend to invade the outer retinal layers and the subretinal space creating retino-choroidal anastomosis (Eliassi-Rad and Green, 1999). The findings of the two previous reports were corroborated in a more recent clinic-pathologic investigation by Powner et al. who additionally reported there is a reduction in collagen type IV, a vital component of the basement membrane which implicating retinal vascular pathology (Powner et al., 2010). More importantly, Powner et al. also reported a markedly reduced expression of Müller cell specific markers in the central macula (Powner et al., 2010).

4.8 Pathophysiology

Gass and Blodi suspected a primary role for the retinal capillary (Gass and Blodi, 1993). Metabolic defects resulting from altered capillary wall would result in increased capillary permeability that generates a state of chronic nutritional deficit in the middle retinal layers and in particular Muller cells and photoreceptors (Gass and Blodi, 1993). A cascade of events is
triggered as a result of the nutritional insufficiency in the retina; whereby, additional architectural and functional changes in the retinal venules would result in the generation of the right-angled venules further accentuating the nutritional deficiency of the middle retinal layers (Nowilaty et al., 2010). This nutritional deficiency would ultimately lead to the death and degeneration of the photoreceptors layer, visual decline and would therefore permit the RPE migration and proliferation to clump in the vicinity of the right-angled venules. With the introduction of modern imaging modalities in MacTel type 2, the view widely held now, is that MacTel type 2 could principally be the results of a neurodegenerative process in the retina. It has now become clear that the earliest changes in MacTel type 2 are manifested in the outer retinal layers. Hyperreflective spots close to the ILM were described in MacTel patients in the pre-clinical stages of the disease (Baumüller et al., 2010). Baumüller et al. showed these spots preferentially develop in the vicinity of zones of IS/OS disruption and intraretinal cystoid spaces (Baumüller et al., 2010). Crystalline deposits reported to exist in 46% of MacTel type 2 patients 60% of which are bilateral (Sallo et al., 2011). These hyperreflective deposits frequently observed in MacTel type 2, are believed to be composed of lipids and to represent remnants of dead Muller cells (Gass and Blodi, 1993). Muller cells secrete vascular endothelia growth factors and perform various important supportive function in the retina (Stone et al., 1995; Provis et al., 2000). Dysfunction or death of Muller cells, is forecasted to result in retinal endothelial cell degeneration (Yannuzzi, Bardal, et al., 2006) or an augmented rate of repeated cycles of cell death and replacement (Green, 1999) as mechanisms to explain the proliferation of retinal capillaries and the neurosensory degeneration manifest as thinning of the outer retinal layers. Chabrel Issa et al. speculated that loss of retinal transparency might also result from Müller cell dysfunction in the view that Muller cells as “optical fibres” or conduits that facilitate light transmission through the retina first reported by Franze et al. (Franze et al., 2007).

Precise pathophysiologic mechanisms for MacTel type 2 are still far from clear at the moment. Precise morphologic measurements in longitudinal studies using novel imaging modalities such as AO in combination with functional testing and most importantly in vitro
investigation should be a powerful approach that would provide elucidative insights into the nature of the basic pathophysiologic processes ultimately responsible for the disease.

4.9 Differential Diagnosis

In early stages of MacTel type 2, some eyes exhibit yellow foveal lesions that could be mistaken for Best’s disease or adult vitelliform dystrophy (Nowilaty et al., 2010). SRNV and macular pigment plaques hold some phenomenological similarities to age-related macular degeneration (AMD). However, the whole mark of AMD, but drusen are absent in MacTel type 2 (Nowilaty et al., 2010). Coat’s syndrome normally presents in male children or juveniles, it is usually unilateral or asymmetrical (Tarkkanen and Laatikainen, 1983). Hereditary haemorrhagic telangiectasia has some bilateral but rare ocular features, could have some similarities. However, it affects all ages and presents with characteristic sub-lingual and epidermal telangiectasia (Horn et al., 1981; Porteous et al., 1992). Sickle cell maculopathy could either be unilateral or bilateral. It normally presents in childhood or early adulthood on background of a long history of sickle cell disease in people with black or Mediterranean ancestry (Graham and Gartner, 1980; Horn et al., 1981). Stargardt’s disease, which presents with characteristic speckle-like appearance in FA images (Lois et al., 2004). Radiation retinopathy (Horn et al., 1981) has an appearance that in part resembles MacTel type 2. As the development of new imaging modalities continue, the body of knowledge on various aspects of MacTel type 2 expands, diagnosis features are better defined.
4.10 Functional Deficits

4.10.1 Visual Acuity (VA)

Clemons et al. investigated the baseline visual acuity of MacTel type 2 at enrolment in the study. She reported that VA was 20/20 or better in 16% of the patients and the mean VA is 20/40 in 522 untreated eyes (better eye 20/32, worse eye 20/50) (Clemons et al., 2010). Patients at the later stages of the disease had lower VA scores (Clemons et al., 2010). Anatomical considerations render visual acuity testing not particularly sensitive for estimating the functional impairment of MacTel type 2. Abnormalities in MacTel type 2 are principally temporal to the fovea while VA essentially tests foveal function and therefore VA may not represent the optimal method to assess macular dysfunction as significant tissue derangements could develop outside the fovea. Watzke et al. reported that 75% eyes from patients with MacTel type 2 had VA drop to below 20/80 s over a follow-up period of 10 to 17 years (Watzke et al., 2005). Charbel Issa et al. described a two-phase decline of visual acuity with clinical progression of MacTel type 2 (Charbel Issa et al., 2010). An initial slower phase with VA not worse than 20/50. He argued that this slow phase might be due to low-grade chronic vascular leakage or the development of hyporeflective cavities in the inner retina. By the time VA is about 20/200 significant atrophy of foveal photoreceptors might have already occurred causing deep paracentral scotomata in addition. A faster rate of loss of VA is normally due to developing macular hole or neovascularization. VA rarely drops to below 20/200 but severe functional impairments are likely to be present.

4.10.2 Fine Matrix Mapping (FMM)

The need for a more accurate measure to estimate visual function lead to the development and increasing use of fine matrix mapping in lieu of Visual acuity testing (VA) which only measures foveal function (central vision) which is only one aspect of visual function. FMM proved itself to be more accurate and useful in the diagnosis and follow up of patients with various retinal conditions. FMM allows the investigator to draw exact topographic maps correlating fundus
details and light sensitivities. Accurate quantification of retinal thresholds over small, discrete retinal lesions and therefore is suitable for longitudinal follow up in focal points of the retina where drusen, deposits and CNV lesions are found (Midena, 2006). Issa et al. demonstrated that parafoveal scotomas in patients with MacTel type 2 topographically correspond to areas with evidence of tissue damage on microscopy and on fluorescein angiography (Issa et al., 2007). Scotomas are immediately surrounded by an area of normal retinal sensitivity (Issa et al., 2007). Based on these observations, Issa et al. suggested angiographic leakage is an antecedent event to functional deterioration in patients with MacTel type 2 (Issa et al., 2007). FMM has proved to possess the necessary sensitivity to detect minute functional changes in retinas of patients with MacTel type 2 in longitudinal studies over short periods of time (Schmitz-Valckenberg et al., 2009). Central visual fields defects in the form of metamorphopsia (positive scotoma) are a common symptom in MacTel type 2. Chabrel Issa et al reported that metamorphopsia in the nasal quadrant of the visual fields was found in 83% of eyes with nonproliferative MacTel type 2 (Charbel Issa et al., 2009). All four eyes with SRNV were reported to have metamorphopsia. Tissue distortion was more pronounced at the nasal and lower quadrants and that the degree of distortion was frequently but not always correlated with the degree of leakage on fluorescein angiography (Charbel Issa et al., 2009). In stages I & II of MacTel type 2, retinal sensitivity is usually preserved. As the disease progresses, sensitivity decline is most evident at about 2 degrees eccentricity in the temporal and temporal inferior quadrants (Issa et al., 2007). In stage III, scotomata may develop depending on the extent of photoreceptor loss. Stages IV and V of MacTel type 2 are characterised by absolute scotomata due to substantial photoreceptor loss at areas corresponding to retinal pigment hyperplasia or neovascularization. Such scotomas could enlarge further to eventually become central scotomas (Issa et al., 2007). In summary MacTel type 2 has a significant impact on visual functioning, and vision-related quality of life functions even when VA is not significantly reduced. The use of fine matrix mapping could be a better alternative to the use of VA to monitor disease progression in longitudinal studies.
4.10.3 Vision-related QoL

One longitudinal study examined the impact of MacTel type 2 on vision-specific QoL using a Rasch-calibrated instrument (Lamoureux et al., 2011). The study reported that during the course of 24-months-period, no significant deterioration in four vision-specific QoL indices in patients living with MacTel type 2 following the initial assessment and no significant decline in VA was observed (Lamoureux et al., 2011). Fine matrix mapping of scotopic rod function was found to be very sensitive in detecting small-scale changes (Schmitz-Valckenberg et al., 2008). In a longitudinal study encompassing functional and morphologic changes in 9 patients with MacTel type 2 assessed over a period of one year with a battery of tests. The tests included repeated scotopic and photopic fine matrix mapping (FMM), photopic microperimetry, visual acuity and imaging studies. Visual acuity and microperimetry changes in one year were found to be well within the test–retest variability (Schmitz-Valckenberg et al., 2009). However, minute structural changes, which included increased dilation of telangiectatic blood vessel, increased hyperfluorescence in late-phase fluorescein angiography and a minor increase in pigment migration. Using a dense grid, FMM demonstrated scotopic dysfunction in one year (Schmitz-Valckenberg et al., 2009). Longitudinal assessments over a longer time period are required to provide more elucidative information on the structural and functional impact of MacTel type 2. In a cross-sectional investigation that compared the vision-targeted HR-QoL with cohorts of normal controls and patients with other blinding conditions, Clemens et al. reported that patients with MacTel type 2 have markedly lowered HR-QoL scores even for patients with other blinding conditions and who have comparable VA scores (Clemons et al., 2010).
Chapter 5

HADs: Prevalence & Distribution

5.1 Overview

Introduction: Idiopathic Macular Telangiectasia Type 2 (MacTel type 2) preferentially affects the temporal area of the macula and leads to metamorphopsia and scotomas that interfere with vision-related quality of life. Earliest changes of the disease evident on fundus autofluorescence (AF) imaging consist of increased autofluorescence temporal to the fovea. Here we study a hitherto undescribed pattern of focal hyperautofluorescent deposits (HADs) in the macula of patients living with MacTel type 2. The aim of this study was to analyse the prevalence and the location of such deposits in comparison to family members and controls.

Methods: A cross-sectional approach was adopted in the examination of CF and AF images of 107 patients, 80 controls and 177 family members. Additionally, 204 AMD patients and 93 patients with DM were examined in a similar manner. Eyes were graded for the presence, location and of the deposits. Grading results were correlated with demographics and clinical parameters.

Results: HADs prevalence was 51% in patients, 38% in controls, 51% in affected family members (AFF) and 27% unaffected family members (UnA) and 26% in possibly affected family members sub-category (PA). The prevalence of deposits on colour fundus images was 75% in patients 78% in controls, 73% in AFF sub-category, 66% in UnA and 78% in PA sub-
category. HADs showed a clear predilection to the temporal parafoveolar region in MacTel type 2 patients. In AMD and DM patients, HAD did not show any spatial predilection. The prevalence of HAD deposits correlated significantly with higher fasting blood glucose levels (6.2mmol/L ± 2 versus 4.9mmol/L ± 1) in the HAD group and the NoHAD group respectively (P < 0.05). Patients in the HAD group exhibited impairment in the oral glucose tolerance test (OGTT) at the 60 and 120 min time points. Blood glucose values were (10.1 mmol/L ± 0.9 and 8.2 mmol/L ± 0.8 respectively. Respective values in the NoHAD group were 7.4 mmol/L ± 0.8 and 6.3 mmol/L ± 0.3 for the 60- and 120 min time points respectively.

Conclusion: HADs are fairly common in patients with MacTel type 2. These deposits show a strong predilection to the temporal parafoveal area of the fundus. Links to impaired glucose metabolism in MacTel type 2 patients suggest possible involvement of the abnormal glucose metabolism. Further investigation of the molecular nature of the deposits and associated tissue derangements is essential in elucidating their role and clinical significance in MacTel type 2.
5.2 Introduction:

Biomicroscopy and fluorescein angiography helped establish the basic features of MacTel type 2 and formed the basis for the widely-used Gass-Blodi disease staging system (Gass and Blodi, 1993). New imaging modalities such as OCT, MPP, MP considerably expanded our knowledge of pathological and physiological processes caused by the disease. In vivo human fundus autofluorescence (AF) is a relatively new spectrophotometric technique. Image is derived from lipofuscin at the level of the RPE. The combination of the blue-green $\lambda_{\text{exc}}$ at 488nm and a short wavelength cut-off filter at 521nm along with the confocal nature of the optics used in the system, ensures that most of the detected emission is derived from lipofuscin (Delori et al., 1995). It has been shown that Bruch’s membrane and drusen have a significant contribution to the fundus autofluorescence signal with $\lambda_{\text{exc}}$ at 488nm. Lipofuscin being identified as the main fluorophore in the fundus (Eldred and Katz, 1988), is the base of autofluorescence imaging. Essentially, an autofluorescence image is a topographical map of the distribution of lipofuscin. The fundus autofluorescence emission is modified by absorption by macular pigment. In MacTel type 2, an early sign of an ensuing disease is the loss of the masking effect of macular pigment on autofluorescence in the fovea. The increased foveal autofluorescence has been attributed to redistribution of MP. The decreased density of MP observed, is thought to explain the observed unmasking in central area on FAF. This is one of the earliest detectable anatomical changes even before vascular derangements are observed (Wong et al., 2009). Multimodal approaches of investigation, established a link between the significantly increased foveal autofluorescence on one hand and retinal atrophy and decreased sensitivity on the other hand. Decreased autofluorescence was related to presence of scotomas on MP (Maruko et al., 2008; Wong et al., 2009). During grading for AF changes it become apparent that an additional pattern of autofluorescent changes in MacTel type 2 exists, one that cannot be explained by redistribution of macular pigment. Many patients exhibit small, focal, hyperautofluorescent deposits (HADs) in the macula an observation that has not been described before. In this cross-sectional study we provided a description for the prevalence and spatial distribution of
HADs in MacTel type 2 patients in comparison with normal age-matched controls and family members. Secondly, we compare the prevalence and patterns of distribution of HAD deposits to drusen in AMD patients and to the phenomenologically similar deposits in diabetes mellitus. Thirdly, a detailed description of HADs prevalence & patterns of distribution and the demographic and clinical parameters of MacTel type 2 patients.
5.3 Methods

5.3.1 Population

Participants in the MacTel study are recruited from 22 different clinical centres located in 7 countries (Australia, France, Germany, Switzerland, Israel, United States and United Kingdom). Ethics approval for the study was sought locally for each centre. At each participating centre, patients were recruited by the principal investigator. Moorfields Eye Hospital Reading Centre (MEHRC), located in London (UK), confirms the diagnosis prior to enrolment. Exclusion criteria included (1) diabetic retinopathy with 10 or more micro-aneurysms and/or small retinal haemorrhages; (2) presence of other confounding ocular conditions that may complicate the evaluation of macular telangiectasia; or (3) known allergies to fluorescein. In addition to patients, participants also included family members and unrelated controls. MacTel patients were selected at random, 107 patients (428 AF and CL images), all controls (80), (320 AF and CL images), 23 affected family members (92 AF and CL images), and 47 possibly affected family members (188 AF and CL images) and 107 unaffected family members (428 AF and CL images). In addition to the participants in the MacTel study, 212 AMD patients (624 AF and CL images) and 95 patients with DM (380 AF and CL images) were examined per subfield for patterns of distribution of HADs.

