Identifying characteristic features of the retinal and choroidal vasculature in Choroideremia using optical coherence tomography angiography

Running head: Choroideremia and OCTA

Alessandro Abbouda MD[1-2], Adam M Dubis PhD[1-2], Andrew R Webster[1-2] MD FRCOpth, Mariya Moosajee[1-2] PhD FRCOpth

[1] UCL Institute of Ophthalmology, 11-43 Bath Street, London EC1V 9EL

[2] Moorfields Eye Hospital NHS Foundation Trust, 162 City Road, London EC1V 2PD

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Corresponding Author:

Dr. Mariya Moosajee
UCL Institute of Ophthalmology,
11-43 Bath Street,
London, UK, EC1V 9EL
Email: m.moosajee@ucl.ac.uk

Abstract

Aims: Using optical coherence tomography angiography (OCTA) to investigate the area with flow in the superficial retinal vessel network (SVRN) and choriocapillaris (CC) layer amongst male subjects with Choroideremia (CHM), female carriers and normal controls to identify vascular changes.

Methods: Images of SRVN and CC layer were acquired in 9 affected males, 5 female carriers and 14 age- and gender-matched controls using the Angiovue software of the RTVue XR Avanti (Optovue, Inc., Fremont, CA).

Results: The mean age was 33 years for affected male CHM patients (median 30 years), 46 years for female carriers (median 53 years) and 39 years for controls (median 38.5). Mean SRVN area ± SD in subjects with CHM was 12.93±2.06 mm^2 in carrier subjects, 15.36±0.60 mm^2 and in controls 15.30±1.35 mm^2 ($p<0.01$). The mean CC area ± SD with flow was 6.97±5.26 mm^2 in CHM subjects, 21.65±0.17 mm^2 in carriers and 21.36±0.76 mm^2 in controls ($p<0.01$). SRVN and CC area with flow showed a negative correlation in CHM subjects with the age ($r=-0.86$; $p<0.003$ and $r= -0.77$; $p<0.01$, respectively). CC area with flow had a positive correlation with SRVN ($r=0.83$, $p<0.001$) Overall, visual acuity had a negative correlation with SRVN and CC area with flow ($r=-0.67$, $p<0.001$ and $r=- 0.57$, $p<0.002$, respectively).

Conclusions: This is the first study to highlight changes in the SRVN in CHM subjects. OCTA detected a reduced area with flow in both retinal and choroidal circulations, and may be a useful tool for monitoring natural history and disease progression in forthcoming clinical trials.

Introduction

Choroideremia (CHM) is an X-linked chorioretinal dystrophy. It was first described in 1872 by Mauthner [1] and is characterized by a progressive atrophy of the choroid, retinal pigment epithelium (RPE) and retina. The estimated prevalence is 1 in 50,000-100,000[2, 3]. The *CHM* gene is located at Xq21.2 and encodes Rab escort protein 1 (REP1)[2, 4]. The classical anatomical description of the choriocapillaris (CC) is a single continuous layer of capillaries forming a network on Bruch's membrane. Each segment of the CC is supplied by an independent terminal choroidal arteriole. The various segments intersect only via the venous channels.[5] The lobules varies in their geometric configuration, having between three to six sides forming an irregular triangular to hexagonal shape. The average size of a lobule is between 620 to 830 µm from venular to venular intersection.[6] It has been proposed that the primary site of degeneration in CHM is the RPE, with a consequent loss of photoreceptors[7]. This concept has been supported by the recent technology acquisition such as optical coherence tomography (OCT).[8, 9] However, before the advent of OCT, the histology of CHM eyes showed that the primary defect presented in the uveal vessels[10] with progressive choroidal thinning towards the transition zone between pigmented and non-pigmented fundus areas. The samples also showed extensive fragmentation of the basement membranes between the pericyte and endothelial cells, with a progressive obliteration of the CC leading to a sharply defined area of simultaneous RPE loss.[10] Flannery *et al* described similar histology in a subject carrier of CHM [11]. The CC was normal in areas with normal photoreceptors, except for widening of the intercapillary pillars, but in those regions with abnormal photoreceptors, choroidal capillaries were fewer in number, had reduced luminal diameter, and fenestrae were sparse. In some areas of intense atrophy, there were no choroidal capillaries.

The superficial retinal vessel network (SVRN) is supplied by the central retinal artery and mainly provides blood flow to the retinal nerve fiber layer and ganglion cell layer. The plexus is spread all over the retina except for three specific areas; the posterior edge of the ora serrata, the fovea avascular zone (FAZ), and the area of retina adjacent to the major arteries.[12] Interestingly, in high myopic patients a correlation exists between decreased choroidal blood flow and reduction of the SVRN.[13, 14] Decreased choroidal blood flow is considered the outcome of increased axial length resulting in ocular elongation stretching the vessels and modifying the retinal microvascular network.[13] In CHM patients, the progressive atrophy of the CC and choroid may directly affect the outer retinal supply and also the retinal vasculature system.

