Prevalence of Ocular Diseases in Human Donor Eyes in New Zealand: A Study Based on Clinical and Histological Imaging

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ABSTRACT

Introduction: Fundus pathology in donor eyes was correlated with cross-sectional Optical Coherence Tomography (OCT) images and histological assessment was performed to determine the prevalence of retinal diseases without the constraints imposed during in vivo clinical imaging.

Material and methods: A fundus camera and OCT imaging system was adapted to enable posterior segment imaging of the entire post-mortem human eye. Retinas from 59 donors (57 retina pairs and two single globes) were imaged in a seven-field imaging format and cross-sectional analysis was done using OCT. To confirm that the signs observed represented true disease incidence analysis of disease markers including gliosis (Glial Fibrillary Acidic Protein), hemichannel expression (Connexin43), Müller cell activation (vimentin) and choroidal endothelial cells (CD-31) and macrophages (CD-68 marker) was performed.

Results: Pathological signs were correlated with clinical diagnoses in eyes from 25 donors (donor ages 45-87 years) but lesions were also found in 23 eyes (donor ages 39- 83 years) with no previously reported clinical diagnosis. Retinas from six donors aged 21-89 years of age were unremarkable. Of all donors, five donors had signs of age related macular degeneration (AMD) and 14 had signs of diabetic retinopathy (DR). Their lesions correlated with OCT and histopathology showed signs of activated microglia, Müller cell hyper-reactivity, increased Cx43 expression and choroidal inflammation. These data indicate that with over 8% of donors showing signs of AMD and 24% of donors showing signs of DR the incidence of AMD may be 1.7 times higher and DR up to 1.6 times higher than clinically reported.

Conclusions: The detection of pathological signs characteristic of AMD and DR in donors suggests a higher prevalence of posterior segment abnormalities amongst New Zealanders donors than previously reported. A more detailed evaluation protocol of the posterior segment in patients will aid detection of lesions that are none the less pathological signs.

Key words: Age related macular degeneration; Diabetic Retinopathy; Human donor tissues; Prevalence.

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INTRODUCTION

The census of visual impairment shows 246 million people across the world are suffering with low vision impairment and 39 million people are legally blind (Pascolini et al., 2012). The most common causes of visual impairments are uncorrected refractive errors followed by untreated cataracts and glaucoma. In developed countries the age-related diseases such as age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the leading causes of visual impairment in the geriatric population. The lack of awareness of symptoms and signs of these diseases and being asymptomatic until advanced stages, are the leading causes of blindness (Rowe et al., 2004). The treatments of these age-related retinal diseases are dependent of the stage of the diseases and areas of involvement (Bressler, 2004; Congdon et al., 2004).Clinical presentation of the diseases are the main source for obtaining information about the prevalence of these diseases in the community (Rydén et al., 2007; Thylefors et al., 1995). The screening programmes are useful in determining the exact numbers of these diseases which are supported by the New Zealand government by Retinal Screening Programmes (Chang et al., 2017; Papali'i-Curtin et al., 2013). The lack of presentation of patients with clinical signs and symptoms of different diseases into clinics cause wrong estimation of prevalence in the regions (Hutchins et al., 2012; Lee et al., 2003). Human donor eyes are the replica of the human eyes as they retain the most of the signs co-relating to ocular diseases (Kalloniatis et al., 2013; Too et al., 2017). The donor tissues help in estimation of the prevalence of these diseases in the community and confirmation of these lesions can be done by immunohistochemistry (Brown et al., 2009; Green et al., 2016). The combined methods help in understanding these diseases and their pathophysiology. These further attribute to developing guidelines for the clinical diagnosis and management of these diseases (Curcio et al., 2011). Systemic health status in association with ocular conditions of human donors are important in research to investigate human diseases and for drug testing procedures.

This study adapted modified techniques of ocular imaging equivalent to the clinical standards in the disease assessment. In addition, immunohistochemistry analysis was performed to support identification of these lesions in the donor eyes. The donor tissue assessments suggest a higher prevalence of ocular diseases in New Zealand in comparison to the results of the conventional population-based studies.

MATERIALS AND METHODS

The human donor tissues used in this research study were provided from New Zealand National Eye Bank, University of Auckland. A total of 58 eyes, 2 single globes were used. The human donor eyes were handled in accordance with the tenets of the Declaration of Helsinki and approved by the Institutional Review Committee of The University of

Auckland and Northern District Human Ethics Committee (NTX/06/19/CPD/AM04). The globes had corneas explanted prior to be used in the research study. The eyes used in the study were all fresh and were obtained less than 8 hours from time of death. The donor tissue information sheet revealed eyes had no known infection or sepsis at the time of death, age, sex, date, cause, and time of death and seldom, systemic/ocular history. The imaging process started with filling up the eyecups with Tear gel (substitute clear fluid) and areas of interest were captured using MICRON IV (retinal imaging system, Phoenix Research Labs, USA) optical coherence tomography (OCT) imager. The areas of interest were selected for cryosectioning based on OCT images. The eyecups were fixed in paraformaldehyde solution and processed after being washed with phosphate-buffered saline. Custom-made eyecup holders were used to place the donor eyes in the upright position for imaging on MICRON IV (Figure 1).