5.3.2 Procedure

Upon signing a consent form, information on age, gender, smoking history (current and past), past medical history including history of diabetes mellitus (DM), hypertension (HTN) and ocular history were collected. Laboratory tests were carried out including tests for carbohydrate metabolism; fasting blood glucose test (FBG), oral glucose tolerance test (OGTT) and glycosylated haemoglobin level (HbA1c), triacylglycerides (TAGs). Fasting lipid profiles, which include total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured. All clinical and laboratory data were entered onto an electronic database at EMMES Corporation, USA.
<table>
<thead>
<tr>
<th>Sub-category</th>
<th>Modality</th>
<th>Clinical Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFF</td>
<td>CF</td>
<td>Loss of retinal transparency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Superficial crystalline deposits</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>Temporal unmasking of autofluorescence</td>
</tr>
<tr>
<td></td>
<td>BLR</td>
<td>Telangiectatic capillaries</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loss of retinal transparency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crystalline deposits</td>
</tr>
<tr>
<td></td>
<td>OCT</td>
<td>Disruption in the IS/OS junction at the fovea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intraretinal pigment migration temporally</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cystoid spaces</td>
</tr>
<tr>
<td>PA</td>
<td>CF</td>
<td>Yellow deposits in the temporal region</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>Increased autofluorescence temporally</td>
</tr>
<tr>
<td></td>
<td>BLR</td>
<td>Loss of retinal transparency temporally</td>
</tr>
<tr>
<td></td>
<td>OCT</td>
<td>No abnormalities in the IS/OS junction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No cystoid spaces</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No intraretinal pigment migration</td>
</tr>
<tr>
<td>UnA</td>
<td>CF</td>
<td>No abnormalities</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>No abnormalities</td>
</tr>
<tr>
<td></td>
<td>BLR</td>
<td>No abnormalities</td>
</tr>
<tr>
<td></td>
<td>OCT</td>
<td>No abnormalities</td>
</tr>
</tbody>
</table>
Following a standardised ophthalmic examination, which includes best-corrected visual acuity (VA), colour fundus imaging (CF) and fundus autofluorescence imaging (FA) were carried, images were obtained in tiff format, anonymised and sent to MEHRC. Once the images were deemed gradable at the MEHRC, the standard grid described before was superimposed using Adobe Photoshop. In brief, the grid is composed of three concentric circles and two radial lines from 1:30 to 7:30 and the 4:30 to 10:30 meridians. The inner, middle and outer circles have a radius of 500µm, 1500µm and 3000µm respectively. Therefore the grid defines nine subfields starting from the middle in a clockwise fashion are: central I, inner temporal II, inner inferior III, inner nasal IV, inner superior V, outer temporal VI, outer inferior VII, outer nasal VIII and outer superior. Corresponding CF and AF images were matched and HAD deposits’ presence was indicated per subfield. Once this was completed and agreement was reached with the senior grader (Irene Leung) and in case of disagreement with the designated senior clinician (Alan C. Bird or Tunde Peto), the presence and the location of HAD deposits was entered into the final database. Once the patient was deemed to have MacTel type 2, first-degree family members were identified and imaged by the participating centres using the MacTel protocol. Those confirmed to have changes related to MacTel were invited to join the study as probands and their first-degree relatives were assessed as well for the condition. Following examination, family members were grouped into three subcategories: I. Affected family members: are individuals who display all the characteristic imaging changes associated with MacTel type 2 but asymptomatic. Those family members were invited to be probands. II. Possibly affected: are individuals who display imaging abnormalities characteristic of MacTel in some but not all imaging modalities and who don’t have clinical symptoms and signs of MacTel type 2. III. Unaffected: are family members who do not fall into either of the two sub-categories mentioned above. i.e. free of clinical signs and symptoms and do not have any imaging abnormalities associated with MacTel type 2 in all imaging modalities.
The standard MacTel grid is composed of three concentric circles and two radial lines and three concentric circles. The inner circle whose geometrical centre is foveola, middle and outer circles have radii of 500µm, 1500µm and 3000µm respectively. Therefore the grid sub-divides the fundus into nine subfields shown in the figure and table above.
5.3.3 Grading for hyperautofluorescent deposits (HADs):

Discrete hyperautofluorescent deposits (HAD) in AF images were identified by their circumscribed borders and elevated autofluorescence intensity compared to the background. In CL images, deposits were identified on the basis of their distinct colour and borders. In both AF and CL images, deposits were counted per subfield. All grading was carried out without any information on the subject’s clinical and laboratory characteristics. Once grading was completed and the database was locked, clinical information from EMMES was obtained. For this analysis, Age is defined as age at the time of enrolment in the study. A subject was defined as having diabetes if he or she had a previous history of diabetes mellitus and was treated with insulin, oral hypoglycaemic agents, diet, or a combination of the above or was newly discovered to have DM at enrolment based on the WHO criteria. Hypertension was defined as a mean systolic blood pressure of $\geq$140 mm Hg, a mean diastolic blood pressure of $\geq$90 mm Hg, a history of hypertension, or the use of antihypertensive medications at the time of enrolment into the study. History of cigarette smoking at the time of the baseline examination was determined as follows: a subject was classified as a non-smoker if he or she had smoked fewer than 100 tobacco cigarettes per lifetime. Ever smoked indicates a person who had smoked more than 100 tobacco cigarettes per lifetime and who no longer smokes. Current smoker is an individual who presently smokes tobacco. Unpaired T-test and Chi-square were done to test significance of differences. Numbers were reported as percentages and means with standard deviations (SD) or with standard error of the mean (SEM).
CHAPTER 5. HADS: PREVALENCE & DISTRIBUTION

5.4 Results

5.4.1 General characteristics and demographics

Table (5.2) shows basic characteristics, past medical history and HAD prevalence in MacTel patients, controls and family members. MacTel type 2 patients, controls and family members were found to have similar characteristics with respect to age and gender composition. The mean age in MacTel patients at the time of enrolment into the study was 58 years ± 0.6 and 59 years ± 1.4 in control individuals. In the family members group, mean ages were 59 years ± 2, 44 years ± 1.6 and 47 years ± 1.6 for the affected, possibly affected and the unaffected subgroups respectively. 34% of MacTel patients have a history of DM significantly higher as opposed to 6% in controls. Affected and possibly affected family members have 13% and 27% prevalence of DM. HTN is also higher among MacTel patients (51%) as compared to controls (29%). History of current smoking is comparable across the different groups and ranged from 10% in controls to 23% in possibly affected family members group. History of past smoking ranged from 29% in the unaffected family members group to 67% in the possibly affected family group. The combined mean VA was lowest in MacTel patient’s patients (69 ± 1.1) compared to controls (86 ± 0.6). In the family members group, affected family members (79 ± 2.1), possibly affected family members (87 ± 1) and the unaffected family members group (87 ± 0.6).
5.4.2 Prevalence of Deposits in CL and AF images

In table (5.2), the prevalence of HAD was 51% in MacTel patients compared to 38% in Controls. In the affected family members sub-group, HAD prevalence was 51% compared to 26% and 27% in the possibly affected and the unaffected groups respectively. Prevalence of deposits in CL images showed no significant variations between MacTel patients (75%), controls (78%), affected family members (73%), possibly affected family members (78%) and unaffected family members (66%).
MacTel patients, controls and family members were found to have similar characteristics with respect to age and gender composition. The mean age in MacTel patients at the time of enrolment into the study was 58 years ± 0.6 and 59 years ± 1.4 in control individuals. In the family members group, mean ages were 59 years ± 2, 44 years ± 1.6 and 47 years ± 1.6 for the affected, possibly affected and the unaffected subgroups respectively. 34% of MacTel patients have a history of DM significantly higher as opposed to 6% in controls. Affected and possibly affected family members have 13% and 27% prevalence of DM. HTN is also higher among MacTel patients (51%) as compared to controls (29%). History of current smoking is comparable across the different groups and ranged from 10% in controls to 23% in possibly affected family members group. History of past smoking ranged from 29% in the unaffected family members group to 67% in the possibly affected family group. The combined mean VA was lowest in MacTel patient’s patients (69 ± 1.1) compared to controls (86 ± 0.6). In the family members group, affected family members (79 ± 2.1), possibly affected family members (87 ± 1) and the unaffected family members group (87 ± 0.6). HAD prevalence was 51% in MacTel patients compared to 38% in Controls. In the affected family members sub-group, HAD prevalence was 51% compared to 26% and 27% in the possibly affected and the unaffected groups respectively. Prevalence of deposits in CL images showed no significant variations between MacTel patients (75%), controls (78%), affected family members (73%), possibly affected family members (78%) and unaffected family members (66%).
OS and OD autofluorescence images of a MacTel type 2 patient. Clinical AF imaging depends on $\lambda_{exc}$ with 488nm, it gives a topographical distribution of fundus autofluorescence. HADs exhibit the typical topographical distribution with a clear predilection to the temporal para-foveal area. HADs are characterised by their distinct autofluorescence that is greater in intensity than the autofluorescence of their immediate niche and that of the fundus. There is also the characteristic “unmasking” and increased autofluorescence in the fovea.
5.4.3 Carbohydrates tests & fasting lipid profiles

Table (5.3) shows laboratory test results for participants at enrolment into the MacTel study. Glycosylated Hb percentage was $(6 \pm 0.1)$ in MacTel patients, controls, and affected and unaffected family members. In the possibly affected family members sub-category, HbA1c percentage was $7 \pm 0.1$. The FBG in MacTel patients was $8\text{mmol}/\text{L} \pm 1.4$ significantly higher than controls $(5\text{mmol}/\text{L} \pm 0.2)$. In the subcategories of family members, FBG concentrations were $5\text{mmol}/\text{L} \pm 0.1$, $5\text{mmol}/\text{L} \pm 0.6$ and $5\text{mmol}/\text{L} \pm 0.8$ in the affected, possibly affected and the unaffected sub-categories respectively. The latter three were not significantly different from controls.

Figure (5.5) illustrates the OGTT test results for MacTel patients at baseline. After an overnight fast for 8–12 hours prior to the tests, in the following morning patients were given a standard liquid glucose dose and venous blood was drawn at 0, 30, 60 and 90-minute-intervals for measurement of serum glucose. The mean initial serum glucose concentration value at 0 minutes was $7\text{mmol}/\text{L} \pm 2$. At 30 minutes, mean serum glucose concentrations were $12\text{mmol}/\text{L} \pm 3$. At the 60 and 90-minutes time points, serum glucose concentrations remained elevated at $15\text{mmol}/\text{L} \pm 5$ and $10\text{mmol}/\text{L} \pm 2$ respectively. In the fasting lipid profile measurement, the total cholesterol value for MacTel patients and controls was $5\text{mmol}/\text{L} \pm 0.2$. In the subcategories of family members, total cholesterol values were $6\text{mmol}/\text{L} \pm 0.4$, $4\text{mmol}/\text{L} \pm 0.5$ and $7\text{mmol}/\text{L} \pm 0.1$ respectively. HDL concentration in MacTel patients was $1.3\text{mmol}/\text{L} \pm 0$ not significantly different from controls $(1.4\text{mmol}/\text{L} \pm 0.1)$. In the category of family members, HDL values were; $1.4\text{mmol}/\text{L} \pm 0.1$, $0.9\text{mmol}/\text{L} \pm 0.2$ and $2\text{mmol}/\text{L} \pm 0.7$ for affected, possibly affected and unaffected family members respectively. Mean LDL values in MacTel patients were $3\text{mmol}/\text{L} \pm 0.1$ not significantly different when compared to $3\text{mmol}/\text{L} \pm 0.2$ in controls. LDL values were; $4 \pm 0.5$, $2 \pm 0.6$ and $4 \pm 0.5$ in the affected, possibly affected and unaffected family members, respectively. Mean TAGs values were $7\text{mmol}/\text{L} \pm 1.3$ in MacTel patients and $2\text{mmol}/\text{L} \pm 0.4$ in controls. In the family members category, mean LDL values were $1\text{mmol}/\text{L} \pm 0.2$, $2\text{mmol}/\text{L} \pm 0.6$ and $2\text{mmol}/\text{L} \pm 0.6$ in the affected, possibly
Figure 5.3 – Autofluorescence and colour fundus images of an AMD an DM patients

Images A and B show drusen in the macula of a patient with AMD, the drusen are mostly hypofluorescent with a few exhibiting isofluorescence or hyperfluorescence. Images C and D are images from the fundus of a patient with DM. The AF image shows presence of hyper-autofluorescence deposits some of which are visible in the accompanying CF image as yellow deposits.
affected and the unaffected sub-categories respectively.

5.4.4 Distribution of deposits in AF and CL images

Figure (5.4): In MacTel type 2 patients, the patterns of distribution of HADs show a predilection to the temporal parafoveal area. In descending order, the highest prevalence was noticed in the inner temporal subfield followed by the central and outer temporal subfields followed by the inner inferior, nasal and superior. The lowest prevalence was noticed in the outer inferior subfield. No such topographical predilection was noticed in control subjects nor in PA and UnA sub-categories of family members. The AFF sub-category of family members, showed a pattern of topographical distribution that closely resembles that MacTel type 2 patients. In AFF, the highest prevalence was seen in the central and the inner temporal sub-fields, but with lower prevalence than in MacTel type 2 patients. Unlike in MacTel type 2 patients, HADs in AMD patients and patients with DM, did not exhibit any distinct patterns of topographical distribution.

Table (5.4), shows the combined distribution per subfield of HADs. In MacTel patients, unlike other categories, subfields II and VI which both make up the temporal parafoveal area show a higher prevalence of deposits (45% and 30%) compared to other subfields in the macula. In other groups, the prevalence of HADs in subfields II and III was; in controls (16% and 21%), affected family members (22% and 3%), possibly affected (17% and 19%) and unaffected family members (15% and 13%) respectively. Unlike MacTel, patients, in controls and family members no clear spatial predilection is evident. In AMD and DM patients, HAD prevalence is much higher but no patterns of spatial predilections were evident. The combined distribution per subfield of deposits in CL images is shown in table (4.5). Deposits in CL images in MacTel patients, did not show predilections to any subfield in the fundus.
In MacTel type 2 patients, the patterns of distribution of HADs show a predilection to the temporal parafoveal area. In descending order, the highest prevalence was noticed in the inner temporal subfield followed by the central and outer temporal subfields followed by the inner inferior, nasal and superior. The lowest prevalence was noticed in the outer inferior subfield. No such topographical predilection was noticed in control subjects nor in PA and UnA sub-categories of family members. The AFF sub-category of family members, showed a pattern of topographical distribution that closely resembles that MacTel type 2 patients. In AFF, the highest prevalence was seen in the central and the inner temporal sub-fields, but with lower prevalence than in MacTel type 2 patients. Unlike in MacTel type 2 patients, HADs in AMD patients and patients with DM, did not exhibit any distinct patterns of topographical distribution.

Figure 5.4 – HADs distribution in the fundus
Table 5.3 – Laboratory test results or participants at enrolment into the MacTel study

<table>
<thead>
<tr>
<th>CHO</th>
<th>MacTel</th>
<th>Control</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>6 ± 0.1</td>
<td>6 ± 0.1</td>
<td>AFF</td>
</tr>
<tr>
<td>BG</td>
<td>8 ± 1.4</td>
<td>5 ± 0.2</td>
<td>PA</td>
</tr>
<tr>
<td>Lipid Profile</td>
<td></td>
<td></td>
<td>Un</td>
</tr>
<tr>
<td>TC</td>
<td>5 ± 0.2</td>
<td>6 ± 0.4</td>
<td>AFF</td>
</tr>
<tr>
<td>HDL</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td>PA</td>
</tr>
<tr>
<td>LDL</td>
<td>3 ± 0.1</td>
<td>4 ± 0.5</td>
<td>Un</td>
</tr>
<tr>
<td>TAGs</td>
<td>7 ± 1.3</td>
<td>1 ± 0.2</td>
<td>AFF</td>
</tr>
<tr>
<td></td>
<td>2 ± 0.4</td>
<td>2 ± 0.6</td>
<td>PA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 ± 0.6</td>
<td>Un</td>
</tr>
</tbody>
</table>

Glycosylated Hb percentage was (6 ± 0.1) in MacTel patients, controls, and affected (AFF), possibly affected (PA) and unaffected family members (UnA). In the possibly affected family members sub-category, HbA1c percentage was 7 ± 0.1. The BG in MacTel patients was 8mmol/L ± 1.4 significantly higher than controls (5mmol/L ± 0.2). In the subcategories of family members, BG concentrations were 5mmol/L ± 0.1, 5mmol/L ± 0.6 and 5mmol/L ± 0.8 in the affected, possibly affected and the unaffected sub-categories respectively. The latter three were not significantly different from controls. In the fasting lipid profile measurement, the total cholesterol (TC) value of MacTel patients and controls was 5mmol/L ± 0.2. In the subcategories of family members, total cholesterol values were 6mmol/L ± 0.4, 4mmol/L ± 0.5 and 7mmol/L ± 0.1 respectively. HDL concentration in MacTel patients was 1.3mmol/L ± 0 not significantly different from controls (1.4mmol/L ± 0.1). In the category of family members, HDL values were; 1.4mmol/L ± 0.1, 0.9mmol/L ± 0.2 and 2mmol/L ± 0.7 or affected, possibly affected and unaffected family members respectively. Mean LDL values in MacTel patients were 3mmol/L ± 0.1 not significantly different when compared to 3mmol/L ± 0.2 in controls. LDL values were; 4 ± 0.5, 2 ± 0.6 and 4 ± 0.5 in the affected, possibly affected and unaffected family members, respectively. Mean TAGs values were 7mmol/L ± 1.3 in MacTel patients and 2mmol/L ± 0.4 in controls. In the family members category, mean LDL values were 1mmol/L ± 0.2, 2mmol/L ± 0.6 and 2mmol/L ± 0.6 in the affected, possibly affected and the unaffected sub-categories respectively.
After an overnight fast or 8–12 hours prior to the tests, in the following morning patients were given a standard liquid glucose dose and venous blood was drawn at 0, 30, 60 and 90-minute-intervals of measurement of serum glucose. The mean initial serum glucose concentration value at 0 minutes was 7mmol/L ± 2. At 30 minutes, mean serum glucose concentrations were 12mmol/L ± 3. At the 60 and 90-minutes time points, serum glucose concentrations remained elevated at 15mmol/L ± 5 and 10mmol/L ± 2 respectively.
In MacTel patients, unlike other categories, subfields II and VI which both make up the temporal parafoveal area show a higher prevalence of deposits (45% and 30%) compared to other subfields in the macula. In other groups, the prevalence of HADs in subfields II and III was; in controls (16% and 21%), affected family members (22% and 3%), possibly affected (17% and 19%) and unaffected family members (15% and 13%) respectively. Unlike MacTel, patients, in controls and family members no clear spatial predilection is evident. In AMD and DM patients, HAD prevalence is much higher but no patterns of spatial predilections were evident.