The purpose of this paper is to identify changes in the SRVN and CC layer *in vivo* in affected male CHM patients and compare it with female carriers and normal subjects by OCTA.

Methods

This is a prospective observational study conducted between June and October 2016 at Moorfields Eye Hospital London (UK). The protocol of this study adhered to the provisions of the Declaration of Helsinki and was approved by the national research ethics committee. Informed consent was obtained from all subjects. The inclusion criteria was a confirmed molecular diagnosis of CHM; and the exclusion criteria included the presence of any other ophthalmic disease. Affected male CHM patients, female carriers, and age-matched controls underwent OCTA imaging (Avanti RTVue XR; Optovue, Inc).[15-17] Macular angiograms (6x6mm) acquired using the Angiovue software of the RTVue XR Avanti (Optovue, Inc., Fremont, CA) were used to detect areas of flow in otherwise static tissue[18] related to the SVRN and CC flow without using fluorescein or indocyanine green dye. The FAZ was

calculated at the superficial retinal layer from a macular angiogram of 3x3 mm using the "no flow function" provided by the same software to identify the area devoid of vessels at the centre of the macula. The SVRN was automatically segmented, using the Angiovue software (Optovue, Inc., Fremont, CA)[19], from the inner limiting membrane with an offset (from the interface reference) of 3 mm to the inner plexiform layer with an offset (from the interface reference) of 15 mm. The CC layer from the RPE with an offset (from the RPE reference) of 30 mm to the deeper choroidal layer with an offset (from the RPE reference) of 60 mm. The segmentations of all examinations were checked before any measurement was performed. In subjects with CHM, the CC segmentation was performed manually [20, 21] for each subject to ensure proper identification of the layer reducing the risk of artefacts. In subjects with CHM, the area with CC was manually corrected by two different observers. Five measurements for each subject were collected by two observers to analyse the CC and inter-observer agreement was determined using the intraclass correlation coefficient (ICC). The average of them was considered for the analysis. The "flow" function in AngioView was used to detect the SRVN and CC area with flow.

All statistical analyses were performed using the Statistical Package for the Social Sciences (version 21.0; SPSS Inc., an IBM Company, Chicago, IL). Continuous variables are presented as the mean ± standard deviation (SD). Normal distribution of data was analysed by the Shapiro-Wilk test. Categorical variables were compared using Fisher's exact test or Chi squared test. Parametric variables between groups were compared using the unpaired t-test. Levene's test was used to verify variance homogeneity. Non-parametric distributed values were analyzed by the Mann–Whitney U test. For the comparison of several related samples, the ANOVA test or the Kruskal-Wallis test were used. Bivariate relationships were evaluated by the Spearman coefficient, or the Pearson analysis, as appropriate. A *P* value <0.05 was considered statistically significant. Taking into consideration the symmetry

between the right and left eye of an individual, the absence of any treatment given, and the non-normal distribution for many continuous variables (such as?), the mean data from both eyes was considered for the statistical analysis as previously suggested.[22, 23]

Results:

A total of 17 eyes from 9 male CHM subjects and 9 eyes from 5 female carriers and 28 eyes from 14 normal subjects (9 men and 5 women) were included in this study. All patients and controls were Caucasian. The demographic features are summarised in table 1. The molecular diagnosis for each subject is reported in table 2. Mean age was 33 years (median 30 years; range 12-57 years) for affected males, 46 years (median 53 years; range 21-64 years) for carriers, and 39 years (median 38.5; range 12-60) for controls. Only one eye of an affected male CHM patient was excluded from the study owing to the low quality of the OCTA images and OCTA artefacts resulting from lack of fixation, and one eye of a female carrier was excluded due to previous choroidal neovascularization. Mean best corrected visual acuity (BCVA) was 0.47±0.31 LogMAR (median 0.17; range 0.0-3.0) for affected males, -0.08±0.00 LogMAR (median 0.0; range -0.04-0.0) for carriers and -0.03±0.00 LogMAR (median 0.0; range -0.08-0.03) for controls. The distribution of the visual acuity was statistically significant different amongst groups. ($p<0.01$)

Summary of SRVN, CC area with flow and FAZ is given in table 1. The difference in SRVN and CC between groups was statistically significant ($p<0.01$, Fig 1 A, B). However, the difference in FAZ between groups was not statistically significant ($p=0.59$). The FAZ had a negative correlation with SRVN ($r= -0.54$, $p<0.003$) and CC area with flow ($r= -0.48$, $p<0.008$).Visual acuity has a negative correlation with SRVN and CC area with flow ($r=-0.67$, $p<0.001$ and $r=- 0.57$, $p<0.002$, respectively).