MICRON IV fundus camera has 50 degrees field of view. These settings allowed us to obtain retinal and OCT images of the central and peripheral retina of the donor eyes. The equipment had spatial resolution of 3 µm for retinal imaging; depth of focus was 20 µm and consisted of 1024 pixels per A-scan. The postmortem retinal images obtained were different from those obtained in vivo. The areas of lesion and retinal layers assessment was done by one ophthalmologist and an optometrist independently. The masking of prior clinical history of the donor eyes was done before the assessment of both clinicians. The histological analyses on these eyes were performed only after

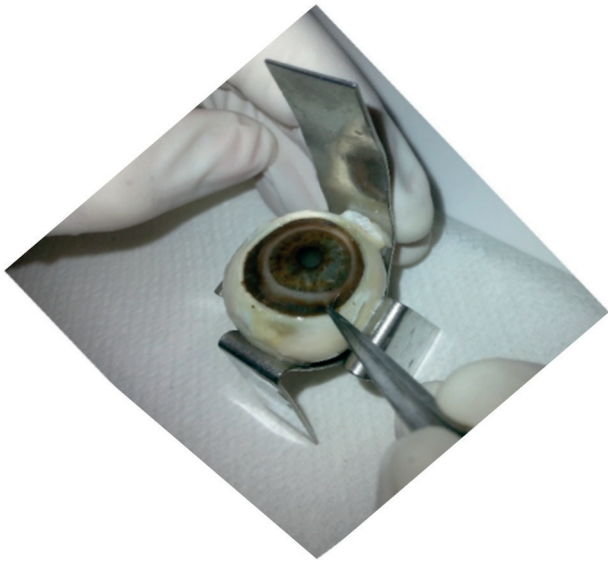

Figure 1A: Custom-made holder used to examine the human donor eyes.

Figure 1B: Fundus imaging system (Phoenix Micron IV).

they met the set grading criterion (as explained in Table 1). National Diabetes Retinal Screening Grading system, Early Photocoagulation for Diabetic Retinopathy Study, The Beaver Dam Eye Study, The Age-Related Eye Disease Study, and An Online Retinal Fundus Image Database for Glaucoma Analysis and Research study grading systems were adopted in this study for grading the donor images. All the retinal images anomalous findings were described and recorded in detail.

Table 1: Clinical grading systems adapted for diagnosis of the diseases in donor eyes.

	Clinical signs	Fundus and optical coherence tomography imaging signs	Reference
NORMAL	No retinal pathology	Clear retina, without any signs	
AMD	Hard drusen	Size of drusen ≤65 µm	(Bird et al., 1995)
	Soft drusen	Size of drusen: 125 µm	
	Hyper pigmentation with any type of drusen	Increased pigmentation (brown / blackish areas)	
	Hypo pigmentation with any type of drusen	Whitish patchy areas	
	Geographic atrophy	Choroidal blood vessels seen at the base of the atrophic area	
	Advance stage/wet AMD	Macular scarring with haemorrhages and laser marks	
DR	Mild Diabetic Retinopathy	Microaneurysms Dot and blot haemorrhages	
	Moderate Diabetic Retinopathy	Blot haemorrhages, deep haemorrhages, pigmented areas with haemorrhages	(Ministry of Health, 2016)
	Severe Diabetic Retinopathy	Laser treatment marks seen in different quadrants; Haemorrhages and retinal detachment	
Glaucoma		Unilateral cup disc ratio ≥0.6:1 Bilateral: Asymmetry ≥0.2:1 Loss or thinning of neuroretinal rim (nerve fibre layer defect) Disc haemorrhage Superior/inferior notching	(Zhang et al., 2010)

Preparation of donor tissues for immunohistochemical analysis

The association of retinal lesions (mild or moderate) with molecular pathology confirmed the diseases. The true disease incidence in these donor eyes were evaluated through performing Immunohistochemistry. As described in Table 2; the following markers used in this study confirmed the lesions. Astrocyte proliferation and hypertrophy (was confirmed by Glial Fibrillary Acidic Protein expression), Müller cell activation in the retina (was confirmed by vimentin labelling), CD31 positive cell numbers (was confirmed by monocytes and neutrophils) and leukocytes (was confirmed by CD45 - common leukocyte antigen positive(Bai, Tang, Ma, Luo, & Lin, 2003; HANNE L. OSTERGAARD*, November 1989) cells). The optic nerve head position was used as the primary reference point to match the lesion locations in both histological and clinical retinal images. At least three sections in each human donor eyes were studied and confirmation of presence of these lesions was done with clinical prospective (examples: drusen in AMD eyes, level of haemorrhages in retina of DR cases).