### Table 5.4 – Combined distribution per subfield (AF)

<table>
<thead>
<tr>
<th>Subfield</th>
<th>MacTel</th>
<th>Controls</th>
<th>Family</th>
<th>DM</th>
<th>AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>34</td>
<td>12</td>
<td>35</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>II</td>
<td>45</td>
<td>16</td>
<td>22</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>III</td>
<td>22</td>
<td>17</td>
<td>0</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>24</td>
<td>16</td>
<td>14</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>V</td>
<td>25</td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>VI</td>
<td>33</td>
<td>21</td>
<td>3</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>VII</td>
<td>17</td>
<td>19</td>
<td>0</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>VIII</td>
<td>20</td>
<td>27</td>
<td>0</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>IX</td>
<td>22</td>
<td>20</td>
<td>3</td>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 5.5 – Combined distribution per subfield (CL)

<table>
<thead>
<tr>
<th>Category</th>
<th>MacTel</th>
<th>Controls</th>
<th>Family</th>
<th>AMD</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AFF</td>
<td>PA</td>
<td>Un</td>
</tr>
<tr>
<td>I</td>
<td>46</td>
<td>50</td>
<td>57</td>
<td>66</td>
<td>57</td>
</tr>
<tr>
<td>II</td>
<td>67</td>
<td>49</td>
<td>59</td>
<td>64</td>
<td>57</td>
</tr>
<tr>
<td>III</td>
<td>36</td>
<td>48</td>
<td>11</td>
<td>62</td>
<td>54</td>
</tr>
<tr>
<td>IV</td>
<td>43</td>
<td>50</td>
<td>32</td>
<td>60</td>
<td>52</td>
</tr>
<tr>
<td>V</td>
<td>39</td>
<td>45</td>
<td>22</td>
<td>57</td>
<td>54</td>
</tr>
<tr>
<td>VI</td>
<td>59</td>
<td>51</td>
<td>32</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>VII</td>
<td>34</td>
<td>49</td>
<td>22</td>
<td>68</td>
<td>55</td>
</tr>
<tr>
<td>VIII</td>
<td>36</td>
<td>48</td>
<td>22</td>
<td>53</td>
<td>55</td>
</tr>
<tr>
<td>IX</td>
<td>28</td>
<td>41</td>
<td>11</td>
<td>57</td>
<td>51</td>
</tr>
</tbody>
</table>

Deposits in CL images in MacTel patients, did not show predilections to any subfield in the fundus.
5.4.5 MacTel patients divided on the basis of HADs (present/absent):

5.4.5.1 Demographics and past medical history

Table (5.6) illustrates Demographics and PMHx of MacTel patients at baseline. Here, we refer to the MacTel group of patients with HAD deposits as $HAD_{group}$ and the group that does not have HAD deposits at baseline as the $NoHAD_{group}$. Prevalence of DM was significantly higher in the $HAD_{group}$ (37%) as compared to the $NoHAD_{group}$ (21%). In the $HAD_{group}$, the percentages of males to females were 42% and 68% respectively, not significantly different from the $NoHAD_{group}$ in which the percentages were; 31% and 69% respectively. HTN prevalence in the $HAD_{group}$ and the $NoHAD_{group}$ was 48% and 52% respectively. Prevalence of smoking in the $HAD_{group}$ and the $NoHAD_{group}$, was 9% and 11% respectively. History of past smoking was reported in 49% and 67% of patients in the $HAD_{group}$ and $NoHAD_{group}$, respectively.

5.4.5.2 Test results of carbohydrate metabolism

In figure (5.6), mean FBG value in the $HAD_{group}$ was $6\text{mmol/L} \pm 2$ significantly higher than $5\text{mmol/L} \pm 1$ in the $NoHAD_{group}$. Oral glucose tolerance test (OGTT) values at the 0- and 30-minutes time points were $5.8\text{mmol/L} \pm 0.2$ and $9.2\text{mmol/L} \pm 0.7$ respectively, in the $HAD_{group}$ higher than in the $NoHAD_{group}$. Mean glucose values at the 0- and the 30-minute time points were; $5\text{mmol/L} \pm 0.1$ and $7.7\text{mmol/L} \pm 0.7$ respectively. At the 60- and 90-minute time points, serum glucose values in the $HAD_{group}$ were; $10.1\text{mmol/L} \pm 0.9$ and $8.2\text{mmol/L} \pm 0.8$ respectively significantly higher than their respective values in the $NoHAD_{group}$; $7.4\text{mmol/L} \pm 0.8$ and $6.3\text{mmol/L} \pm 0.3$.

5.4.5.3 Fasting Lipid Profile

Table (5.7) shows the fasting lipid profile for MacTel patients divided on the basis of HADs. Mean values for the total cholesterol concentrations in the $HAD_{group}$ and $NoHAD_{group}$ were $5.4\text{mmol/L} \pm 0.2$ and $5.6\text{mmol/L} \pm 0.1$ respectively. In the $HAD_{group}$, mean HDL value was $1.4\text{mmol/L} \pm 0.04$ compared to $1.4\text{mmol/L} \pm 0.04$ in the $NoHAD_{group}$. Mean LDL values
were 3mmol/L ± 0.14 and 3.4mmol/L ± 0.1 in the HAD\textit{group} and NoHAD\textit{group}, respectively. The mean TAGs concentrations in the HAD\textit{group} was 8 ± 1.7 compared to the NoHAD\textit{group} in which it was 3.6mmol/L ± 0.4.
The table shows the characteristics of MacTel patients at baseline when divided on the basis of having or not having HADs at baseline. Here, we refer to the MacTel group of patients with HAD deposits as \textit{HADgroup} and the group that does not have HAD deposits at baseline as the \textit{NoHADgroup}. Prevalence of DM was significantly higher in the \textit{HADgroup} (37\%) as compared to the \textit{NoHADgroup} (21\%). In the \textit{HADgroup}, the percentages of males to females were 42\% and 68\% respectively, not significantly different from the \textit{NoHADgroup} in which the percentages were; 31\% and 69\% respectively. HTN prevalence in the \textit{HADgroup} and the \textit{NoHADgroup} was 48\% and 52\% respectively. Prevalence of smoking in the \textit{HADgroup} and the \textit{NoHADgroup}, was 9\% and 11\% respectively. History of past smoking was reported in 49\% and 67\% of patients in the \textit{HADgroup} and \textit{NoHADgroup}, respectively.
Fasting blood glucose (FBG) values: Mean FBG value in the *HAD group* was 6mmol/L ± 2 significantly higher than 5mmol/L ± 1 in the *NoHAD group*. Oral glucose tolerance test (OGTT) values at the 0- and 30-minutes time points were 5.8mmol/L ± 0.2 and 9.2mmol/L ± 0.7 respectively, in the *HAD group* higher than in the *NoHAD group*. Mean glucose values at the 0- and the 30-minute time points were; 5mmol/L ± 0.1 and 7.7mmol/L ± 0.7 respectively. At the 60- and 90-minute time points, serum glucose values in the *HAD group* were; 10.1mmol/L ± 0.9 and 8.2mmol/L ± 0.8 respectively significantly higher than their respective values in the *NoHAD group*; 7.4mmol/L ± 0.8 and 6.3mmol/L ± 0.3.

**Figure 5.6** – Carbohydrate test values or MacTel patients divided on the basis of HADs
Table 5.7 – Fasting lipid profile of MacTel patients divided on the basis of HADs

<table>
<thead>
<tr>
<th></th>
<th>HADs</th>
<th>NoHADs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>Mean 5.4 ± 0.2</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>n</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>HDL</td>
<td>Mean 1.4 ± 0.04</td>
<td>1.4 ± 0.04</td>
</tr>
<tr>
<td>n</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>HDL</td>
<td>Mean 3 ± 0.14</td>
<td>3.4 ± 0.1*</td>
</tr>
<tr>
<td>n</td>
<td>88</td>
<td>92</td>
</tr>
<tr>
<td>TAGs</td>
<td>Mean 8 ± 1.7</td>
<td>3.6 ± 0.4*</td>
</tr>
<tr>
<td>n</td>
<td>94</td>
<td>83</td>
</tr>
</tbody>
</table>

Mean values of the total cholesterol concentrations in the HAD group and NoHAD group were 5.4mmol/L ± 0.2 and 5.6mmol/L ± 0.1 respectively. In the HAD group, mean HDL value was 1.4mmol/L ± 0.04 compared to 1.4mmol/L ± 0.04 in the NoHAD group. Mean LDL values were 3mmol/L ± 0.14 and 3.4mmol/L ± 0.1 in the HAD group and NoHAD group, respectively. The mean TAGs concentrations in the HAD group was 8 ± 1.7 compared to the NoHAD group in which it was 3.6mmol/L ± 0.4.
5.5 Discussion

This study highlights the prevalence and distribution of HAD deposits in patients with MacTel type 2 in comparison with HAD deposits in AMD and DM. The prevalence of HAD deposits is higher in MacTel patients when compared to the categories of controls and family members. In addition, the pattern of distribution in the fundus displays a strong predilection to the temporal parafoveal area in MacTel type 2 patients. Family members and controls had a significantly lower prevalence of HAD deposits with no distinctive fundal distribution patterns. The prevalence of HAD deposits in affected family members generally resembles that of patients.

We found that prevalence of HAD deposits on AF and on CF images is strongly bilateral. From previous work, it is known that MacTel type 2 range of pathological changes is bilateral (Gass and Blodi, 1993; Yannuzzi et al., 2006; Helb et al., 2008), although it could display unilateral asymmetry in disease development (Clemons et al., 2010). Even in cases where it appears unilateral, close examination often reveals subtle changes are often evident on close inspection. MacTel type 2 leads to visual loss, which is eventually bilateral (Clemons et al., 2010). Fundus autofluorescence imaging (AF) which mainly images distribution of LF which is the main retinal fluorophore, produces images with a characteristic central dark area caused by a combination of weakening RPE melanin autofluorescence and maximal absorption by macular pigment at 520nm. FAF signal displays an interesting asymmetry; being maximal between 7-13 degrees off the fovea decreasing towards the periphery, closely resembling rod distribution (Holz et al., 2001). RPE reportedly have two main fluorophores, melanin that starts to form on around day 27 of intra-uterine life (Yamada et al., 2006) and the more intensely autofluorescent LF. LF which starts to accumulate after birth (Dorey et al., 1989) is particularly enriched in PUFAs. This group of lipids, known to be abundant in photoreceptor outer segments, are prone to oxidative processes. PUFAs are thought to have the same spectral properties as RPE.

As a technique applicable in clinical ophthalmology, autofluorescence is a relatively new
diagnostic tool. Autofluorescence imaging was not part of the original imagining character-
isation used in the classification of idiopathic macular telangiectasia by Gass and Blodi (Gass and Blodi, 1993). With the introduction of fundus autofluorescence imaging as a diagnostic tool, it was quickly noticed that the earliest evidence for pathology in MacTel type 2 are subtle and generalised increases in foveal autofluorescence, proceeding all anatomic and vas-
cular changes (Wong et al., 2009). In addition the loss of macular pigment, which follows along with the changes in RPE, pigment fluorescence and/or fluorophore composition (Wong et al., 2009). The generalised autofluorescence changes were attributed to changes in the macular pigment composition, fluorophore content (Zeimer, 2010) and rod photoreceptor loss (Schmitz-Valckenberg, Holz, et al., 2008)

In this study, we report a pattern of fundus autofluorescence changes in MacTel patients that was not described before (see figure 5.2, and results are summarised in figure 5.4). The pattern we report here is circumscribed focal hyperautofluorescent deposits that are charac-
terised by their distinct predilection to the temporal parafoveal area of the fundus. This area has been suggested as a “nidus” for early morphological changes in MacTel type 2. Abujamra et al. reported that the affected area in MacTel type 2 is predominantly the inferior temporal and inferior nasal quadrants (Abujamra et al., 2000). The reported work came from a CF and FA study on a total of 11 patients with MacTel type 2.

In AMD, global characteristics of fundus autofluorescence changes with excitation at 488 nm were classified into 9 distinct patterns (Bindewald, 2005). Although not specifically looking into drusen autofluorescence, drusen exhibited increased, decreased and same fluorescence as the fundus and none of these patterns showed any type of regionalisation (Bindewald, 2005). Lengyel and co-workers, described an association between drusen with hyperautofluorescent activity and honeycomb capillary pillars (Lengyel, 2004) potentially indicating a link between this sub-class of drusen and the capillary on the one hand and the AMD pathology on the hand. Contrary to doubts expressed by some investigators (Lois et al., 2002), it was shown that drusen autofluorescece mirrors early functional changes in AMD (Meyerle et al., 2007; Midena et al., 2007). Cotton wool spots, which characterise the proliferative phase of diabetic
retinopathy, are morphologically similar to HADs on autofluorescence imaging in that both exhibit distinct autofluorescence activity. Cotton-wool spots, which represent areas of axonal disruption secondary to the ischaemia caused by the microvascular changes of deranged CHO metabolism in patients with DM, do not exhibit fundus topographical predilections. The predilection of the HADs in MacTel type 2 patients seem to be unique to the disease in that it favours areas of pathology of the disease and not just an unrelated random phenomenon in the fundus.

This predilection of the temporal region is further corroborated by the work of other researchers (Barthelmes, 2006; Issa et al., 2007). The spatial association of the focal pattern of HAD we describe here with the temporal region known for early changes in MacTel type 2 would lead to the assumption that HAD deposits form part of the imaging characteristics of MacTel type 2. The mean age and gender composition of the population in our study and is close to that reported previously (Clemons et al., 2010b). In brief, our clinical and sociodemographic data is exemplary of data on MacTel type 2 patients previously reported.

The high prevalence of diabetes mellitus and hypertension, which is far above what is expected from the background has also been reported before (Clemons et al., 2010), corroborating previous reports (Chew et al., 1986). This study shows for the first time that the links to deranged carbohydrate metabolism applies more precisely to a subgroup of MacTel patients with hyperautofluorescent deposits (HAD). A possible role for deranged glucose metabolism is the causation of autofluorescent changes is suggested by studies which showed that drusen autofluorescence has a distinct shorter wavelength than LF (Hammer et al., 2007). Our own data pooled from 95 patients with diabetes mellitus demonstrated a very high prevalence of hyperautofluorescent lesions manifested in FA images.

It was also shown that Maillard-reactions favoured under diabetic conditions and in ageing (Sell and Monnier, 1989) produce AGEs which have strong autofluorescent activity (Hammer et al., 2007). He reported that the signal associated with AGEs excitation at 488nm is >500nm and generally leads to a green shift in the autofluorescence of the fundus mostly derived from LF (Hammer et al., 2007). Maillard or nonenzymatic browning reaction have been identified
as causative factors for some of the changes occurring in collagen as a result of ageing and diabetes mellitus. Reducing sugars react non-enzymatically with free amino groups of proteins to form insoluble, highly cross-linked, yellow products with distinct autofluorescent properties (Sell and Monnier, 1989). Studies on in vivo non-enzymatic glycosylation reactions demonstrated an age-dependent increase in collagen-linked fluorescence with \( \lambda_{\text{Em}_{\text{max}}} \) at 440nm (with \( \lambda_{\text{exc}} \) at 370nm) in skin (Monnier, Vishwanath, et al., 1986), dural tissue (Monnier et al., 1984) and in dentine tissue where these reactions have been linked to dental caries. Experimentally, incubating collagen with sugars such as glucose, glucose-6-phosphate or ribose mimicked these effects. Involvement of the Maillard reaction in these changes was further corroborated in DM by the observation that nonenzymatic glycosylation was increased in diabetic and ageing collagen in patients with insulin-dependent type I diabetes mellitus (IDDM) (Monnier, Elmets, et al., 1986). AGEs receptors RAGE, AGE R1, R2 and R3 have been found to be significantly upregulated in RPE cells overlaying deposits suggesting a strong association with drusen formation (Yamada et al., 2006). AGEs have also been identified in AMD (Hammer et al., 2007). Considering the high prevalence of diabetes mellitus in the MacTel population (31%), and the fact that prevalence of HADs is associated with derangements in the indicators of carbohydrate metabolism (HbA1c, FBG and OGTT).

The extent of the contribution of the AGEs to the overall fundus fluorescence in vivo was however called into question by other authors. Schweitzer et al. reported that the excitation maxima for the principal fluorophores in the fundus (A2E, FAD, AGE) were in the range of 440-450nm (Schweitzer et al., 2007). Selective excitation of fundus fluorophores could, in principle, be achieved by varying the excitation wavelength. AGEs \( \lambda_{\text{exc}} \) Max is 370nm is maximally excited at 370nm but because AGEs, like LF, are a mixture of fluorophores, there is still a substantial degree of excitation at 470nm with an emission maximum peak at 523nm (Schweitzer et al., 2007). This is very close to the excitation used in standard AF cameras. It’s therefore safe to assume here, in our experiment in which we used \( \lambda_{\text{exc}} \) at 488nm, that it is possible some of the over all autofluorescence emanated from AGEs. Laser lights of shorter wavelengths are subject to a substantial absorption by the intervening media,
however, the precise percentage of this absorption or other circumstances that may modify
the absorption and to what extent could the posterior back of the eye could still be excited
by shorter wavelengths, remain outstanding questions.

In our population, we postulate that the autofluorescence of HADs in MacTel type 2
patients could in part be due to autofluorescence that originates from the cross-linked collagen
fibres as a result of the non-enzymatic Maillard reactions derived from collagen in drusen
and Bruch’s membrane but also possibly from the lens. However, the prevalence of HADs
deposits in MacTel patients is much higher than the prevalence of diabetes mellitus or deranged
carbohydrate metabolism, which leads to the assumption that AGEs and collagen cross-linking
as a result of Maillard reactions could only partially account for the autofluorescence of HADs
in MacTel type 2 patients. In addition to the generalised autofluorescence attributed to LF
pigment, oxidised poly-unsaturated fatty acids from partially digested rods outer segments
(Kopitz et al., 2004), glycosylated proteins (Stitt, 2001), Schiff-base products of all-trans-
retinal and ethanolamine (Radu et al., 2004) and co-factors of cellular energy metabolism
have all been demonstrated to contribute to the intrinsic tissue autofluorescence.

In our work, we demonstrated not only that HADs are a common finding in MacTel type
2 patients but also are associated with the temporal parafoveal area of the fundus. In AFF
sub-group, HADs also occur and more interestingly, they exhibit a slight but clear predilec-
tion to the temporal parafoveal region. This finding potentiates clinical application as a
prognostic biomarker tool for early detection for the transition from asymptomatic to overt
disease. Next, we will be exploring in detail the characteristics of HADs using a multimodal
approach to elucidate specific features on their effect on the choroid circulation via fluorescein
angiography, their size and local effects on retinal architecture using high-definition volumet-
ric OCT-Spectralis. Despite, its inherent limitations, VA are still the main outcome measure
used to estimate visual function in patients with MacTel type 2. VA could remain relatively
unaffected in the presence of substantial architectural disruption in the retina. Therefore, clin-
ically, there is a need for a sensitive, credible and easily measured prognostic biomarker. The
postulated pathophysiological link of HADs to MacTel type 2 and practicality and ease of AF
imaging merits a prospective longitudinal investigation for HADs as a candidate prospective biomarker against clinical endpoints. A determination of the identity of the fluorophore (s) responsible for the focal autofluorescence pattern is crucial for the identification of the pathologic processes involved. Measurement of fluorescence spectra could give valuable clues on the excitation, emission spectra and lifetimes of the fluorophore (s) involved that could help in the molecular identification.