Analyzing only affected males, the age had a positive correlation with the size of the FAZ ($r=0.75$; $p<0.01$) and it was negatively correlated to the SRVN and CC area with flow ($r=-0.86$; $p<0.003$ and $r=-0.77$; $p<0.01$, respectively. Fig 1 C-D.) Moreover, CC area with flow had a positive correlation with SRVN ($r=0.83$, $p<0.001$). A high degree of reliability between the 2 observers measuring variables from OCTA was seen; the ICC was 0.99 with a 95% confidence interval from 0.99 to 1.0 ($p<.0.01$). Examples of SRVN and CC area with flow in each group of patients are shown in Fig. 2 and 3.

In order to use the mean value between eyes, linear regression analysis was performed to determine symmetry between right and left eyes for OCTA variables. This test showed a strong degree of symmetry between eyes for FAZ ($r^2=0.74$; $p<0.01$), SRVN ($r^2=0.62$; $p<0.01$) and CC area with flow ($r^2=0.98$, $p<0.01$).

Discussion

To the best of our knowledge, this is the first paper to report the analysis of the SRVN and CC area of flow using OCTA in subjects with CHM and identify differences between affected males, female carriers and controls. Recently, Spaide *et al* reported that the visualization of flow in individual choriocapillary vessels is below the current resolution limit of OCTA, but areas of absent flow signal are identified as "flow voids". The automatic flow function provided by OCTA shows the area with perfusion based on the bright intensity of each layer providing an estimation of the area perfused [24] . The instrument is not able to visualize the flow but it can furnish important information related to the normal vessels anatomical distributions. Previous papers [25] [26] described that the CC integrity and RPE are strictly related and the CC atrophy was correlated to RPE degeneration. A similar finding

was reported by a recent paper that analysed the CC integrity in CHM subjects by OCTA. [27] The authors identified the CC density was significantly lower in CHM subjects than in female carriers and controls but they did not analyse the CC area with flow. In both subjects and carriers, CC density was significantly greater underlying regions with photoreceptor preservation as opposed to regions with photoreceptor loss.[27] The polarized nature of the RPE is essential for the health of the inner retina and CC. [28, 29] Apical and basolateral RPE secrete different molecules, for example soluble vascular endothelial growth factor (VEGF) isoforms, released from the basolateral membrane, maintain the vitality of the CC and the integrity of the CC fenestrations, which disappear with VEGF depletion and lead to atrophy[30-32,28] We can assume that the reduction in the secretion of VEGF following RPE disruption can lead to a progressive CC atrophy that increases the instability of the RPE. The main function of the CC is to deliver oxygen and remove metabolites from the RPE and the outer retina, which has the highest metabolic demand of all biological tissues[33]. The CC is the only route for metabolic exchange in the retina within the FAZ. We identified a negative correlation between the CC area with flow and the FAZ area. This means that the reduction in the CC area with flow may be associated with an enlargement of the FAZ, which may be related to a reduction in the visual acuity outcome. The identification of flow area change in subjects with CHM may be important for management and therapeutic endpoints.

Interestingly, the RPE has been found to regenerate together with CC in some animal models, such as in the rabbit following iodate-induced retinopathy. [34-36] Majji and colleagues [34, 36] developed a surgical model of CC atrophy by surgically debriding the RPE in rabbits. The RPE regenerated in a centripetal manner, covering the wounded area by day seven post injury followed by CC revascularization by 4-8 weeks. Using this model, the area of RPE cell loss can be controlled and the effects of pharmacological agents on CC can be measured. The

identification of the molecular trigger could be beneficial for potential targeted regenerative treatment in human subjects and OCTA may be a good instrument to follow subjects during is regenerative phase.

In this study the SRVN was found to show a significantly lower area of capillary plexus with flow in affected males compared to female carriers and controls, suggesting that ischaemic conditions affects the outer retinal layers. This parameter was associated with an enlargement of FAZ, but did not reach statistical significance amongst the different groups. Abnormalities in the superficial capillary plexus were reported in a recent paper that analysed Stargardt's disease by OCTA[37] but this feature was expected as the disease affects the central retina. It remains unclear why we observe superficial retinal vascular changes in CHM when the central retina tissue is still preserved. It may be related to the reduction in the CC area with flow, influencing a reduction in the superficial retinal circulation to maintain a balance between both circulatory networks. Vascularity of the retina is not related to the volume of tissue but to metabolic need[38], so reduction in photoreceptor density and subsequent ganglion cells can be the cause of constriction of capillary network. A different assumption can be based on the reduced levels of VEGF secretion from the RPE. The lack of VEGF can down regulate both the growth of the retinal capillary network and the CC vessels. In subjects with diabetes, a reduction of the superficial capillary network has been reported[39] and it was associated with a decrement of contrast sensitivity and visual field defects.[40, 41] We can assume that changes in the capillary network may play a role in CHM subjects causing similar loss of function. Moreover, a reduction of blue color discrimination amongst patients with CHM have been noted in line with the loss of photoreceptors in the peripheral retina area (Personal communication with MM)where the short wave cones are more highly

represented.[42] Similar colour vision impairment is reported in type II diabetic patients preceding vascular alterations in the peripheral retina.[43]