Immunohistochemical labelling: Both retinal and choroidal tissues were collected onto Superfrost Plus slides (Labserv, Auckland, New Zealand). The slides were stored at −20 °C until required for immunolabeling. The process of labelling started with defrosting the frozen sections at room temperature for 10 minutes. Then the slides underwent a wash in 0.1 M phosphate-buffered saline. Following the wash, the sections were blocked at room temperature for 30 minutes with 6 % normal goat serum (Sigma-Aldrich Corp., USA), 1% bovine serum albumin, and 0.5% Triton X-100 in 0.1 M phosphate-buffered saline. A solution of 3 % normal goat serum, 1 % bovine serum albumin, and 0.5 % Triton X-100 in 0.1 M phosphate-buffered saline was used to prepare the primary antibody solution. Table 2 shows the list of antibodies used. The slides were incubated in the primary antibody solution for overnight. Some tissues were used as part of control experiments. The control slides underwent an incubation process without primary antibody included in the blocking solution, followed by incubation with secondary antibodies. After overnight incubation, these slides were washed thrice in 0.1 M phosphate-buffered saline every 15 minutes. The secondary antibodies used were goat anti-rabbit or goat anti-mouse Alexa 488 or Alexa 594 (Life Technologies, USA). The secondary antibodies were diluted to 1:500 and the tissues were incubated with these solutions for 3 hours at room temperature. Then the slides were washed thrice with 0.1 M phosphate-buffered saline every 15 minutes and were incubated with 1 μg/mL 4′, 6-diamidino-2-phenylindole (Sigma-Aldrich Corp.) in 0.1 M phosphate-buffered saline for 15 minutes. After completion of the incubation process in both primary and secondary antibody solutions, the sections were washed and mounted in an anti-fading medium (Citifluor, UK), and were coverslipped. The coverslips were sealed with nail polish at the edges of the slides.

Table 2: Primary antibodies used in this research study.

Antibody	Production	Host	Working dilution	Company	Catalogue Number	Immunogen	Reference
Anti - Glial Fibrillary Acidic Protein	Monoclonal, clone G-A-5	Ms	1:1000	Sigma-Aldrich, USA	C9205	Purified Glial Fibrillary Acidic Protein from pig spinal cord.	(N. M. Kerr et al., 2010)
Anti CD45	Monoclonal, clone OX-1	Ms	1:20	BD Pharmingen, USA	550566	CD45-enriched glycoprotein fraction from Wistar rat thymocytes	(Ishida et al., 2003)
Anti CD31	Polyclonal	Rb	1:100	Abcam, USA	Ab28364	Synthetic peptide corresponding to C- terminus of mouse CD31	(Dong et al., 2012)
Anti-Vimentin	Monoclonal	Ms	1:1000	Sigma	C9080	Purified vimentin from pig eye lens	(Shen et al., 2010)

Image analysis and quantification

Olympus FV1000 confocal laser scanning microscope and FV-10 ASW 3.0 Viewer and Adobe Photoshop CS6 softwares were used to study the Immunohistochemical labelling of the sections. Three donors in each group were imaged. To quantify the images, Image J software (Image J 1.45s software –Wayne Rasband, National Institute of Health, USA) binary image application was used. Equal threshold settings were applied all images. Data were plotted as the % area labelled by the marker in a 100 μm^2 area. For Glial Fibrillary Acidic Protein, the data is reported as % labelling per image (250 µm x 250 µm area). For vimentin, the data refers to the % area of marker expression between the inner plexiform layer and retinal pigmented epithelium. The ganglion cell layer was not included in the assessment in order to avoid inclusion of astrocytes in the Müller cell labelling estimate (astrocytes also label with vimentin). The lesions which were not clearly assigned to any disease (like AMD or DR pathology) have confirmed labelling in immunohistochemistry. High magnification single confocal images of 1024 x1024 pixels were used to count the positive cells in Immuno's. Only 4′, 6-diamidino-2-phenylindole stained nuclei surrounded by positive CD31 or CD45 label were counted. Data was plotted as mean ± standard error of the mean.

Statistical Analysis

One–way analysis of variance followed by a Bonferroni post-hoc test was used to perform statistical analysis. $p < 0.05$ was considered to indicate statistically significant differences in diseased tissues. Graph Pad Prism 7 (Graph Pad Software, USA) was used to plot the data.

RESULTS

The donor eyes obtained were aged from 21 to 89 years old (average 70 ± 13.1 years). The ethnicity information sheets of these donors revealed 90.5% were Caucasians and 9.5% of Indian's of which 79% were males and 21% were females. Six donor eyes of age 21-82yrs were categorised as normal as they showed no posterior segment pathologies. In (Figure 2) correspondence is seen through the normal retinal images, OCT and immunohistochemical labelling. Figure 2A and 2B shows normal central and peripheral retinas. Figure 2C shows OCT images of normal posterior segment layers. Figure 2D -2F shows normal cell type marker expressions in a 75-year-old normal patient. Retinal layer expression for the markers was seen in Figure 2D GFAP (ganglion cells), vimentin (Müller cells marker; Figure 2E). In choroid, Figure 2F -2G shows CD31 positive cells and CD45 labelled leukocytes, respectively.