5.6 Conclusions

1. HADs are a characteristic finding in AF images of patients with MacTel type 2 and affected family members of MacTel type 2 patients.

2. The topographical distribution of HADs in the fundus of patients with MacTel type 2 shows a clear preference to the temporal parafoveal area.

3. Deposits in the fundus of patients with MacTel type 2 are much more easily observed in AF images than in CF images.

4. Links between MacTel type 2 and DM have long been suspected. We demonstrated that defects in the CHO metabolism are more precisely associated with patients who have HADs.
Chapter 6

HADs Characterisation

6.1 Overview

Purpose: To provide a descriptive analysis of the hyper-autofluorescent deposits (HADs) and associated retinal anatomic alterations in patients with idiopathic macular telangiectasia type 2 (MacTel type 2).

Design: Cross-sectional retrospective case-control study (CS-CCS). Volunteers underwent a multi-modal imaging procedure that included fundus autofluorescence imaging (FAF), colour fundus imaging (CF), fluorescein angiography (FA) and spectral-domain optical coherence tomography (SD-OCT).

Participants: 15 MacTel type 2 patients, 10 AMD patients, 10 DM patients, 7 affected family members of patients with MacTel type 2 and 5 normal control volunteers. A total of 1440 deposits were individually analysed in detail.

Methods: Deposits were individually examined for size, location and appearance in colour fundus images (CF), autofluorescence images (AF) and fluorescein angiography images (FA). Morphometric measurements were performed on deposit scans using high definition volumetric SD-OCT. Data from deposits and from the morphometric study of the retinal anatomic architecture were analysed and compared across the different clinical categories and with measurements made on scans from normal controls at the same degree eccentricity.
Main Outcome Measures: Autofluorescence intensity of deposits and other characteristics in multimodal images in addition to associated retinal morphologic alterations in SD-OCT.

Results: In MacTel patients, 63% of the deposits are hyperautofluorescent (HADs) while the rest 37% are isofluorescent (IFDs). In AMD patients, drusen deposits are predominately either isofluorescent (31%) or hypofluorescent (53%). 82% of the deposits in FMs are hyperautofluorescent. In MacTel patients only 63% of HAD are detectable in CF images compared to 87% in controls. In AMD patients, all the deposits are seen in CF images (100%). Only 10% of the deposits in patients with MacTel showed a fluorescein-induced fluorescence in the early-phase of the fluorescein angiography procedure as compared to 98.6% in AMD patients. Typically, late-phase fluorescein angiography image shows evidence of hyperfluorescence in the immediate vicinity but not including the HAD’s location. On SD-OCT scans, HADs appear as characteristic hyperreflective thickenings of RPEs encroaching on the photoreceptor outer segment layer.

Conclusions: Hyperautofluorescent deposits in AF images are hyperreflective on SD-OCT. These deposits are anatomically located within the RPE layer and some are encroaching on the IS/OS junction and outer photoreceptor layer. It is suggested here that HADs are an early manifestation of photoreceptor and/or RPE death, which due to an underlying neurodegenerative process at the heart of the pathogenesis of MacTel type 2.
CHAPTER 6. HADS CHARACTERISATION

6.2 Introduction

The body of knowledge on the histologic changes of macular telangiectasia has dramatically increased with the use of several new imaging techniques. One of the several new imaging techniques, fundus autofluorescence (AF) revealed increased foveal autofluorescence is an early anatomic change in MacTel type 2 (Wong et al., 2009). The increased autofluoresence possibly due to a change in the fluorophore milieu of the fovea anticipates clinical and angiographic changes. In our previous work, we described the hitherto unreported pattern of focal hyperautofluorescent deposits (HAD) in MacTel type 2. These deposits typically occur at the temporal region and show a degree of autofluorescence higher than the immediately surrounding fundus. HAD prevalence increased with the clinical progression of MacTel type 2 down the Gass-Blodi classification. The goal of the present study was to provide a descriptive characterisation of HAD in MacTel type 2 patients and to study details of the consequent structural abnormalities in the outer retina. Modern SD-OCT could now attain a resolution of 3-4 micrometers. In normal healthy retinal tissues it commonly resolves 4 bands in the outer retina outside of the central fovea. The outer retina starts at the external limiting membrane (ELM), which is the junction between Muller cells and photoreceptors (Drexler et al., 2003). The following hyper-reflective band is typically considered the junction between the inner segments (IS) and outer segments (OS) of the photoreceptors (IS/OS) (Ko et al., 2005; Srinivasan et al., 2006). The third band is commonly referred to as either the OS tips, the thickest and outermost highly reflective band has commonly been attributed to the retinal pigment epithelium RPE-Bruch membrane complex (Huang et al., 1998; Drexler et al., 2003). The application of OCT imaging in MacTel type 2 patient revealed a wealth of tissue architectural derangements at all clinical stages of the disease. To summarise OCT findings in macular telangiectasia type 2: Overall retinal thickness varies significantly between different MacTel type 2 patients and does not bear any clinically important relationship to observed hyperfluorescence visible on FA (Hageman and Mullins, 1999; Bottoni et al., 2010). Loss of the normal pit in the fovea has been described at all stages of the disease, in more severe cases a complete flattening has
CHAPTER 6. HADS CHARACTERISATION

been observed (Yannuzzi et al., 2006; Sanchez et al., 2007). Thinning and disruption of inner segment/outer segment photoreceptor junction (IS/OS) more readily visualised by UHR-OCT and outer retinal atrophy is a common and consistent finding (Gaudric et al., 2006; Paunescu et al., 2006; Cohen et al., 2007) evidence that the primary cause for reduction of VA is neuronal tissue loss. Unlike macular edema, the IS/OS disruption correlates with visual acuity and accelerates with the clinical progression of the disease (Paunescu et al., 2006; Cohen et al., 2007; Sanchez et al., 2007) In advanced cases substantial tissue loss is strongly evident by the presence of fluid filled cyst-like structures at the foveola and inner retinal layers in 50-100% Stage 3 or higher (Gaudric et al., 2006; Paunescu et al., 2006; Yannuzzi et al., 2006). Although rare, it was reported these cysts could span the entire width of the retina (Koizumi et al., 2007). VA in eyes with cysts vision is typically between (20/40 to 20/70). Disease progression and an accelerated rate of visual loss had been linked to enlargement of macular cysts (Yannuzzi et al., 2006). The present study aimed at providing a descriptive characterisation of the hyperautofluorescent deposits (HADs) seen mainly in the temporal parafoveal area of patients with MacTel type 2 in AF images. A multimodal approach was employed to provide a link between HADs and structural changes evident at SD-OCT scans of patients with MacTel type 2.
CHAPTER 6. HADS CHARACTERISATION

Figure 6.1 – Retinal Architecture in SD-OCT Spectralis

[Photocredit(HeidelbergEngineering)]
6.3 Methods

6.3.1 Human Subjects

Tenets of the Declaration of Helsinki were strictly followed. Participants form part of Macular Telangiectasia study was provided written informed consent after explanation of the nature and possible consequences of the study. Similar regulations were followed for patients with DM, AMD and normal control volunteers. 15 MacTel patients, 7 AFF, 10 AMD patients and 5 control subjects were studied. Colour fundus images (CF), autofluoresence (AF), fluorescein angiography images (FA) and OCT-Spectralis images were obtained for participants. Individuals with history of ocular surgery or other diagnosed retinal abnormalities were excluded. The deposits were individually counted in superimposed images; 15 MacTel type 2 patients had 578 deposits; 5 controls (155); 10 AMD patients (312) 7 AFF (328).

6.3.2 Correlative point-to-point AF-CF-FA imaging analysis:

The definitions of categories and details of grading per subfield in AF and CF images were elaborated in sections (5.3.1, 5.3.2 and 5.3.3). Autofluoresence images and corresponding colour and early-phase fluorescein angiograms were precisely superimposed on each other and a standard MacTel type 2 grid was superimposed on the images. Images were adjusted using the layers function in Photoshop (Adobe Photoshop CS5). Superimposed images are separately saved as tiff files. Using the stack function, images are opened in ImageJ software (version 1.46p, National Institute of Health, USA). Image scales were imported and images were then manually calibrated using vascular markings for precise point-to-point correlation of the partially transparent images. After precise correlation, 100% transparency was restored. Using an ROI manager in ImageJ, drusen were identified and manually and encircled. Measures of area, standard deviation as well as location of the deposit/drusen by subfield was registered. By fading-in and fading-out different layers of images in the stack, the characteristics of the deposits/drusen in the different imaging modalities (AF, CF and FA) were revealed and recorded. Data was exported in an Excel file and statistical analysis was performed using Stata 11.
CHAPTER 6. HADS CHARACTERISATION

Area of the deposit, its location per subfield, early-phase fluorescein hyperfluorescence, appearance in the CF images and its juxtaposition to retinal capillaries were registered.

6.3.3 Spectral-domain optical coherence tomography (SD-OCT)

High-definition volumetric scans of the macula were acquired with the eye tracking function using the OCT-Spectralis (Heidelberg Engineering, Software version 1.1.6.3, Heidelberg, Germany).

The Spectralis machine employs a dual laser system; a broadband superluminescent diode at 870nm as a light source to generate two-dimensional topographical scans sequenced along the Z-axis at a rate of 40,000 B-scans per second. The images consist of $1536 \times 496$ pixels. The three-dimensional “volume scan” is a computer construct of the sequential two-dimensional images. Optical depth resolution is $7 \mu m$ with digital resolution enhancement reaches $3.5 \mu m$. Considerable speckle noise reduction in the SD-OCT scans is achieved by the use of scanning lasers retinal imaging and SD-OCT allows for real-time tracking of eye movements and real-time averaging of OCT scans. The Spectralis images are coupled to infrared cSLO imaging.

The OCT images were recorded by qualified photographers. Volumes were $6 \text{ mm} \times 6 \text{ mm}$ and consisted of 145 B-scans with a step size of $34 \mu m$. Images were exported as tiff files, anonymised and sent to the MEHRC. Longitudinal reflectivity profiles (LRPs) centred on the drusen/deposit and on either side were obtained using the “Plot Profile” function of ImageJ software (ImageJ version 1.44p, LIH, USA). LRP data was then exported to PeakFit software (Sigmaplot Inc) for the precise peak identification. A Fourier deconvolution/ filtering algorithm which depends on a Gaussian response was applied for the deconvolution process.

In the analysis of the identified LRP peaks, the nerve fibre Layer (NFL), the External Limiting Membrane (ELM), Inner Photoreceptor Layer (IPL), Inner/Outer photoreceptor Junction (IS/OS), Outer Photoreceptor Layer (OPL), RPE interdigitation (RI) and RPE/Bruch’s Interphase (RBI) were identified. The outer retinal segment was identified as the peak-to-peak distance between external limiting membrane (ELM) to the RPE/Bruch’s Interphase (RBI).
AF and CL (and FA, not shown here) images are first superimposed on each other in multiple layers in Photoshop (Adobe Photoshop CS5). Images are precisely point-to-point correlated by manual adjustment in partially transparent layers. The vascular patterns are used as landmarks for precise correlation of the images. After correlation is precisely made, full transparency is restored and images are saved as separate tiff files. A stack is created by superimposing the AF, CL and FA images on top of each other using the stack function of ImageJ software (version 1.46p, National Institute of Health, USA). Deposits/drusen are then manually encircled in the AF image, marked and counted as ROIs which are then saved separately using the ROI function of ImageJ. In the stack, the AF image is then replaced by CF image and the ROIs are automatically added to the CF image. Each marked ROI is then examined in the CF image for the appearance of the deposit/drusen in question. The same is repeated by adding the FA image and examining the ROIs one at time for the hyperfluorescence of drusen/deposits in question.
The thickness of the outer segment was considered as the FWHM of the complex of the peaks composed of ELM to the RBI.

Deposits were identified on the Spectralis scans by the point-to-point correlation of the accompanying infrared (IR) image with the AF image as described previously. Vascular patterns were used as reference points for the deposit identification process. LRP Measurements were made at the centre of the deposit/drusen and on both sides. Positions of the deposits/drusen relative to the RPE layer and the degree of hyperreflectivity of the deposits/drusen (relative to RPE) were noticed. Retinal architecture and in particular the status if the IS/OS junction over the deposit/drusen area and immediate deposit/druse-free vicinity was noticed. The measurement was compared with measurements performed on control individuals with the same degree of eccentricity for each individual deposit/drusen studied.
6.4 Results

6.4.1 Autofluorescent activity of the deposits

In table (6.1): In MacTel patients, 63% of the deposits are hyperautofluorescent while the rest 37% do not appear to have a fluorescent intensity that is distinguishable from the background autofluorescence of the fundus (isofluorescent). In AMD patients, the vast majority of the deposits are either isofluorescent (31%) or hypofluorescent (53%). 82% of the deposits in FMs are hyperautofluorescent as compared to 17% and 2% that were isofluorescent or hypofluorescent. 57% of the deposits in control subjects were isoautofluorescent, 40% hyperautofluorescent and only 3% were hypoautofluorescent.

6.4.2 Autofluorescence and induced fluorescence of deposits

With respect to HADs in patients with MacTel type 2, table (6.3) shows that only 63% are detectable in CF images compared to 88% in controls. In AMD patients, all the deposits are seen in CF images (100%). Only 10% of the deposits in patients with MacTel showed hyperfluorescence in the early-phase of the fluorescein angiography (FA) procedure as compared to 100% of the deposits in AMD patients. In MacTel patients, 52% of the deposits are juxtaposed to retinal capillaries compared to 41% in controls, 51% in AMD patients and 66% in affected family members of MacTel patients.

6.4.3 Location

In the SD-OCT scan in figure (6.6), in MacTel patients, the deposits lie immediately below a retinal vessel and appear as a characteristic thickening in the hyperreflective bands representing RPE cell layer (the RPE interdigitation and RPE/Bruch’s membrane complex). The deposits show a degree of hyper reflectivity similar to that of the RPE layers in the neighbouring areas not affected by the circumscribed spot. HADs in MacTel appear to invade the photoreceptor outer segment layer leading to a local displacement of the inner/outer photoreceptor segment interphase. In AMD patients (figure 6.9) the deposits lie in the sub-RPE space and appear
hyporeflective in comparison to the bands representing RPE reflectance. In MacTel deposits which have the same intensity degree as the RPE layer. In AMD patients, the drusen deposits invade the sub-RPE space leading to stretching and arching of the overlaying RPE in a dome-shaped manner above the deposit. The anatomic integrity of the photoreceptor outer segment layer is compromised.

In figure (6.7), an SD-OCT scan of the same MacTel type 2 patient, showing the presence of a hyperreflective thickening at the RPE level that invaded the outer photoreceptor band (A). Image (B) shows a longitudinal LRP across the deposit also shown in (C). Image (D) shows the LRPs across the deposit and on either side of it. In image (E) is a LRP of a healthy control subject at the same degree of eccentricity as the deposit. In figure (6.8), at the outer retinal segment, there is a hyperreflective deposit just external to the trough representing the outer photoreceptor layer a peak. The deposit appears to invade the area where there is normally the outer photoreceptor trough (indicated with the red arrows). The outer retinal segment, which spans from the External Limiting Membrane (ELM) to the RPE/Bruch’s Membrane Complex is clearly thickened at the centre of the deposit compared to its thickness on both sides of the deposit.

In figure (6.10) In the SD-OCT scan, unlike MacTel type 2, the drusen deposits lie in the sub-RPE space and appear hyporeflective compared to MacTel deposits, which have the same intensity degree as the RPE layer. The deposit invades the sub-RPE space leading to stretching of the overlaying RPE layer that stretches above the deposit. The anatomic integrity of the photoreceptor outer segment layer is compromised. Figure (6.11) shows a LRP of an SD-OCT scan across a drusen. It exhibits an inward displacement of the RPE and ELM. There is also a widening of the sub-RPE space where the druse is deposited. The druse itself is fairly hyporeflective. Image (B) shows a longitudinal LRP across the deposit also shown in (C). Image (D) shows the LRPs across the deposit and on either side of it. In image (E) is a LRP of a healthy control subject at the same degree of eccentricity as the deposit.
6.4.4 The “basket phenomenon:

Figure (6.5) shows correlative point-to-point correlation AF-FA Images. Image (A) shows a HAD in the temporal parafoveal area with its characteristic elevated hyperautofluorescence compared to its vicinity. In image (B), the deposit appears as a dark “filling defect” surrounded by a “basket-like” rim of hyperfluorescence in the early-phase FA. Dilated and incompetent leaky capillaries form the basket meshwork hosting the deposit. This phenomenon could be due to the fluorescein leakage and/or staining at the rim of the deposits as a result of a disruption of the anatomical/functional integrity of the outer blood-retinal barrier consequent to disruption of the tight junctions between RPE cells.