Studying changes over time between vascularization and functional tests may provide useful data for these subjects. The relationship between age and CC area with flow was previously described by Mullins *et al* who reported increased choroidal non-perfusion with age.[44] In affected male CHM patients, we found that the age is an important factor for the reduction of the CC area perfused. This result is in line with the evolution of the disease and its progression over the time.

Our analysis presents some limitations, including its small sample size for each category due to the rare disease status of CHM. There is a lack of standardization of OCTA analysis protocol, highlighted by no previous studies using OCTA to analyse the difference in CC between male CHM patients, female carriers and normal controls. This is a prospective observational study that allows us to report these features, however further larger studies are required, in addition to natural history studies from a young age to monitor the changes in the CC over time. The manual selection of the CC layer was fundamental to select the correct layer and to analyse the area with flow area avoiding the inclusion of deep choroidal vessels. The automatic segmentation was not reliable in affected males compared to female carriers and controls, as it often included different layers. Furthermore, we excluded subjects with poor fixation leading to the exclusion of more advanced stages of the disease. OCTA is able to detect normal capillary flow between 0.4-3 mm/s[45, 46] and this value includes the CC blood velocity that is estimated to be around 0.48 to 2.45 mm/s[47-49] but it may be limited by areas where blood flow is lower than 0.4 mm/sec,[45, 46] and where shadowing from overlying retinal

vessels cause an over representation of areas without perfusion. In addition, the data reported on the SRVN included both small vessels and the capillary network. The software provided does not allow differentiation between these two systems. A 6X6 mm analysis was preferred to the 3X3 mm analysis because the quality of the images showed less artefacts especially for patients with difficulty in maintaining fixation and it allows us to image outside of the central retinal islands where the CC is atrophied and corresponding SRVN is reduced.

In conclusion, OCTA provides an estimation of the CC blood status even if it remains challenging. It shows blood flow impairment in both superficial and CC layer in CHM subjects. It does play an important role for better understanding the pathogenesis of choroideremia, allowing the application for new therapeutic strategies and monitoring of disease progression.

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Conflict of Interest

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Summary Box

What was known before:

- It is established that Choroideremia induces changes in choroidal circulation and in the outer retinal layer in affected male subjects.

What this study adds:

- This is the first study to identify reduced area with flow in the superficial retinal vessel network in the peripheral area of the retina where the choriocapillaris is fully atrophied.
- OCTA may be a useful tool for monitoring natural history and disease progression in forthcoming clinical trials.

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Titles and legends to figures:

Figure 1: Boxplots illustrating the distribution of (A) superficial retinal vessel network (SRVN), and (B) choriocapillaris (CC) area with flow amongst CHM affected males, carriers and controls. The mean ranks are represented by *horizontal lines* in the gray boxes. Error bars represent the minimum and maximum value. Kruskal Wallis analysis shows statistical significant difference for SRVN ($p=0.01$) and CC flow area ($p=0.00$). Mann Whitney U test significance between pairs of groups are reported in the figure. (*=$p<0.05$) Scatter diagrams show relationship between age of participants and (C) SRVN area with flow and (D) CC area with flow amongst (o) CHM affected males, (▲)female carriers and (+) controls.

Figure 2: Optical Coherence Tomography Angiography (OCTA) images of CHM affected male, female carrier and control. Macular angiograms 6x6 shows (A, C, E) superficial retinal layer and (B, D, F) CC layer with automatic flow detection highlighted in yellow.

Figure 3: Optical Coherence Tomography Angiography (OCTA) images of two affected male patients with CHM at different stages of the disease. (A) Patient 7, age 36 c.116+1G>A. The remaining CC tissue can be identified in the OCTA scan because it is arranged in a honeycomb-like pattern. (*) Clear detection of (α) Sattler's layer of intermediate vessels in the middle and (β) the outermost Haller's layer with large vessels is possible when the CC is absent. (B)Patient 1 age 54 with c.877 C>T; p.R293X, shows a small area of choriocapillaris (CC) and deep choroidal vessels compared to a larger area of CC showed in (A). (C-D) The OCT segmentation of the CC with the flow detection is represented by red spots. (E-F) The area of CC with flow analysis highlighted in yellow is reported for both cases.