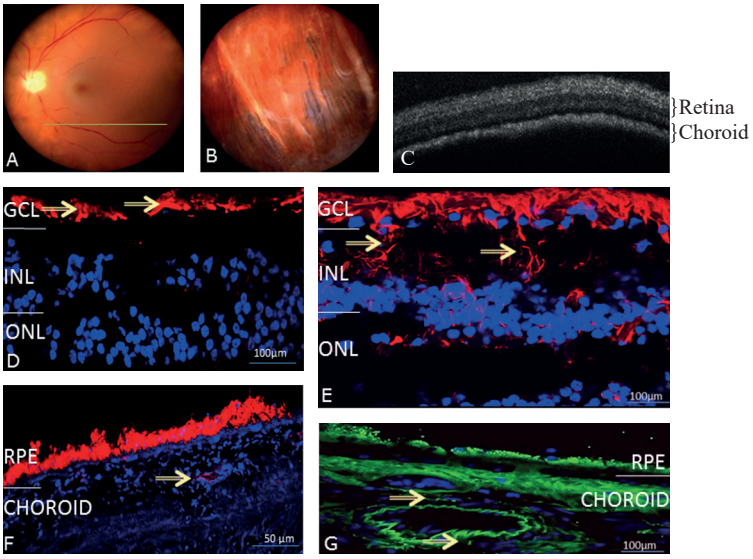

Figure 2: (Figure 2A, B): Retinal images of central and peripheral fundus seen in (C) OCT showing retina and choroidal normal layers. (D) Expression of normal levels in the ganglion cell layer (GCL) (GFAP, red). (E) Vimentin labeling (red, arrows) indicating normal labelling of Müller cells. (F) CD45 labeling leukocytes and showing no inflammation. (G) CD31 (green) labeling endothelial cells in choroidal cells.

Scale bars = 100 and 50µm.

GCL = Ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer; OPL = Outer plexiform layer; ONL = Outer nuclear layer.

Forty-seven donors eyes with ocular pathology is summarised in Table 3, of which only 24 donors had documentation that verified prior diagnosis on donor information sheet.

Of the confirmed lesions in the donor eyes, 37% of them had lens related pathology such as pseudophakia or cataracts in one or both eyes. Of these, nine donors only had lens conditions with no other posterior segment diseases. Six donor eyes had lesion's which could not be categorised using pathology grading scales. Figure 3 shows the grading classification used in lens assessments. The grading of the lens opacifications was not included in the scope of the study.

Table 3: Lesions detected in donor eyes through Ocular imaging (fundus imaging and optical coherence tomography).

Lesions	Ocular disease	Total number of eyes affected	Previously diagnosed	Reported disease prevalence in the population ±	Disease prevalence among donors*
Microaneurysms, haemorrhages, laser treatment marks	DR	14	4	19%	24%
Drusen, macular scar, haemorrhages	AMD	5	3	7.6%	8.5%
Cataract Intra ocular lens	Cataract and Pseudophakia	22	13	30%	37%
Cupping > 0.5, nerve fibre layer defects	Glaucoma	3	1	2%	5%
Others	Corneal graft and iris abnormalities	3	3	-	5%
Undiagnosed, drug deposits	Unconfirmed lesions	6		-	10%
None	Normal eyes	6		-	10%

± Epidemiological studies from literature (Danesh-Meyer, April 2014; Ministry of Health, 2013; Papali'i-Curtin et al., 2013; Worsley et al., 2015).

*Total cumulative prevalence includes two groups of donor tissues in this study.

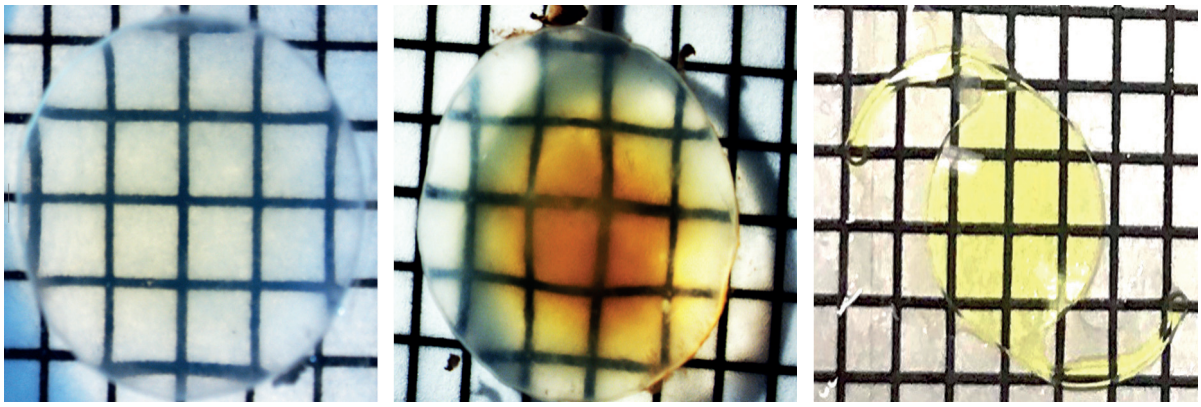

Figure 3: Stereoscopic images of human donor lens.