6.4.5 Cross-comparison of HADs, drusen and sub-retinal drusenoid deposits

In figure (6.12) images A, B and C represent an HAD, sub-retinal drusenoid deposit and druse, respectively. HADs in MacTel type 2 invade the area where there is normally the outer photoreceptor trough and cause a disruption of the line representing the IS/OS junction. The outer retinal segment is clearly thickened at the centre of the deposit compared to its thickness on both sides of the deposit. Drusen on the other hand, occupy the sub-RPE space and appear hyporeflective compared to MacTel deposits, which have the same intensity degree as the RPE layer. The invasion of the sub-RPE space leads to stretching of the overlaying RPE layer and distortion of the of the photoreceptor RPE layer in addition to the thinning of the outer retina. Sub-retinal drusenoid deposits, have a characteristic conical appearance underneath the retina but above and separate from the RPE layer.
### Table 6.1 – Deposits relative autofluorescence intensity

<table>
<thead>
<tr>
<th>Category</th>
<th>Deposits Autofluorescence Intensity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Same</td>
<td>Increased</td>
</tr>
<tr>
<td>MacTel</td>
<td>212</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>37%</td>
<td>63%</td>
</tr>
<tr>
<td>Controls</td>
<td>89</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>57%</td>
<td>40%</td>
</tr>
<tr>
<td>AMD</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>31%</td>
<td>16%</td>
</tr>
<tr>
<td>FMs</td>
<td>54</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>17%</td>
<td>82%</td>
</tr>
<tr>
<td>Total</td>
<td>491</td>
<td>881</td>
</tr>
<tr>
<td></td>
<td>34%</td>
<td>61%</td>
</tr>
</tbody>
</table>

In MacTel patients, 63% of the deposits are hyperautofluorescent while the rest 37% do not appear to have a fluorescent intensity that is distinguishable from the fundus (isofluorescent). In AMD patients, the vast majority of the deposits are either isofluorescent (31%) or hypofluorescent (53%). 82% of the deposits in FMs are hyperautofluorescent as compared to 17% and 2% that were isofluorescent or hypofluorescent. 57% of the deposits in control subjects were isofluorescent, 40% hyperautofluorescent and only 3% were hypofluorescent.
In MacTel patients only 63% of HAD are detectable in CF images compared to 88% in controls. In AMD patients, all the deposits are seen in CF images (100%). Only 10% of the deposits in patients with MacTel showed hyperfluorescence in the early-phase of the fluorescein angiography (FA) procedure as compared to 100% of the deposits in AMD patients. In MacTel patients, 52% of the deposits are juxtaposed to retinal capillaries compared to 41% in controls, 51% in AMD patients and 66% in affected family members of MacTel patients.
Images A, B and C represent AF, CF and early-phase FA angiography images of a patient with MacTel type 2. In the AF images there are numerous focal HADs in the temporal parafoveal area. In the corresponding CF image, these HADs appear as drusen-like yellow deposits. There is leakage of the fluorescein dye in early phase around the hyperautofluorescent deposits presumably due to an architectural disruption at outer blood-retinal barrier at the level of the tight junctions between RPE cells causing extravasation of the fluorescein dye. The HADs clearly do not stain with the fluorescein dye.
The figure shows correlative point-to-point correlation AF-FA Images. Image (A) shows a HAD in the temporal parafoveal area with its characteristic elevated hyperautofluorescence compared to its vicinity. In image (B), the deposit appears as a dark “filling defect” surrounded by a “basket-like” rim of hyperfluorescence in the early-phase FA. Dilated and incompetent leaky capillaries form the basket meshwork hosting the deposit. This phenomenon could be due to the fluorescein leakage and/or staining at the rim of the deposits as a result of a disruption of the anatomical/functional integrity of the outer blood-retinal barrier consequent to disruption of the tight junctions between RPE cells.
In (A) there is a circumscribed hyperautofluorescent deposit (HAD) which shows autofluorescence intensity greater than that displayed by the immediately surrounding fundus. The deposit is not visible in the accompanying CF image (B). On the fluorescein angiography image, late-stage hyperfluorescence surrounds but does not stain the deposit itself (C). In the SD-OCT scan, the deposit lies in close proximity to a retinal vessel. It appears as a characteristic thickening in the bands representing RPE interdigitation and RPE/Bruch’s membrane complex. The deposit seems to have invaded the photoreceptor outer outer segment layer leading to a local inward displacement of the inner/outer photoreceptor segment interphase.
Figure 6.7 – SD-OCT scan of a MacTel patient

An SD-OCT scan showing the presence of a hyperreflective thickening at the RPE level that invaded the outer photoreceptor band (A). Image (B) shows a longitudinal LRP across the deposit also shown in (C). Image (D) shows the LRPs across the deposit and on either side of it. In image (E) is a LRP of a healthy control subject at the same degree of eccentricity as the deposit.
In the outer retinal segment, there is a hyperreflective deposit just external to the trough representing the outer photoreceptor layer a peak. This deposit’s peak is located where there is normally the outer photoreceptor trough (indicated with the red arrows). The outer retinal segment which spans from the External Limiting Membrane (ELM) to the RPE/Bruch’s Membrane Complex is thickened at the centre of the deposit compared to its thickness on both sides of the deposit.
Figure 6.9 – Multimodal Images of drusen in an AMD patient

In the colour image (CF) of an AMD patient (A), there is a large number of drusen deposits of varying sizes. In the accompanying autofluorescence image (C)-in comparison to fundus autofluorescence- some drusen are hyperfluorescent, some are hypofluorescent but the majority are isofluorescent and therefore are not visualised in the autofluorescent image. In the SD-OCT scan, unlike MacTel type2, the deposits lie in the sub-RPE space and appear hyporeflective compared to MacTel deposits which have the same intensity degree as the RPE layer. The deposit invades the sub-RPE space leading to stretching of the overlaying RPE layer that stretches above the deposit. The anatomic integrity of the photoreceptor outer segment layer is compromised.

[Photocredit: (Spaide and Curcio, 2010)]
An SD-OCT scan across a drusen with an inward displacement of the RPE and ELM. There is also a widening of the sub-RPE space where the druse is deposited as a relatively hyporeflective area (A). Image (B) shows a longitudinal LRP across the deposit also shown in (C). Image (D) shows the LRPs across the deposit and on either side of it. In image (E) is a LRP of a healthy control subject at the same degree of eccentricity as the deposit.

**Figure 6.10** – SD-OCT scan of an AMD patient
Figure 6.11 – Longitudinal light reflectivity profiles of the druse above

The outer retinal segment is thinner displaced upward (to the left in the graph). There is a compromise in the integrity of both photoreceptor inner and outer layers (red and blue arrows respectively) at the site of the drusen when compared to the LRPs of the areas on both sides of the deposit.
CHAPTER 6. HADS CHARACTERISATION

Figure 6.12 – Different types of sub-retinal deposits

Images A, B and C represent an HAD, sub-retinal drusenoid deposit and druse, respectively. Image A featuring a typical HAD in a MacTel type 2 patient. In the magnified image (the bottom row), the HAD (blue arrow) is also hyperreflective on SD-OCT and it represents a focal thickening of the the normally hyperreflective line representing the RPE layer (red arrow). Image B features subretinal drusenoid deposits (red arrow), which appear as a conical deposit below the retina but characteristically above and separate from the RPE layer (blue arrow). Image C features a typical AMD druse. The druse itself (blue arrow) is hyporeflective and is characteristically located beneath the the RPE cell layer (red arrow).

Photorecredit for images B & C: (Spaide and Curcio, 2011)
6.5 Discussion

Optical coherence tomography (OCT) is a robust, non-contact, non-invasive established ophthalmologic diagnostic imaging technology that provides in vivo images of retinal sections. It is employed diagnostically in various conditions. High-resolution volumetric SD-OCT scans allows the visualisation of the microscopic details of the retinal architecture. SD-OCT produces clearly discernible reflective layers. An analysis of the reflectivity of these layers as a function of scan-depth results in a longitudinal reflective profile curve (LRP) with several peaks each representing a particular anatomic layer. Using a multimodal approach for the investigation of the hyperautofluorescent deposits (HADs) in MacTel patients, this study determined these hyperautofluorescent deposits are hyperreflective on high-resolution SD-OCT scans and are located in the outer retinal layer at the IS/OS and RPE levels. These deposits, which are easily recognised in autofluoresence images (AF) in the stages preceding the development of clinical visual loss, could be employed as an imaging biomarker for early diagnosis and as an antecedent biomarker for progression. Histological studies are required to determine the precise chemical nature of these deposits to help unlock how they fit in the pathophysiological spectrum of the disease. In AMD patients the PRL thickness is reduced over drusen (Schuman et al., 2009). The degeneration of the photoreceptor outer segments may be complete. The characteristic hyperreflective haze in the photoreceptor outer layer reported in AMD patients might be an early sign of neurological damage (Schuman et al., 2009). Abnormal vessel growth and loss of integrity of the photoreceptor layer was associated with a reduction in visual acuity (Paunescu et al., 2006b). In MacTel patients, the presence of hyperreflective plaques is associated with a number of morphologic observations. Hyperreflective plaques associated with retinal atrophy were described in patients at different stages of the disease using TD-OCT (Chen and Lee, 2007). SD-OCT, which has a spatial resolution of 4 micrometer allows for cell-level morphologic assessment of individual retinal cell layer changes. Superficial retinal hyperreflective plaques on the NFL layer were described and attributed to crystals resulting from Muller cells degeneration. Other causes for the presence of hyperreflection MacTel pa-
tients are RPE proliferation and migration (Yannuzzi et al., 2006a) (Yannuzzi et al., 2006), neovascularization (Barthelmes et al., 2008). In the inner retina highly hyperreflective were reported (Sanchez et al., 2007). This is the first study that provides a detailed morphologic description of the hyperautofluorescent deposits observed in MacTel type 2 patients on AF images and make a link to morphological changes evident on SD-OCT scans. Crucially, the HAD changes we observed in the fundus of patients with MacTel type 2, pre-date any evidence of abnormality in OCT scans.

Nutritional deprivation of middle retina layers in MacTel patients was proposed a probable mechanism to explain the degeneration and subsequent atrophy of Müller cells and the photoreceptor cells. Given he reported increased hyperreflectivity of the temporal parafoveal area in MacTel patients (Barthelmes et al., 2008; Bottoni et al., 2010), it is not inconceivable that the hyperautofluorescent deposits observed in this study represent a stage in MacTel’s pathophysiology whereby, the initial pre-clinical degenerative process is only manifest on autofluorescence images before causing enough tissue architectural damage detectable on OCT. This is the first study that provides a detailed morphologic description of the hyperautofluorescent deposits observed in MacTel patients on AF images and make a link to morphological changes evident on SD-OCT scans. Crucially, the HAD changes we observed in the fundus of patients with MacTel type 2, pre-date any evidence of abnormality in OCT scans. The hyperautofluorescence would then result from deposition and pathological stress at the RPE-photoreceptor complex.

Disruptions in the dynamics of blood flow in retinae of MacTel type 2 patients were considered an early sign of the disease. Subtle hyperfluorescence in the early-phase of FA is a universal early finding in the course of MacTel type 2. Right-angled retinal vessels and sub-retinal pigmented plaques are among the commonest findings on FA in MacTel type 2 patients in the deep retinal circulation (Yannuzzi et al., 2006a). Dilated and incompetent veins and arteries also characterise the disease. Possibly in response to ischaemia, three types of anastomosis were described in patients with MacTel type 2; retino-retinal anastomosis (RRA) (Yannuzzi et al., 2006b), retinal-sub-retinal anastomosis (RSR) and retino-choroidal anastomosis (Nowilaty
et al., 2010). These observations lead to the assumption in the ophthalmic community that MacTel type 2 is primarily a vascular disease. This partially stemmed from lack of sensitive imaging modalities that were able to detect earlier changes in the neurosensory retina. Our multimodal investigation demonstrated that HADs are hosted in a “basket” of telangiectatic incompetent capillary meshwork. HADs themselves appear as dark filling defects in the FA image. We postulate that several reasons could explain this phenomenon, it impossible that also a combination of reasons could be behind that rather than one single factor. An attractive possibility, this phenomenon could be due to the fluorescein leakage and/or staining at the rim of the deposits due to disruption of the anatomical/functional integrity of the outer blood-retinal barrier as a direct result of the disruption of the tight junctions between RPE cells. Another possibility could that the detection systems used in the FA imaging, optimised for the detection the fluorescein dye, have a sensitivity threshold much higher than that associated with the comparatively much weaker autofluorescence emanating from the HADs. Therefore, HADs being below the detection threshold of the system would appear as dark “filling defects” surrounded by a rim of highly fluorescent fluorescein dye. A third possibility is the physical exclusion of the dye by the deposit, which would result in the fluorescein leaking from the incompetent telangiectatic capillaries to mark the perimeter of the zone of physical exclusion. Any of these three reasons could explain the “HADs basket phenomenon” seen on point-to-point correlative AF-FA imaging analysis, we postulate that these three likely explanations are not mutually exclusive and therefore a combination of any two or possibly all the three could be behind the observed “HADs basket phenomenon”.

The finding that HADs are deposits within the RPE layer encroaching on the photoreceptor outer segment suggests an attractive postulate. The expansion of the RPE cell volume and accumulation of materials could be a result of an abnormality in the RPE phagocytic machinery due to a loss of polarity. Such a hypothesis was first advanced by Spaide and Curcio in the context of their explanation for the accumulation of conical-shaped sub-retinal drusenoid deposits (Spaide and Curcio, 2010). RPE cells ordinarily phagocytose material at their apical surface, and it could be hypothesised the accumulation of the subretinal deposits could reflect a
global abnormality of phagocytosis through the loss of polarity. Each RPE’s apical membrane invaginates 30-50 photoreceptor outer segments (POS). A fraction estimated between 10-15% of the outer segment of each photoreceptor cell is phagocytosed by the apposing RPE each day (Young and Bok, 1969; Newsome et al., 1987). This continuous processing of the shedding of the POS places enormous metabolic strains on RPE cells. In MacTel type 2 we hypothesise that the progressive accumulation of HADs is a result of a transportation and/ or an enzymatic deficiency of the RPE rendering it incapable of disposing of the engulfed photoreceptor outer segments. The consequences of that could be the reduction in the functionality of the RPE cells and focal death of the associated photoreceptor cells. Molecular and histologic characterisation of the nature of these deposits should give decisive answers.

In summary, this study established the characteristics of the observed hyperautofluorescent deposits in MacTel type 2. The study also established the previously unrecognised link between the focal hyperautofluorescence deposits (HADs) and distinct structural alterations by using a multimodal imaging approach, qualitative and quantitative analysis. Focal deposits in MacTel patients, unlike other diseases known to be characterised by deposition, are mostly HADs and do not display an induced hyper-fluorescence in the late-phase of the fluorescein angiography procedure. HADs are characterised by hyperreflectivity on SD-OCT and represent circumscribed plaques that cause disruption at the IS/OS junction, invasion of the photoreceptor outer segment and thickening of the RPE layer. Histological and functional investigation is required to determine the chemical nature of the deposits and the functional effects on vision. The current approach relying on AF imaging, FA, CF and SD-OCT images lacks the necessary sensitivity to give conclusive answers, however, it does prove the worthiness of further investigation of the hyperautofluorescent deposits using AO, histology, fine matrix mapping and other methods to uncover the molecular processes for a better understanding of the pathophysiologic processes involved in the progression of the disease.
6.6 Conclusions

1. Using a correlative AF and high-definition OCT Spectralis approach, we demonstrated that HADs are hyperreflective deposits within the RPE layer that also occupies the photoreceptor outer segment and disrupts the IS/OS junction.

2. AF imaging exhibits a greater degree of sensitivity in detecting HADs as compared to CF imaging.

3. Our point-to-point correlative AF-FA imaging analysis demonstrated the “HADs basket phenomenon”. In early-phase FA, HADs appear as dark “filling defects” surround by fluorescein hyperfluorescence. The probable explanation is likely to be the disruption of the tight junctions between the RPE cells at the level of the outer border of the blood-retinal barrier. Other possibilities are also likely.
Chapter 7

HADs Progression

7.1 Overview

Introduction: MacTel type 2 is a slowly progressive neurodegenerative disorder whose pathologic processes run for decades before significant changes are evident. The current study was carried out with dual objectives in mind, first to study patterns of evolution of HADs in MacTel type 2 patients over a 5-year-period of follow up and secondly to study the association of the HADs with the clinical progression of the disease.

Conceptual points: A dual approach was pursued for the study of progression. “Deposits Temporal progression” refers to change in the count and or number of the deposits either increase or reduction over a period of 5 years of follow up in the study. “Deposits in the Clinical Progression of MacTel type 2” is percentage change in the prevalence of the deposits in AF and CL images with the clinical progression of MacTel type 2 according to the Gass-Blodi classification system.

Study design: A longitudinal retrospective approach was followed. 53 MacTel type 2 patients underwent autofluorescence imaging at both baseline and at 5 years of follow up. Participants were examined for patterns of change of deposits at these two time points in both AF and CL images. The outcome measures were: “Regression”, “increase”, “no change”, “deposits count”, “subfield”, “CL”, “AF”, the five stages of the Gass-Blodi classification. AF
and CL images from baseline and 5 year visits were precisely superimposed on each other; the standard MacTel grid was used to identify the subfields. The initial step was the assessment of the predominant type of change between the initial visit and the 5-year-visit were made for each subfield independently. Secondly, counts and prevalence of deposits per subfield were determined.

Results: At the 5-year-visit, 51% of the HADs deposits exhibited regression, 36% did not change between baseline and 5 years, 13% increased in size and/ or count. In CF images 67% of the deposits did not show evidence of change, nearly equal proportions showed either increase or regression. At all Gass-Blodi stages, the prevalence of deposits in CF images was larger than in AF images. At sage I, the prevalence of HADs was 20%, it increases to 74% in stage II to peak at 76% in stage III and subsequently, regresses to 54% and 42% at stages IV and V respectively. There was a strong correlation between the HADs prevalence in the inner and outer temporal region with stages of progression according to Gass-Blodi classification ($R^2 = 0.94$ and 0.77 for the inner and outer temporal subfields respectively).

Conclusion: The close association of the prevalence of HADs with both the area of pathology and clinical stages of MacTel type 2 makes them an attractive candidate imaging prognostic or surrogate biomarker for predicting the likely future course of the disease and monitoring progression and/ or effects of therapeutic interventions.
7.2 Introduction

MacTel type 2 is a slowly progressive disease and visual disturbances can manifest insidiously and progressively over the course of many years or even decades. Knowledge on the impact and progression of MacTel type 2 is scarce partially because longitudinal studies have not been conducted over a sufficiently long time and partially due to this long course of the disease. One longitudinal study that examined MacTel type 2 impact on vision-specific QoL using a Rasch-calibrated instrument (Lamoureux et al., 2011). The study reported that in a course of 24-months-period, no significant deterioration in four vision-specific QoL indices in patients living with MacTel type 2 following the initial assessment and there was no significant decline in visual acuity was observed (Lamoureux et al., 2011). Fine matrix mapping of scotopic rod function was found to be very sensitive in detecting small-scale changes (Schmitz-Valckenberg et al., 2008). In a longitudinal study encompassing functional and morphologic changes in 9 patients with MacTel type 2 assessed over a period of one year with a battery of tests. The tests included repeated scotopic and photopic fine matrix mapping (FMM), photopic microperometry, visual acuity and imaging studies. Visual acuity and microperometry changes in one year were found to be well within the test–retest variability (Schmitz-Valckenberg et al., 2009). However, minute structural changes, which included increased dilation of telangiectatic blood vessel, increased hyperfluorescence in late-phase fluorescein angiography and a minor increase in pigment migration. Using a dense grid, FMM demonstrated scotopic dysfunction in one year (Schmitz-Valckenberg et al., 2009). Longitudinal assessments over a longer time period are required to provide more elucidative information on the structural and functional impact of MacTel type 2. Generalised increases inl autofluorescence intensity of the fovea of patients with MacTel type 2 predate anatomic and vascular changes (Wong et al., 2009). These changes are reflective of macular pigment loss, which follows along with the changes in RPE, pigment fluorescence and/or fluorophore composition (Wong et al., 2009). To date, there is no report of a longitudinal study of the changes in fundus autofluorescence of MacTel patients. Such a study particularly if conducted as part of a multimodal investigation that combines AF
imaging to newer imaging modalities such as MPP and AO, should give elucidative insights.

This retrospective cross-sectional study aimed at the identification of phenomenological patterns of change of the hyperautofluorescent deposits over a 5-year-period of follow up. It also aimed at investigating the relationship of the prevalence of the HADs with the widely-recognised clinical stages of progression of MacTel type 2 according to Gass-Blodi classification (Gass and Blodi, 1993).

7.3 Methods

The use of the term “progression” in the context of deposits in MacTel type 2 implies two completely different concepts. Therefore two different approaches were followed to explore the two concepts. “Deposits Temporal progression” refers to change in the count and or number of the deposits either increase or reduction over a period of 5 years of follow up in the study. “Deposits in the Clinical Progression of MacTel type 2” is percentage change in the prevalence of the deposits in AF and CL images with the clinical progression of MacTel type 2 according to the Gass-Blodi classification system (Gass and Blodi, 1993). Below, the methodology for each approach is described separately.

7.3.1 Temporal Progression Study

7.3.1.1 Subjects

Tenets of the Declaration of Helsinki were strictly followed in the study. Participants from the Macular Telangiectasia study were provided with written consents after explanation of the nature of the study. Participants in this study on the patterns of change of HAD deposits over time are volunteers who have completed five years of follow up and whose AF and CL images are available and are of good quality at both the initial visit and the 5 year visit.
7.3.1.2 Parameters

The parameters used in the study are; deposits count, subfield, CL & AF images, initial and 5-year-visits.

7.3.1.3 Definitions of outcome measures

- **“Regression”**: a subfield was marked for “regression” when a 5th year visit image is superimposed on an initial visits shows that there was a reduction in the size and/or count of the deposits in the subfield in question.

- **“Increase”**: is defined as an increase in count and/or size of the deposits per subfield in a 5th year visit image when precisely superimposed on an initial visit image.

- **“No change”**: is indicated when both the count and size of the deposits between the initial and 5th year visit images, shows no change i.e. nor regression neither increase.

7.3.1.4 Grading procedure

The definitions of categories and details of grading per subfield in AF and CF images were elaborated in sections (5.3.1, 5.3.2 and 5.3.3). The details of correlative point-to-point AF-CL imaging analysis were elaborated in section (4.3.2). AF and CL images from baseline and 5 year visits were precisely superimposed on each other using vascular patterns to match the images through the function of alteration of the degree of transparency in Adobe Photoshop (CS5, Adobe Systems Incorporated, San Jose, USA). The standard MacTel grid (whose description and dimensions were described in the methodology section 5.3.3) was used to identify the 9 areas of the fundus.

The initial step was the assessments on the predominant type of change between the initial visit and the 5-year-visit were made for each subfield independently. Layers carrying images from the initial visits were faded in and out on the background of the image from the 5-year-visit. The fading-in and fading-out of the images was repeated for each subfield independently and an assessment of the predominant type of change was made for each subfield. This
procedure was carried out in both AF and CL images from OSs and ODs. Assessment for change was made for no change, regression of deposits and increase in size and or count per subfield.

After completion of the initial assessment step in all the images and using the fade-in fade-out function in superimposed images of the initial and 5-year-visits, counts of deposits per subfield were made. The counts were made independently for each subfield in AF and CL images from OSs and ODs. Data on both the assessment for the predominant type of change and counts per subfield of the deposits was entered in an Excel spreadsheet.

7.3.1.5 Statistical Analysis

Data was collected in Excel sheets (MO 2011) and transported analysed for analysis in Stata Program (Version 11, StataCorp LP 4905, Texas USA). Percentage tables were used to analyse data for predominant type of change and Chi-square test was used to identify significance. Counts per subfield were converted to percentage prevalence per subfield. Prevalence was compared in subfields between the initial and 5-year-visits and Chi-square test was used to identify significance between the two time points. Bilateral symmetry in the prevalence of deposits per subfield was tested using Cohen’s Kappa statistic in AF and CL at both the initial and 5 years time point.

7.3.2 Deposits in the Clinical Progression of MacTel type 2

7.3.2.1 Subjects

Diagnosed MacTel type 2 patients from among the Participants in the Macular Telangiectasia study were clinically assessed and the degree of the progression of the disease was classified into one of the five categories of the Gass-Blodi Classification (Gass and Blodi, 1993). Patients were then randomised within each clinical category. AF and CL images were picked at random from each clinical category representing one of five clinical stages of progression in the Gass-Blodi classification.
The images are point-to-point correlated using vascular markings in Photoshop as described in section (4.4.3). The ROIs representing the drusen/deposits (red, at baseline image) are transferred to the 5 years AF image. In the 5 years, AF image, new deposits/appeared (yellow ROIs). An estimate of count of deposits per subfield is made and compared between the AF images at baseline and at 5 years. Change per subfield is then registered as either, (1) Progression: if there is an increase in deposits count and/or size (the case in the image above), (2) Regression: if there is decrease in size and/or count or disappearance of the deposits or (3) No change: if the 5-year-AF image is similar to the baseline AF image in deposits count and size.

**Figure 7.1** – Grading for progression
7.3.2.2 Parameters

Parameters used in this part of the study are; deposits count, subfield, CL & AF images, the five stages of the Gass-Blodi classification.

7.3.2.3 Outcome measures

Prevalence of deposits in AF and CL images per subfield in both eyes at all stages of the Gass-Blodi classification.

7.3.2.4 Grading procedure

The definitions of categories and details of grading per subfield in AF and CF images were elaborated in sections (5.3.1, 5.3.2 and 5.3.3). The details of correlative point-to-point AF-CL imaging analysis were elaborated in section (4.3.2). AF and their corresponding CL images were precisely superimposed on each other using vascular landmarks and the transparency and the layers functions in Photoshop (CS5, Adobe Systems Incorporated, San Jose, USA). The standard MacTel grid (described before) was superimposed on the image stack to identify the 9 subfields of the fundus. As described before, counts of deposits independently per subfield were made in AF and CL images.

7.3.2.5 Statistical Analysis

Data gathered in Excel spreadsheets were exported to Stata (Version 11) where counts per subfield were converted to percentages for each subfield. Percentages were then calculated for each subfield per stage of Gass-Blodi classification. Pearson’s correlation coefficient was used to assess the strength of the association between MacTel type 2 clinical progression on the Gass-Blodi classification and the prevalence of the deposits in AF and CL in each subfield of the fundus.
7.4 Results

7.4.1 Patterns of change at 5 years

Figure (7.2) shows an autofluorescence and its accompanying colour fundus image (CF). The images taken at the baseline visit show presence of HADs deposits mostly in the inner and outer temporal areas of the fundus. HAD deposits also appear in the accompanying CL image, however, are harder to visualise. At the 5-year-follow up visit, most of the deposits in the area regressed and some have completely disappeared. The degree of contrast between the deposits and the surrounding fundus has reduced indicating a reduction in the degree of autofluorescence of the deposits. The accompanying CL image shows no detectable change.

No change in deposits in the follow up period is exemplified in figure (7.2). Despite an increase in the background fundus autofluorescence in the central and inner temporal fields, there was no change in the appearance of most of the deposits indicated by the arrows between the baseline and 5-year-visits in this patient. Similarly no change is evident in the accompanying colour images.

Figure (7.3) illustrates an increase in the size of a pre-existing deposit. In some patients, there was an increase in the size of existing deposits between the baseline visit and the 5-year-visit. Change is easier to manifest in the AF images.

A mixed pattern of change during the course of five years is exemplified in figure (7.4). Images taken at the baseline visit show presence of numerous HADs deposits in all subfields of the fundus. HAD deposits are readily visualised in the accompanying colour images. In images taken at the 5-year-visit, there is a mixed pattern of change. The temporal area shows an increase in the count of the HAD while the nasal area shows a reduction. In figure (7.5) two high-definition SD-OCT scans centered on the HAD deposit at 90 degrees show a considerable focal deposition and thickening at the IS/OS junction exhibited by increased thickness of the hyperreflective line denoting invasion of the photoreceptor outer segment.
CHAPTER 7. HADS PROGRESSION

7.4.2 Assessment of patterns of change

7.4.2.1 Overall Percentage change

An overall assessment of change in deposits in AF and CL images is provided in figure (7.6). Superimposed AF images taken at baseline and at 5 years of follow up showed a regression in 51% of the deposits. 36% of the deposits, did not change with respect to size or count while in 13% an increase in count and/or size was evident. In CF images, two thirds of the deposits (67%) did not differ in terms of size and count between the two time points; nearly equal proportions of the deposits have increased (count and/or size) or regressed during the same time.

7.4.2.2 Deposits and MacTel type 2 Clinical Progression

Figure (7.7) illustrates the overall prevalence of deposits in AF and CL images with the disease progression. At all stages of Gass-Blodi classification, the overall prevalence of the deposits in CL images is higher than that in AF images. In CL images at stage I of the Gass-Blodi classification, the prevalence was 60% and increased to 92% at stage II. Subsequently, it remains essentially unchanged at 91%, 90% and 100% at Gass-Blodi stages III, IV and V respectively. The prevalence of HAD deposits increases from 20% at Gass-Blodi stage I to 74% at stage II and peaks at 76% in stage III. At Gass-Blodi stages IV and V, the prevalence is 54% and 42% respectively. Figure (7.8) illustrates the Pearson’s correlation coefficient ($R^2$) values for HAD prevalence and clinical progression of MacTel type 2 according to the 5 stages of the Gass-Blodi classification. The correlation values were significant at the inner and outer temporal areas (subfields II and VI, respectively). At subfield VI $R^2 = 0.94$ indicating a very high degree of correlation and a high degree of correlation was observed at subfield II ($R^2 = 0.77$). In subfields I, V, VII and IX of the retina, there were low degrees of correlation between HAD prevalence and the clinical progression of MacTel type 2. Figure (7.9) illustrates the Pearson’s correlation coefficient ($R^2$) values for deposits in CL images and the 5 stages of the clinical progression of MacTel type 2 according to the Gass-Blodi classification. The correlation values
were significant at the inner, outer temporal areas and inner nasal areas (subfields II, VI and IV respectively). \( R^2 \) values indicated a very high degree of correlation (0.94) at subfield VI and high degrees of correlation; 0.89 and 0.77 at subfields IV and II respectively. In subfields V, VII and VIII, only a low degree of correlation was observed (\( R^2 \) values between 0.25 – 0.32). In subfields I, III and VIII there were almost negligible degrees of correlation.
An autofluorescence and its accompanying colour fundus images taken at baseline and at 5 years. The images taken at the baseline visit show presence of HADs deposits mostly in the inner and outer temporal areas of the fundus. HAD deposits also appear in the accompanying CL image, however, are harder to visualise. At the 5-year-follow up visit, most of the deposits in the area regressed and some have completely disappeared. The degree of contrast between the deposits and the surrounding fundus has reduced indicating a reduction in the degree of autofluorescence of the deposits. The accompanying CL image shows no detectable change.
Figure 7.3 – No change in HADs

An autofluorescence and its accompanying colour fundus images taken at baseline and at 5 years. Despite an increase in the background fundus autofluorescence in the central and inner temporal fields, there was no change in the appearance of most of the deposits indicated by the arrows between the baseline and 5-year-visits in this patient. Similarly no change is evident in the accompanying colour images.
Figure 7.4 – Increase in count and size of HADs

An autofluorescence and its accompanying colour fundus images taken at baseline and at 5 years. The images taken at the baseline visit (A, B and C) show presence of numerous HADs deposits mainly at the central and temporal parafoveal subfields of the fundus. At the 5-year-visit, there is a large increase in both count and size of most existing HADs. Most HADs are not readily visualised in the accompanying colour images.
Figure 7.5 – High-definition volumetric SD-OCT scan of the HAD deposit
High definition volumetric SD-OCT of the image shown in figure (7.4). Two intersecting scans at 90 degrees centred on the HAD shown at the middle of the red box. The scan shows a considerable focal deposition and thickening of the hyperreflective line representing the IS/OS junction and invasion of the photoreceptor outer segment.
Figure 7.6 – 5-year-change in deposits in autofluorescence and colour fundus images

Superimposed AF images of HAD deposits taken at 5 years of follow up showed regression of 51% of the deposits when compared to images taken at baseline. 36% of the deposits, did not change with respect to size or count while in 13% showed increases in count and/or size over a period of 5 years of follow up. In CF images, two thirds of the deposits (67%) did not differ in terms of size and count between the two time points; nearly equal proportions of the deposits have increased (count and/or size) or regressed during the same time.
The figure illustrates the overall prevalence of deposits in both AF and CL images at all areas of the fundus. At all stages of Gass-Blodi classification, the overall prevalence of the deposits in CL images is higher than that in AF images. In CL images at stage I of the Gass-Blodi classification, the prevalence was 60% and increased to 92% at stage II. Subsequently, it remains essentially unchanged at 91%, 90% and 100% at Gass-Blodi stages III, IV and V respectively. The prevalence of HAD deposits increases from 20% at Gass-Blodi stage I to 74% at stage II and peaks at 76% in stage III. At Gass-Blodi stages IV and V, the prevalence is 54% and 42% respectively.
The figure illustrates the Pearson’s correlation coefficient ($R^2$) values for HAD prevalence and clinical progression of MacTel type 2 according to the 5 stages of the Gass-Blodi classification. The correlation values were significant at the inner and outer temporal areas (subfields II and VI, respectively). At subfield VI $R^2 = 0.94$ indicating a very high degree of correlation and a high degree of correlation was observed at subfield II ($R^2 = 0.77$). In subfields I, V, VII and IX of the retina, there were low degrees of correlation between HAD prevalence and the clinical progression of MacTel type 2.
The figure illustrates the Pearson’s correlation coefficient ($R^2$) values for deposits in CL images and clinical progression of MacTel type 2 according to the 5 stages of the Gass-Blodi classification. The correlation values were significant at the inner, outer temporal areas and inner nasal areas (subfields II, VI and IV respectively). $R^2$ values indicated a very high degree of correlation (0.94) at subfield VI and high degrees of correlation; 0.89 and 0.77 at subfields IV and II respectively. In subfields V, VII and VIII, only a low degree of correlation was observed ($R^2$ values between 0.25 – 0.32). In subfields I, III and VIII there were almost negligible degrees of correlation.
Table 7.1 – Summary of deposits prevalence in AF and CL images correlated with Gass-Blodi clinical stages

<table>
<thead>
<tr>
<th>Subfield</th>
<th>AF</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>II</td>
<td>0.77</td>
<td>0.77</td>
</tr>
<tr>
<td>III</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>IV</td>
<td>0.03</td>
<td>0.89</td>
</tr>
<tr>
<td>V</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>VI</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>VII</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>VIII</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>IX</td>
<td>0.27</td>
<td>0.26</td>
</tr>
</tbody>
</table>
7.5 Discussion

The current work has two objectives, first to study the evolution of the HAD in AF and CL images in MacTel patients over a period of 5 years of follow up. Secondly, to study the association of the HAD deposits in MacTel patients to the stages of clinical progression of the disease. The term “progression” in the context of idiopathic macular telangiectasia refers to a linear somewhat interlocking cascade of morphologic and functional deterioration steps starting from asymptomatic occult ocular abnormalities that eventually ends in sub-retinal exudation, haemorrhage and neovascularization functionally manifest as rapid and severe visual loss (Al-Shamsi et al., 2010). Gass and Blodi proposed the most commonly used classification system today which identified five stages of clinical severity (Gass and Blodi, 1993), this was later reviewed by Yannuzzi et al. and simplified by reducing the first four stages into the non-proliferative phase and the last stage into the proliferative phase divided into proliferative and non-proliferative (Yannuzzi et al., 2006b). The use of the term “progression” in the context of deposits in MacTel type 2 implies two distinct concepts. Evolution and change of the deposits over time and how do deposits relate to the cascade of morphologic events that characterise the clinical progression of the disease. In our use, the phrase “Deposits Temporal progression” refers to change in the count of the deposits at the 5-year-visit compared to the baseline visit. It also includes as assessment made by comparing these two time points for change over five years with the designations; regression, no change and increase. “Deposits in the Clinical Progression of MacTel type 2” is percentage change in the prevalence of the deposits in AF and CL images with the clinical progression of MacTel type 2 according to the Gass-Blodi classification system (Gass and Blodi, 1993). Our data on the assessment of deposits temporal progression over a five-year-period demonstrates that 51% and 13% of the deposits have regressed in AF and CL images respectively. 67% and 36% of the deposits in CL and AF images did not change after 5 years of follow up. Change in the prevalence of deposits in CL and AF images over the 5-year-period was mixed. There were increases in some areas while reduction in prevalence occurred in others. However, the overall magnitude
of change was very small. Despite the evidence for increase in the count and prevalence of the HAD deposits in some subfields, the results overwhelmingly indicate that the mixed pattern of change between subfields after 5 years is the rule. While this might look perplexing at first, however, bearing in mind that when patients are first enrolled in the study, they are often at different clinical stages of the disease and therefore have different stages of tissue damage. Rates of clinical progression and pathophysiologic changes vary between individual patients over 5 years, therefore, we postulate this also in part reflect the mixed pattern of change we observed.