Figure 3A: Normal; Figure 3B: Hard brown cataract seen; Figure 3C: Intraocular lens.

An example of drug deposits (donor information sheet revealed the cause of death to be pancreatic cancer and had undergone cancer treatment) seen in fundus images and OCT likewise shows confluent white patchy deposits in the vitreous. Figure 4 shows eyes with lesions which are placed in the undiagnosed category.

Eight percent of the donor eyes had age-related macular degeneration changes such as Drusen, retinal pigment epithelium damage. These lesions were histologically confirmed with increased expression of the markers. Figure 5A and 5B are the retinal changes of AMD and corresponding OCT images of the AMD donor

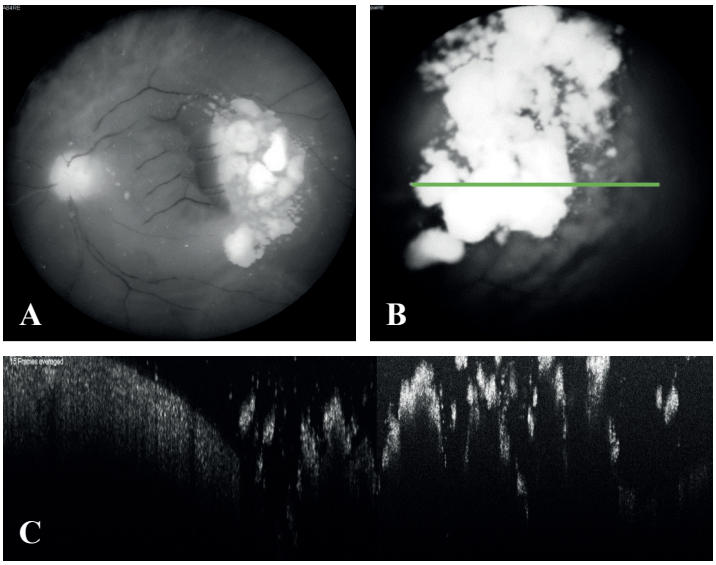

Figure 4: (A) and (B) show drug deposits in central and peripheral retinas corresponding (C) OCT images.

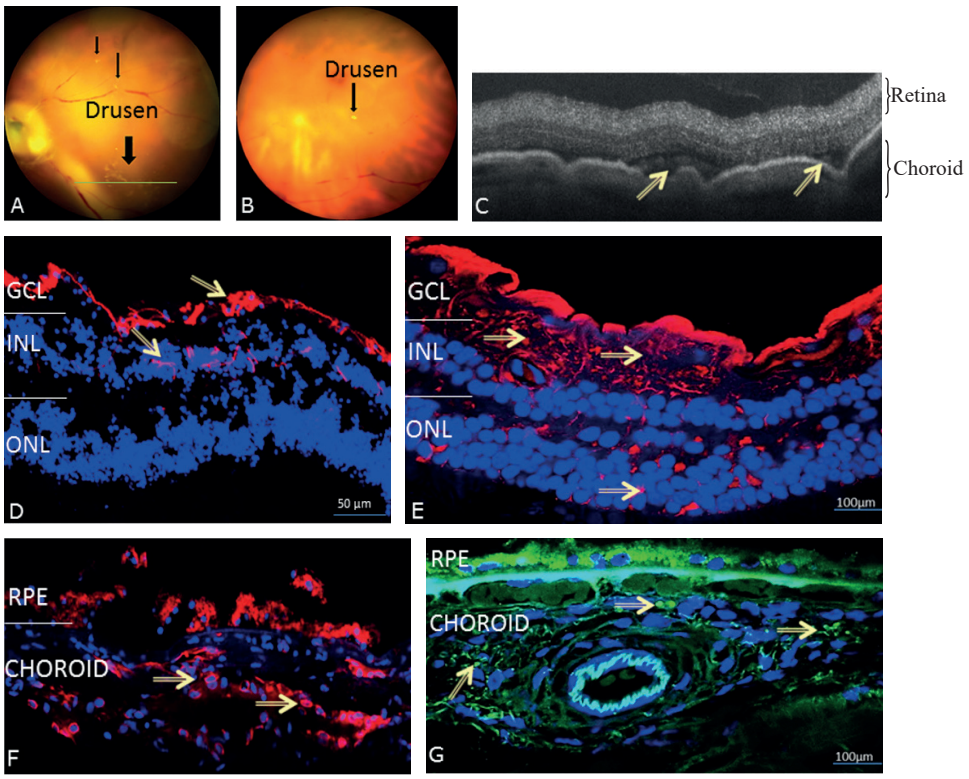

Figure 5: (A, B) images show signs of AMD in the central and peripheral retina. (C) corresponding drusen changes noted in OCT imaging (arrow). (D, E) shows increased expression of GFAP and Vimentin labelling in the retinal layers. (F, G) shows CD45 labelled leukocytes and CD31 labelled monocytes and neutrophils (green) labelling in the choroid of AMD tissues. Scale bars = 100, 50 and 25 µm.