Lack of dramatic changes in HAD deposit over a period if 5 years is hardly surprising. MacTel type 2 is a slowly progressive disease whose pathologic processes run for decades before significant changes could occur. Schmitz-Valckenberg et al. investigated structural and functional changes over a period of one year in the same population in our study. He assessed cone and rod impairment using fine-detailed visual functioning mapping. Tiny differences on Diabetic Retinopathy Study charts or MP1 were detected at one year, well within the margin of the sensitivity of the instruments (Schmitz-Valckenberg et al., 2009). Therefore, in addition to patients being in different stages of the disease, which will have a diluting effect on the magnitude of averaged changes, it is also presumed that during a period of five years, some changes might take place but would be hard to detect with the sensitivity of the current instruments. Our previous study on the prevalence of the HAD deposits in MacTel type 2 patients, has established two important observations; HAD deposits characterise MacTel patients and affected family members and that the temporal region of the fundus is disproportionately favoured for the development of the deposits. Our work on the characterisation of the HAD deposits in MacTel patients showed the involvement of the OS-RPE interphase as the site for the development of autofluorescent deposits, which might indicate remnants of dead photoreceptors. Based on these two observations, it is assumed here that HADs are related to the pathophysiology of the disease and therefore should show a correlation with the clinical progression of MacTel type 2. This assumption implies that prevalence of the deposits and/or distribution should show a correlation with MacTel’s progression. In order to test this
assumption, we designed “Deposits in the Clinical Progression of MacTel type 2” in which prevalence of the deposits in AF and CL images was determined by subfield and in patients from the different stages of MacTel type 2.

Our results show that the overall prevalence of the HAD deposits in AF images, i.e. the prevalence of the deposits in all areas of the fundus collectively showed an remarkable pattern. At stage I of the Gass-Blodi classification, HAD prevalence is 20% and ascends to a plateau at stage II (74%) and III (76%). After stage III, the prevalence is reduced to 54% and 42% at stages IV and V respectively. The correlation between the prevalence of HAD deposits and the stages was strongest at the temporal region ($R^2$ values were 0.77 and 0.94 for the inner and outer temporal regions respectively). It is interesting and potentially important that the HAD deposits start to appear at stage I of the disease and peak at stage II of the Gass-Blodi classification. During stage I which is asymptomatic, only slight parafoveal greying could be seen on biomicroscopy (Al-Shamsi et al., 2010). At stage II, MacTel is characteristically asymptomatic or if there are any symptoms, usually very mild visual disturbance. The only evidence on biomicroscopy is a loss of parafoveal retinal transparency. There is no telangiectatic capillaries or superficial refractile crystals (Yannuzzi et al., 2006). Therefore, in principle, an increase in the count of the focal HAD deposits in stage II and I could signal or anticipate an ensuing acceleration of the disease processes and therefore signals a clinical a progress to the next stage.

The plateau phase in the prevalence of HAD deposits which lasts during stages II and III. At stage III most patients become symptomatic (Gass and Blodi, 1993). This is the stage where there is a peak in the prevalence of HAD.

At stage IV, there is a reduction in the prevalence of HAD deposits that continues in stage V of the disease. It is not possible to speculate from the data the reduction in the prevalence of HADs occurs prior to the progression to stage IV. Stage IV is the last stage of the non-proliferative phase of MacTel type 2 (Yannuzzi et al., 2006) and is clinically characterised by the prominence of the RPE hyperplasia and clumps around the right-angled vessels (Al-Shamsi et al., 2010). The advent of retinal autofluorescence imaging allowed an unprecedented access
to monitor changes in RPE cell layer. In the most commonly used setting in clinical practice, laser light of 488nm is used to excite the retinal fluorophore. Emission is thought to derive mostly from lipofuscin, which is largely contained within the RPE layer (Schmitz-Valckenberg et al., 2008; Lois and Forrester, 2009). The application of fundus autofluorescence imaging in MacTel type 2, lead to a quick realisation that a generalised increase in foveal autofluorescence is among the earliest manifestations of anatomical change in MacTel type 2 (Wong et al., 2009). Clinically, it is characterised by an accelerated rate of visual decline. It is hypothesised here that there is an accelerated disease process at the end of stage III that results from the death of photoreceptor cells which are then deposited as remnants at the OS-RPE junction where the appear as hyper-reflective spots on Spectralis OCT scans and hyperautofluorescent deposits in AF images. Therefore, the HADs are an external manifestation of the underlying disease process. Patterns of focal increase in autofluorescence have also been noticed. In our work on the prevalence of these patterns, we demonstrated not only that these patterns are associated with MacTel patients, but also are associated with the temporal parafoveal area of the fundus. In our work on the characterisation of the HAD, it was demonstrated that these deposits are debris at the OS-RPE junction resulting from the death of the photoreceptors. At the moment there is a lack of credible clinical measures for the progression of MacTel type 2. Visual acuity (VA) could remain relatively intact in the presence of significant imaging alterations and impairments of functions such as reading. Therefore, there is a need for a sensitive, credible and easily measured and quantifiable biomarker. HADs in AF images appear as an attractive potential imaging biomarker that could not only describes disease progression but is also related to pathophysiological events. However, any potential biomarker has to be proven against clinical endpoints.
7.6 Conclusions

1. With the clinical progression of MacTel type 2, the overall HAD prevalence exhibits a peak at stages II and III prior to the evident decline in the visual function.

2. The association between MacTel type 2 clinical progression and the prevalence of HAD is strongest in the temporal parafoveal area where the disease manifests itself.

3. HADs in the temporal parafoveal area pre-date the appearance of tissue architectural changes evident in the same locations on OCT, which justifies their further investigation as potential antecedent biomarkers for progression.
Chapter 8

General Discussion

8.1 Discussion

Fundus autofluorescence imaging (AF) recently gained wide application in clinical ophthalmology as a robust non-invasive diagnostic tool. It depends on the stimulated emission mainly derived from the excitation of the lipofuscin (LF) molecules contained within the RPE cells (Schmitz-Valckenberg et al., 2004). Numerous factors affect the emission spectrum and consequently information AF images could provide, for instance, the wavelength of the exciting laser and the presence of other fluorophores in collagen and drusen and other pathologies which have been demonstrated to affect the spectral properties and therefore contribute significantly to the fundus autofluorescence (Marmorstein et al., 2002; Hammer et al., 2007). The normal pattern of fundus autofluorescence image reflects both the distribution of lipofuscin (LF) and the modifying effects of other fluorophores and chromophores. In a typically normal AF image, the optic nerve head appears dark as it is devoid of RPE and LF. Vasculature appears dark because of absorption of the signal by the red blood cells and blood components. The fovea is darker due to the masking effect of the luteal pigments (lutein and zeaxanthin). The parafoveal area exhibits a relatively decreased autofluorescence intensity caused by a combination of increased melanin deposition and lower density of LF granules at RPE cells in the central retina. Abnormalities in the autofluorescence in MacTel type 2 became evident soon
after the introduction of the technique in clinical ophthalmology. The earliest evidence of a supervening MacTel type 2 is subtle increases in central AF signal. These subtle increases are observed long before the more overt angiographic, MP changes or changes on OCT are evident (Wong et al., 2009). The subtle changes are attributable to early anatomic changes preceding vascular abnormalities, these changes could be due to the loss of macular pigment (Helb et al., 2008) and consequently its unmasking effect on autofluorescence and/or quantitative and qualitative changes in fluorophore (s) content of the affected areas (Wong et al., 2009) Hereby, we describe a new pattern of fundus autofluorescence change in MacTel type 2. It consists of focal increases of autofluorescence signal that shows a clear predilection to the temporal region. These hyperautofluorescent deposits (HADs). Multimodal examination, revealed that HADs are hyper-reflective on high-resolution OCT Spectralis scans. HADs were localised to the outer retinal layer at the level of the IS/OS junction and some appear to be within the RPE cell layer. We postulate here that the pathophysiological correlate of these hyperautofluorescent hyper-reflective deposits (HADs) is a focal accumulation of fluorophores. The location on high-definition Spectralis scans demonstrates a compromise in the integrity and invasion of the trough representing the photoreceptor outer segment. The location of the deposit suggests the fluorophore that gives rise to the focal hyperautofluorescent pattern is likely to be the shed photoreceptor outer segments and in case of RPE proliferation, lipofuscin accumulation. Lipofuscin is a generic term for a complex mixture of polyunsaturated fatty acids and modified proteins. LF is composed of at least 10 different fluorophores (Eldred and Katz, 1988). A2-E, which is the major golden-yellow emitting component fluorophore of, arises as an undegradable metabolic end product arising as a result of RPE digestion of the rods outer segment (ROS) enriched in the polysunsaturated fatty acids (PUFAs). We hypothesise here, that the HHDs are focal accumulations of PUFA or LF resulting from degeneration and apoptosis of the photoreceptors and/or reduced functional capacity of the RPE cell layer to phagocytose and process the outer segments. We identified two patterns of HHD deposits. One pattern is an accumulation external to a morphologically intact RPE layer represented by the major thick hyper-reflective line. In the second pattern, there is a focal expansion of
the hyper-reflective line representing the RPE cell layer. We propose an accumulation of the
ROS external to the RPE possibly due to reduced ability of the RPE to phagocytose in former
example and a reduced functional capability of the RPE to process the phagocytosed ROS
segments and the consequent expansion of the RPE lysosomal fraction in the latter example.
Spectral-domain OCT (SD-OCT) examination revealed loss of the integrity of the junction of
the photoreceptor inner and outer segments (IS/OS) line (Barthelmes et al., 2008; Charbel
Issa et al., 2008). However, the lack of good visualisation of photoreceptors by SD-OCT meant
that uncertainties persisted. Our observations have detailed the probable temporal sequence
in which HADs deposits fit into the pathophysiologic cascade of events that characterise the
clinical staging of MacTel type 2. Our hypothesis corroborated by evidence that the these
focal HADs deposits favour the temporal region which is implicated in MacTel type 2 early
pathophysiologic changes (Abujamra et al., 2000). Even more so, the prevalence of the depos-
ts in the temporal region shows a very strong correlation with the clinical stage of the disease.
HAD deposits, increase in prevalence at the early stages of the disease to peak at stage III of
the Gass-Blodi classification when the disease is characteristically clinically silent. Prevalence
decreases in stages IV and V when visual impairment is evident. Our multimodal investigation
demonstrated that areas of HADs are surrounded by a ring-like pattern of hyperfluorescence
in the early phase of fluorescein angiography (FA). HADs are predicting factors that precede
the development of these vascular abnormalities. Ooto et al. described a similar ring-like
pattern of fluorescein hyperfluorescence, have recently used a combined AO-SLO approach to
study such leaks. They demonstrated that FA leakage areas which appear temporally as dark
ring-like structures that correspond to areas of cone loss (Ooto et al., 2011). Autofluorescence
in the fundus is a phenomenon also expressed by other types of deposits in a variety of clinical
and physiologic conditions. Drusen, which are extracellular complexes of lipid and proteins
(Crabb et al., 2002) some of which have considerable autofluorescent activity (Marmorstein et
al., 2002; Hammer et al., 2007).

The international Fundus Autofluorescence Classification Group (IFAG) devised a system
whereby autofluorescent patterns in AF images are classified into 6 morphologically distinct
patterns (Bindewald, 2005). Einbock et al. studied autofluorescent patterns in AF images from 124 patients with soft drusen based on the IFAG classification and reported that drusen could exhibit an increased, decreased or no autofluorescence intensity compared to the fundus. One of the 6 identified patterns, the reticular pattern could be seen in macular or peripheral zones as patchy, linear or lace-like (Bindewald, 2005). Follow up of patients indicated that reticular pattern, which also exhibits phenotypical metamorphosis, is associated with a high risk of progression to age-related macular degeneration with visual loss (Einbock et al., 2004).

Lengyel et al. reported that autofluorescent drusen in aged eyes, have strong association with the honeycomb pillars of the choriocapillaris (90.2%) (Lengyel, 2004). However, the significance of this association is not clear. Although LF is the dominant fluorophore in the retina, the origin of drusen autofluorescence is not entirely clear (Solbach et al., 1997). Different candidates have been proposed. Oxidised polyunsaturated fatty acids (PUFA) originating from phagocytosed rod outer segments (Kopitz et al., 2004) is one such candidate. Unlike AMD drusen, the focal patterns of HADs deposits in MacTel type 2 are almost exclusively hyperautofluorescent. Our data indicate that a phenotypical metamorphosis takes place in the HAD deposits over time. Our 5-year-follow up period, showed there is a regression and loss of autofluorescence in some deposits. At stages IV and V of the clinical progression of MacTel type 2, there is an accelerated loss of autofluorescence of the HAD deposits that coincides with a clinically overt visual decline. We postulate that this reduction of autofluorescence is resultant from the focal death and/or removal of photoreceptors and/or RPE at these foci. This assumption could explain the the reduction in visual function that occur at stages IV and V after the peak in the prevalence of HAD deposits and also the potential clinical utility of the phenomenon as a prognostic biomarker or an antecedent biomarker.

Numerous lines of evidence from different pathologies suggest that hyperautofluorescent deposits are due functional derangements of the photoreceptor outer segment-RPE complex. Stargardt disease (fundus flavimaculatus) is a hereditary juvenile macular degeneration that results in blindness. The most common form of the disease (STGD1) is autosomal recessive due to a mutation in ABCA4 (Allikmets et al., 1997). The ATP-binding cassette (ABC)
transporter gene superfamily encodes membrane proteins that are involved in substrate translocation across the plasma membranes (Higgins, 1992). The mutation results in excessive accumulation of LF and in particular its N-retinylidene-N-retinylethanolamine (A2E) component (Cideciyan, 2004). The deposits in Stargadt’s disease on FAF show confluence with increased autofluorescence signal intensity in the initial stages of the disease. As the disease progresses, the signal intensity decreases as well as texture changes occur due to the destruction of the Photoreceptor-RPE complex (Cideciyan, 2004). Using SD-OCT, Querques and colleagues studied the flecks characteristic of Stargaadt’s disease; found that type 1 deposits are located above the RPE (Querques et al., 2009).

The royal college of surgeons’ rats (RCS) have a genetic defect that prevents phagocytosis of photoreceptor outer segments by RPE. When their retinas were compared to retinas from normal rats, it was found that LF accumulation is substantially reduced (Katz et al., 1986). Further evidence for the origin of LF comes from studies the spectral properties of photoreceptor-RPE complex. Boulton et al. reported that FAF, A2E in cells and RPE lipofuscin emission spectra all have emission maxima between 590-620nm and display a characteristic red shift (Boulton et al., 1990). Hyperautofluorescent deposits are primarily located in the temporal parafoveal region in MacTel type 2 patients in an area associated with the earliest pathological changes of MacTel type 2. Fluorescence of the fundus in MacTel type 2 patients is likely to be emanating from a mixture of different fluorophores linked to the pathological processes of the disease. Discrimination of these fluorophores and the identification of their unique spectral signatures could potentially be a powerful diagnostic tool for monitoring and anticipating the progression of MacTel type 2 long before it is evident on other imaging modalities. Association of the focal patterns of HADs with both the temporal parafoveal area and the clinical progression of MacTel type is an added reason that merits their further tissue study. These results suggest that the involvement of the photoreceptor-RPE complex is an early change in the pathogenesis of MacTel type 2. Probing into the formation of the hyperautofluorescent deposits might be vital for the understanding MacTel type 2 metabolic problems in the photoreceptor-RPE-choroid complex. The ease and practicality of detecting
HAD deposits make it an attractive candidate as a surrogate imaging biomarker for MacTel progression.
8.2 Methodology Critique

8.2.1 Design

The basic design of this study was cross-sectional aimed at establishing a “snap shot” of the prevalence of the focal pattern of hypersutofluorescent deposits (HADs) in MacTel patients in comparison to controls and family members. To this basic design, a retrospective longitudinal element has been added to provide a tentative insight into the possible causative correlates of HADs over a 5-year-period. While valuable in giving an insight into differences between groups in the phenomenon of interest, cross-sectional studies do not offer credible clues into causation. In our study, this downside of cross-sectional studies was tempered down by adding the retrospective cross-sectional element to study via studying change over a 5-year-period of follow up. This was further substantiated by a detailed multi-modal investigation of HADs in MacTel patients and in other groups. While we believe, this study is sufficient to establish beyond reasonable doubt the clinical value and pathophysiologic significance of HADs in MacTel type 2, we recommend a range of approaches and techniques to invigorate our findings (please see section on future directions 8.3).