eyes. Figure 5D shows increased expression of GFAP and Vimentin expression was also increased in outer retinal layers indicating Müller cell activation (Figure 5E). In Figure 5F and 5G, increased expression of both CD31 positive monocytes and neutrophils and CD45 positive leukocytes was noted.

Diabetic retinopathy was confirmed in 14 donors and they had signs of cotton wool spots, multiple haemorrhages or treatment done with laser photocoagulation. Based on the location of the haemorrhage in the retinal layers, the classification of the diseases was done. Example, if the location of the haemorrhage is in inner retinal layers then it is suggestive of hypertensive retinopathy, and if deep haemorrhages were noted then classified as diabetic retinopathy (Shechtman et al., 2008). Figure 6A, 6B shows DR donor eyes with multiple haemorrhages and Laser treated areas in the central and peripheral retina. Figure 6C

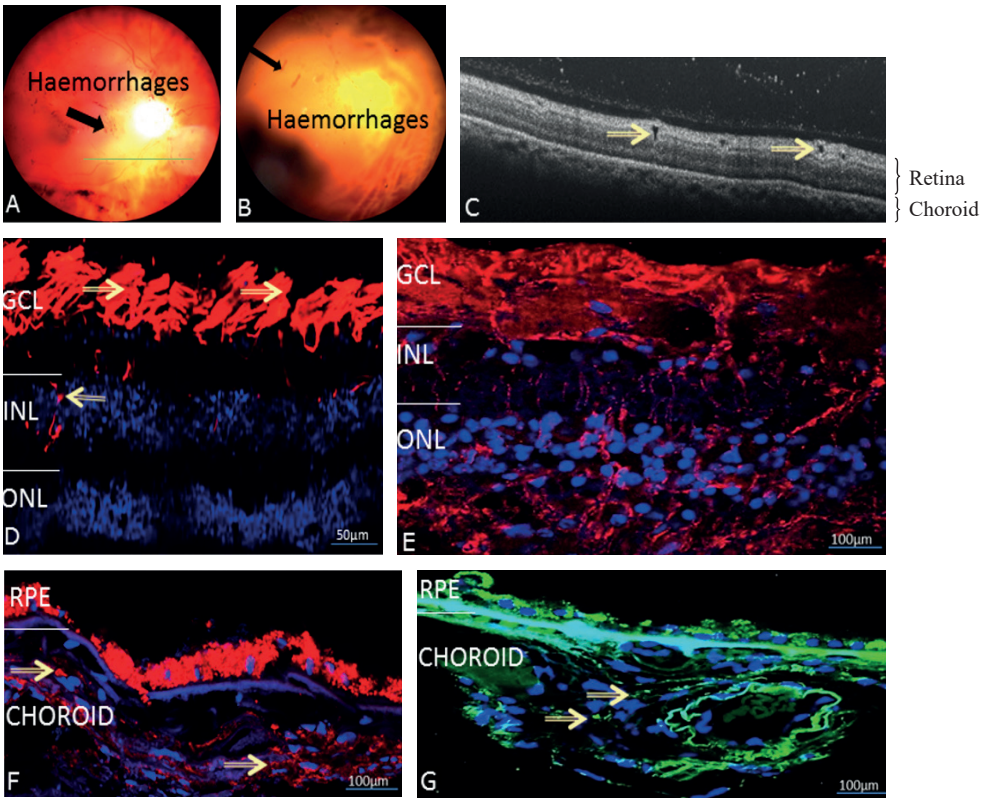

Figure 6: (A, B) DR donor eyes fundus images. (C) OCT corresponds to the retina. GFAP expression (D) (red); (E) (red, arrows) indicating increased vimentin labelling. (F, G) CD45 and CD31 endothelial cell marker expression in the choroid. Scale bars = 50µm.

OCT confirms the presence of haemorrhages in the inner retina layers suggestive of DR. Figure 6D, 6E shows immunolabeling with GFAP and Vimentin markers in the retinal layers. Figure 6F and 6G, respectively shows increased numbers of CD31 and CD45 positive cells in the choroid.

Donor eyes of glaucomatous changes were classified with asymmetrical cup: disc ratio of >0.2 in both eyes and/or unilateral cup: disc ratio ≥0.6:1; disc haemorrhages, inferior or superior notch and/or thinning in neuro retinal rim changes of optic nerve head, characteristic peripapillary atrophy and/or with generalized, or localized retinal nerve fiber thinning or defects. Three donor eyes were categorised with >0.6 cup disc ratio as glaucomatous eyes. Literature suggests increased GFAP and connexin 43 labelling was noted in retina and optic nerve head areas (Nathan M. Kerr et al., 2011). Senthilkumari et al worked extensively on the histological changes of glaucomatous

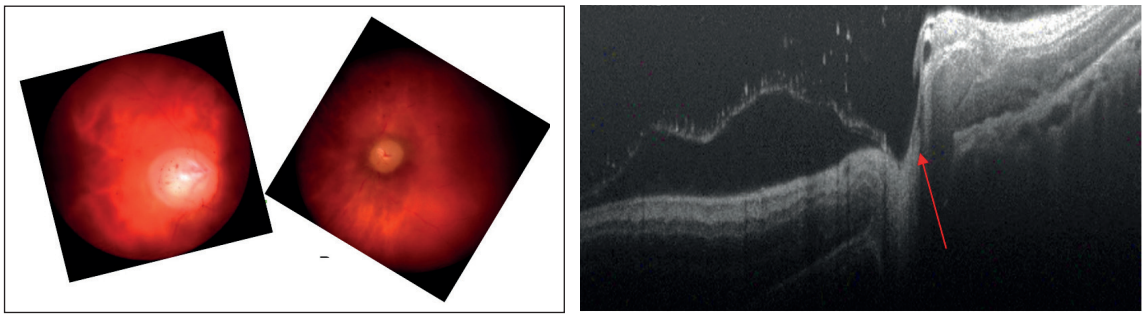

Figure 7: (A, B) shows central retinal regions of right and left eyes with cup: disc ratio 0.8:1 and 0.2:1 cup: disc ratio and no other signs, suggestive of glaucomatous changes in the right eye. (C) OCT shows disc haemorrhages corresponding to the right eye retinal images.

donor eyes (Senthilkumari et al., 2015). Figure 7 shows donor eyes with vertical cup: disc ratio ≤0.6 with no focal retinal rim thinning seen.

The comparison of expression of inflammatory markers is shown in retina and choroids of the normal and diseased donor eyes in Figure 8. Given that mild clinical signs were noted in the human donor eyes they were confirmed as true pathology increased GFAP expression (n=5 AMD lesions, n=5 DR lesions), ($p< 0.01$ and $p< 0.001$) and Vimentin labelling in the AMD donor lesion areas ($p<0.0001$) and DR eyes ($p<0.0001$). In the choroids, there were significantly more CD31 and CD45 positive cells in damaged areas ($p<0.001$ for both markers) was noted. Inflammation was noted more in choroids than in the retina of both diseases.

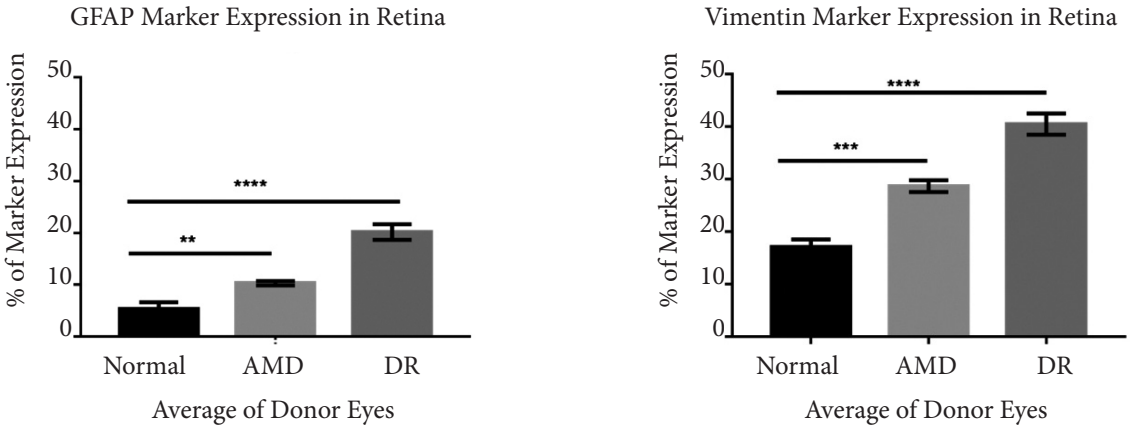

Figure 8: Quantitative analysis of Glial Fibrillary Acidic Protein (astrocyte marker, A) and for vimentin (Müller cell marker, B). **** = $p \leq 0.0001$ and *** = $p \leq 0.01$.