8.2.2 Fundus Autofluorescence Imaging (AF)

FA image shows the topographical distribution of the intensity of the signal representing the recorded emission spectrum. Intensity is represented in grey scale values (arbitrary values, 0–255) per pixel. Low pixel values (dark) illustrate low intensities, and high pixel values (bright) illustrate high intensities. An absolute quantification of AF intensity is not possible with the current detection systems. Meaningful topographical comparisons of auto fluoresce intensity remain relative and only possible within the same image. Interpretation of autofluorescent patterns is interpretor-dependent and therefore differences between different interpreters are common, especially in borderline changes. Other limiting factors include the attenuation of the exciting laser signal and emission spectrum by the intervening media (cornea, lens and vitreous) (Einbock et al., 2004). Artefacts could develop due to eye movement during ima-
ging. A major disadvantage of the current design of the widely used fundus autofluorescence imaging system is their dependence solely on the blue-green excitation light ($\lambda_{\text{exc}}$ with 488nm) and a wide-pass filter with short wavelength cut-off point at 521nm to act as a barrier to the excitation wavelengths (Von Rückmann et al., 1995)

This setting optimised only for the detection of autofluorescence emission from lipofuscin (Eldred and Katz, 1988; Delori et al., 1995). Ocular fluorophores, under both physiologic and pathologic conditions have widely different excitation and emission characteristics. The choice of the exciting laser determines the range of physiological and pathological features highlighted in AF images (Hammer et al., 2007). A further loss of information occurs due to the filter whose cut-off point set at 521nm in the emission detection systems. Effectively, this system is set to detect fluorophores whose excitation and emission properties fall between the limits determined by the Stoke’s shift for the exciting laser and the cut-off point of the filter, out of these perimeter is either not excited or filtered off. Therefore we conclude that the current range of autofluorescent changes observed in AF images of patients with MacTel type 2 may not represent the full range of pathophysiological changes that could be detected with a better design of AF cameras that allows multi-wave length excitation to produce topographical AF images and analysis of focal lesion with multiple wavelength of excitation.
CHAPTER 8.  GENERAL DISCUSSION

8.3 Future Directions

Introduction of AF imaging for monitoring patients with MacTel type 2, added unique information to the time line of the evolution of the disease. The earliest pathological changes consist of anatomic changes due to and/or quantitative and qualitative changes in fluorophore(s) content of the affected areas (Wong et al., 2009). The consequent “unmasking effect” of loss of macular pigment is detected in AF as a global increase in central AF signal long before vascular and other changes are evident on other modalities. In our study, we provided evidence for a hitherto undescribed pattern of changes in fundus autofluorescence. This focal pattern consists of deposits at the IS/OS junction and thickening of the RPE layer is associated with the temporal parafoveal area of the fundus and correlates well with the clinical progression of MacTel type 2. Establishment of the clinical value of this focal pattern of autofluorescence requires an understanding of the molecular nature and chemical properties of the component fluorophores. Determination of the precise spectral properties of fluorophores necessitates their chemical isolation in human MacTel type 2 tissue and from animal models of MacTel type 2. Isolation would then help elucidate their structure and mechanism of formation, which will go a long way into fitting them into the pathologic cascade of events in the photoreceptor/RPE/choroid complex that is ultimately responsible for the development and the progression of MacTel type 2. Study of the phenotypical change and reduction in autofluorescence of the HAD deposits over time in MacTel type 2 requires the study of the amount, configuration and the biophysical environment of the fluorophore, any of which could be altered by disease processes. The properties of fluorescence anisotropy and fluorescence lifetime could add very valuable information and help distinguish fluorophores of interest from the numerous other fluorophores in the fundus. Previous work established that unique spectral signatures are associated with Bruch’s membrane and RPE both in normal and AMD tissue (Hammer et al., 2007). Autofluorescence of the fundus in MacTel patients is likely to be emanating from a mixture of different fluorophores linked to the pathological processes of the disease. Information gained from the isolation of these fluorophores and the identification of
their unique spectral signatures could then be applied in *in vivo* diagnosis for monitoring and anticipating the progression of MacTel type 2. Currently, the most commonly used setting of AF cameras in clinical ophthalmology, relies on excitation of the fluorophores in the fundus with a singular laser light of $\lambda_{\text{exc}}$ with 488nm. There is a limited range of fluorophores and features that could be highlighted with $\lambda_{\text{exc}}$ at 488nm.

Development of new autofluorescence imaging systems will boost both the quality and the range of information obtainable from AF images. Previous work indicated that different physiological and pathological features in fundus are highlighted in AF images by the choice of the exciting laser light (Hammer et al., 2007a). We propose the exploration of the potential for the use of shorter and longer wavelengths for excitation in a multi-excitation instrument merits investigation. Provided safety and practicality issues are satisfactorily addressed, introduction of lasers with capabilities for $\lambda_{\text{exc}}$ at 364nm, 405nm, and 543nm would broaden the range of the detectable physiologic and pathologic abnormalities to include a whole host of new fluorophores. We also propose an introduction of a function that in addition to the producing images of the fundus would allow for focal/ localised multi-spectral analysis in the fundus. Such function, would allow the clinician or a scientist to study in detail the spectral properties of localised lesion such as drusen to settle any doubts on their nature.

Inclusion of such features in a new design of fundus autofluorescence cameras would undoubtedly be faced by technical challenges such as increased absorption of shorter wavelengths by the intervening media (lens, vitreous and cornea). Such challenges could be overcome by the use of 405nm instead 364nm laser, which is subjected to lesser absorption. The use of such systems would tremendously expand the range of physiologic and pathologic information obtainable in vivo for a clinician. The ease and practicality of detecting HAD deposits in addition to the and the non-invasive nature of makes it an attractive candidate as a surrogate imaging biomarker for MacTel progression and for the evaluation of animal models of the disease.

This study, which essentially followed a cross-sectional retrospective longitudinal design established a temporal “snap shot” of the prevalence of the focal pattern of hypersautofluorescent
deposits in MacTel type 2 patients in addition to change over a 5-year-period. A hint to the possible causation came from combining data obtained from the comparison of MacTel type 2 patients with other categories (controls, family members, DM and AMD patients) and comparison of patients with and without HAD, to data from multi-modal characterisation of HADs. Construction of a causation chain in which HADs, their antecedent changes and clinical consequences are explained within MacTel’s pathophysiological cascade, dictates the design of a prospective longitudinal study.

A prospective longitudinal study would allow the investigation of phenomenon and consequences into the future (Menard, 2002). A prospective study of HAD can potentially provide data to test our hypotheses that HADs are early signs of photoreceptor death and therefore anticipate visual decline after a peak in prevalence at stage III of Gass-Blodi classification. Conclusive answers to this question, is imperative in establishing HADs as a singular or part of a battery as an antecedent imaging biomarker for the progression and/or acceleration of the pathology in MacTel type 2. A combination of adaptive optics (AO) and fundus autofluorescence imaging (FA) for the study HADs could provide in vivo data to test our hypothesis that HADs are remnants of dead photoreceptors in the retina. Although our SD-OCT data clearly shows HADs are located at the IS/OS junction and therefore are likely to represent areas of photoreceptor apoptosis, the resolution is not sufficient to make conclusions on the type of photoreceptors (rods versus cones). Recently, Ooto et al. using a prototype of adaptive optics coupled to a confocal scanning laser ophthalmoscope (AO-SLO) identified dark regions in the cone mosaic in eyes with MacTel type 2 (Ooto et al., 2011). These dark regions corresponded to areas of disruption in the IS/OS on SD-OCT images representing photoreceptor abnormalities to the areas of leakage on FA (Ooto et al., 2011). In order to establish—with a reasonable measure of certainty—that HADs are in fact remnants of dead photoreceptors whose autofluorescence arises from partially oxidised PUFAs, it is imperative to design an AO-AF study. Such a tool could be incorporated into a prospective longitudinal study that aims at establishing a pathophysiological causal links between HADs antecedent and consequent changes.
Part IV

Overarching Discussion
Chapter 9

Overarching Discussion

Idiopathic macular telangiectasia type 2 (MacTel type 2) is a disease characterised by a slowly progressive neurodegenerative process that primarily affects the temporal area of the central retina. To-date no effective therapy that proved efficacy in slowing or modifying the rate of progression of the disease. Introduction of novel imaging modalities lead to the notion that a neurodegenerative process at IS/OS junction and RPE layer is an early manifestation of the disease. Despite the accumulating body of knowledge on the morphologic changes associated with the disease progression, to-date, there is no validated prognostic measure for the disease progression. A prognostic biomarker that has the capability to predict the future course of the disease and the response to therapy will be very valuable for both the clinician and in therapeutic trials. Recent progress in structure-function correlation derived from high-resolution spectral-domain OCT demonstrated that neurodegenerative changes are an early manifestation of the disease. Subtle increases in foveal autofluorescence representing early anatomic changes have been found are also recognised in the disease. We have demonstrated the new hitherto undescribed focal pattern of autofluorescence increase in the temporal parafoveal area. Based on our findings we postulate that this pattern is closely related to the pathophysiology of MacTel type 2. It is argued here that hyperautofluorescent deposits (HADs) could potentially act as useful prognostic biomarker for disease progression either singularly or as part of a battery of indicators that could collectively attain the required sensitivity and specificity.
for the disease. A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal physiologic or pathologic processes or pharmacological responses to a therapeutic intervention (Bielekova and Martin, 2004). A successful candidate biomarker should fulfil criteria that includes, biological rationale, clinical relevance, practicality, correlation with disease activity and Sensitivity and specificity (Bielekova and Martin, 2004). It is possible that an ideal biomarker that ticks all the boxes will never be found, but it is argued here it is worth investigating HADs for potential use as an imaging biomarker either singularly or as part of a battery of other indicators.

(i) Biological rationale: Our detailed descriptive characterisation of the hyperautofluorescent deposits (HADs) demonstrated these deposits which are also hyperreflective in spectral-domain OCT are disruptions of the IS/OS junction and represent deposits that invade the photoreceptor outer segment and expansion distress of the RPE cell layer. Multi-modal characterisation of the deposits has also demonstrated that during the early phase, fluorescein dye diffuses around the deposit areas without staining the deposits themselves potentially indicating that deposits could act as physical barriers to fluorescein. These observations corroborate our SD-OCT findings and indicate that there is likely to be an anatomical and/or a functional disruption of the outer blood-retinal barrier due to disruption at the level of the tight junctions between RPE cells. HADs represent an early external manifestation of a process of a neurodegenerative process that leads to photoreceptor outer segment death in large deposits; there is also RPE stress and enlargement. (ii) Clinical relevance: The potential for HADs as biomarkers requires a demonstration of its clinical relevance via the demonstration of a position in the chain of morphologic associations characterising MacTel type 2. We found that the prevalence of HADs in temporal parafoveal area shows a very strong correlation with the clinical progression of MacTel type 2 according to Gass-Blodi classification. This observation drawn from our retrospective longitudinal study needs to be substantiated by a prospective longitudinal investigation. While our work uncovered important associations between HADs and MacTel type 2 clinical progressions, an ideal prospective longitudinal study would seek to look into the association between HADs and functional deficits of patients as measured by
vision-related QoL, visual acuity or fine matrix mapping (microperometry). Certain desirable cut-off points could act as surrogate clinical biomarkers and milestone goals for therapeutic interventions in clinical trials or warning signs for significant visual deteriorations.

(iii) Practicality: The practical attractiveness of the use of autofluorescence imaging as a diagnostic tool and for HADs as a potential biomarker, rests in its convenience for both the patient and clinician. The non-invasive nature of the imaging procedures makes it particularly attractive as an imaging biomarker. Serial follow up of HADs would be facilitated and changes over time could easily be monitored. (iv) Correlation with disease activity: Potential biomarkers are required to demonstrate a relationship to disease activity either directly (clinical) or para-clinically. AF images of HADs count or size in the fovea could potentially relate to disease activity. Our work on progression demonstrated that HADs attain peak prevalence at stage II of Gass-Blodi staging system and show a significant regression just prior to the appearance of visual decline in stage V. We hypothesise such a decline- after a peak- in the prevalence of HADs is due to accelerated death of photoreceptors. The loss of photoreceptors renders RPE cells devoid of the fluorophores that give rise to autofluorescence emission. More investigation would need to prove the accuracy of the use of HADs as a para-clinical measure of disease progression. (v) Correlation with disability/prognosis: A prospective longitudinal study that includes an assessment of functional deficits based on point-to-point correlation with HADs over a sufficiently long period of time, would ultimately be required for the demonstration of a correlation of HADs as biomarker with the visual impairment over multiple for determining meaningful clinical surrogacy. In one such prospective longitudinal study, Steffen Schmitz-Valckenberg et al who studied the same population as in our study reported measurable FMM in the space of one year (Schmitz-Valckenberg et al., 2009). Given the prolonged course of the disease, the time duration may not be sufficient for measurable changes to be manifest in HADs, however, careful point-to-point analysis of the Schmitz-Valckenberg data along with our AF images has the potential of elucidating the link between HADs progression and functional changes.

No single candidate is expected to satisfy all the conditions to be the “perfect” biomarker.
In the absence of a gold standard biomarker for the clinical progression of MacTel type 2 or as a surrogate for clinical endpoints that could be applied for monitoring disease activity and for the evaluation of therapeutic interventions. It is imperative to consider the evaluation of HADs to find out if they possess the necessary sensitivity for a biomarker. It would be necessary to find out if quantifiable change as detected using our approach could be used in a longitudinal setting and whether it possess enough sensitivity to reflect clinically relevant endpoints, for instance two scores on vision-related QoL. In summary, HADs possess the necessary mechanistic relevance to the pathophysiology of MacTel type 2 as numerous evidence indicate that HADs as measured by autofluorescence imaging, represent an early sign of photoreceptor and / or RPE neurodegenerative process. Important questions remain to be answered in sufficiently empowered prospective longitudinal study concern the sensitivity and specificity of HADs for the use in the clinical evaluation of patients MacTel type 2 and whether HADs attributes such as size, location intensity of autofluorescence etc could have prognostic significance. Establishment of HADs as a prognostic or a surrogate biomarker would help the decision-making process regarding efficacy of therapeutic interventions and risk stratification for disease progression and visual decline for patients with MacTel type 2. Fundus autofluorescence imaging entails the acquisition of a topographic autofluorescence emission map using excitation with a particular wavelength. Spectral emission optical contrast between healthy and diseased tissues is achieved through the quantitative differences in their intensities. Fundus autofluorescence imaging in the form most in use in clinical ophthalmology, employs a blue-green laser excitation light at 488nm the detection system consists of a wide-pass filter that has a wavelength cut-off point at 521nm (Von Rückmann et al., 1995). This system was optimised for the detection of autofluorescence emission emanating from lipofuscin (Eldred and Katz, 1988; Delori et al., 1995). The downside of excitation with a singular laser that fluorophores excitable at shorter wavelengths will not be excited and therefore potentially important information on the status of the Bruch’s membrane and deposits are missed. Ocular fluorophores, under both physiologic and pathologic conditions have widely different excitation and emission characteristics. The choice of the exciting laser determines the range of physiological and pathological features
highlighted in AF images (Hammer et al., 2007). A further loss of information occurs due to
the filter whose cut-off point is set at 521nm in the emission detection systems. Effectively, this
system detects fluorophores whose excitation and emission properties fall between the limits
determined by the Stoke’s shift for the exciting laser and the cut-off point of the filter, out
of these perimeter is either not excited or filtered off. Therefore we conclude that the current
range of autofluorescent changes observed in AF images of patients with MacTel type 2 may
not represent the full range of pathophysiological changes that could be detected with a better
design of AF cameras that allows multi-wave length excitation to produce topographical AF
images in combination with focal analysis of lesions with multiple wavelengths of excitation.
Our work has demonstrated that at $\lambda_{\text{exc}}$ with 488nm, a significant contribution to the over-
all fluorescence spectrum is likely due to and bearing the spectral signatures of the Bruch’s
membrane and drusen. This contribution augments with $\lambda_{\text{exc}}$ at 364nm as a larger number
of fluorophores is excited. The identified unique spectral signatures of drusen and Bruch’s
membrane could potentially be employed for a non-invasive diagnostic tool and could as well
be useful for assessing animal models for AMD and other diseases that in which drusen or
drusen-like deposits and/or derangements of Bruch’s membrane form part of the disease phe-
nomenology. A new design of fundus autofluorescence cameras, one that enables the creation
fundus emission maps with multiple excitation wavelengths would greatly expand the range of
the pathological and physiological phenomena explored by fundus autofluorescence imaging.
Introduction of a focal analysis option whereby spectral information could be generated from
a focal point would greatly enable precise analysis of focal abnormalities such as drusen or
deposits and would provide elucidative insights into their molecular structure and fluorophore
content. With the current setting of AF imaging, distinction between the different structures
of the fundus rests mainly on the intensity differences, which is presumed to be due to concen-
tration, and distribution differences between the fluorophores excitable with that particular
wavelengths. We have demonstrated that there is another possible explanation for the differ-
ences in autofluorescence intensity in ocular tissue. We found that there is a strong relation
between the metal content of drusen and autofluorescence intensity with $\lambda_{\text{exc}}$ at 488nm. A
large proportion of the difference in autofluorescence intensity between drusen that underlie their autofluorescence heterogeneity could be explained by the quantitative differences in their metal content. Recent advances in the understanding of the interaction between fluorophores and metals have shown that both fluorescence intensity and lifetimes of a fluorophore are radically modified by the presence of metals in the immediate niche of the fluorophore (Geddes C.D. et al., 2003). Autofluorescence intensity depends on the sum of its quantum yield and lifetime (Lakowicz, 2006) both of which are increased by fluorophores proximity to metals (Geddes C.D. et al., 2003). Metal fluorophore interaction can have dramatic effects on the fluorophore’s detectability as the enhancement of intensity could increase the intensity by a factor up to a million times (Geddes C.D. et al., 2003). Bruch’s membrane thickening is associated with the deposition of extracellular material that reportedly contain pathophysiologically high concentrations of Zn (Lengyel, Flinn, Peto, et al. 2007). We have demonstrated that soft drusen, a risk for AMD; quantitatively contain larger concentrations of zinc than hard drusen which do not constitutes a risk for AMD development. We have also demonstrated that drusen and there is mineralization with Ca and other metals in the sub-RPE deposits. The role of metals in modifying autofluorescence properties in biological tissue is an unexplored area at the moment. Given the increasing adoption and application of autofluorescence imaging in clinical ophthalmology and other areas of investigation in medicine, it is imperative to closely examine the role of the metals and trace elements in the modification of autofluorescence properties both in health and in disease.
Bibliography


44. Bressler, S.B., Maguire, M.G., Bressler, N.M., Fine, S.L., 1990. Relationship of drusen and abnormalities of the retinal pigment epithelium to the prognosis of neovascular


in pathologic deposits associated with aging and age-related macular degeneration. Proceedings of the National Academy of Sciences 99, 11830.


oxidation products in human and monkey retinas. Investigative ophthalmology & visual science 38, 1802.


of the National Academy of Sciences of the United States of America 101, 5928.


Archives of Ophthalmology 126, 330.


283. Sell, D.R., Monnier, V.M., 1989a. Structure elucidation of a senescence cross-link from


407–412.