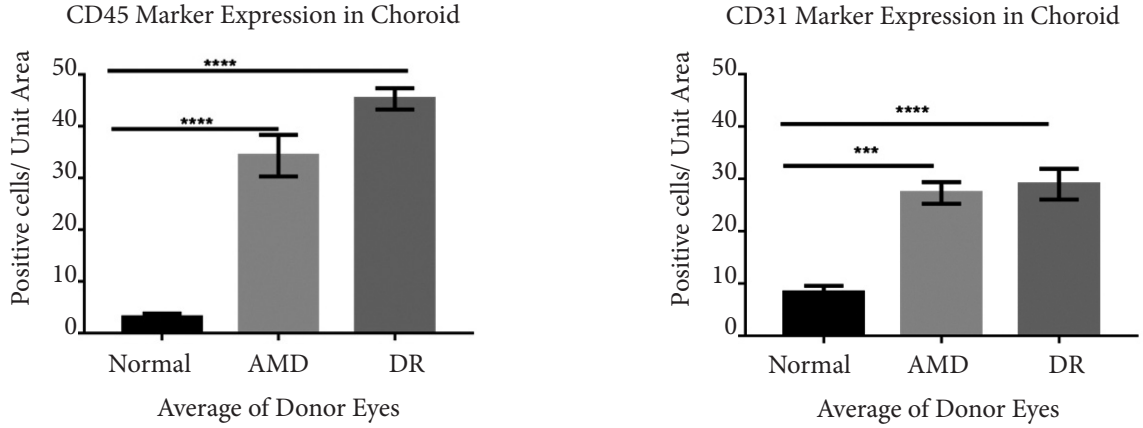

Figure 9: Quantitative counts of immunolabeled positive cells for the leukocytes CD45 marker (A) and the monocyte/neutrophil marker CD31 (B). **** = $p \leq 0.0001$ and *** = $p \leq 0.001$.

DISCUSSION

To calculate the prevalence of diseases in the communities conducting large epidemiological studies and screenings are the basic tools and methods used (Wong et al., 2008). Nevertheless, post-mortem eyes are the replica of the clinical signs observed in the diseased patient's eyes. Literature shows evidence on the effect of time to surgery, delay in corneal tissue samples processing and outcome of corneal transplantation surgery (Keane et al., 2013). The New Zealand Eye Bank, published in 2011 shows that these methods are the key points to evaluate the outcome of the success rate of corneal transplantations (Patel et al., 2011). In this study, we have shown estimation of prevalence of diseases with a combinational method of using clinical and immunohistological techniques. We adapted modified clinical imaging to obtain retinal and OCT images of the donor eyes. We have studied a small sample size for analysis and noticed a higher prevalence of lens conditions (37% of donors) and retinal pathologies (24% DR and 8% AMD). Awareness of the diseases in the donor families contributes to one of the causes of the high prevalence of diseases noted (Williams et al., 2013). No skewing of the data was noted due to the above reasons and no sampling was affected. Wellington region study revealed 22% of diabetic patients had some form of diabetic retinopathy, and the Northland diabetic screening population presented had 19% of them (Hutchins et al., 2012; Lily YL Chang, 2017; Papali'i-Curtin et al., 2013). This study concluded, 24% of all donors had DR related lesions supporting the regional New Zealand study data. Literature shows that combined techniques like clinical imaging such as OCT and immune histological assessments can confirm the presence of ocular

diseases (Brown et al., 2009, Curcio, 2005)). Modified large view techniques are useful for obtaining and confirming the lesions in the donor eyes(Bagheri et al., 2012; Ghazi et al., 2006). Studies done based on histopathological analysis of human donor eyes confirmed the technique's usefulness in revealing and confirming the glaucoma disease in donor eyes (Senthilkumari et al., 2015). In this study, using modified fundus imaging with molecular marker assessments confirmed the unidentified lesions with ocular diseases as supporting the literature(Feit-Leichman et al., 2005; Klein et al., 2014; Wilding et al., 2015).

The molecular level analysis of these disease like AMD and DR shows changes in the cellular levels of retina with increased expression of GFAP, Vimentin (astrocytes and Muller cells) in both the diseases (Abcouwer, 2011, Trivino et al., 1996, Kaur et al., 2008, Ramirez et al., 2001)). Wu et al also showed an increased expression of GFAP and Müller cells in early stages of AMD disease (Wu et al., 2003). However, increased expression of GFAP was noted in donor eyes and they were indicative of vascular abnormalities in DR eyes (Mizutani et al., 1998, Amin et al., 1997). Thus, this study concludes similarly that histological assessments of the post-mortem tissues has been a good indicator corresponding with the OCT and fundus lesions in these eyes. Unsurprisingly, GFAP and vimentin were higher in DR (an inner retina disease) than in AMD (primarily an outer retina disease). The first step of immune defence in the body is to produce leukocytes, this study focused on studying these markers in choroids of AMD and DR eyes (Chimen et al., 2015). Previous studies show evidence of increased glial activity in retina and loss of endothelial cells (Mullins et al., 2011) increased CD45 labelling in AMD and DR eyes ((Huang et al., 2013, Colak et al., 2012; Zeng et al., 2008, Madigan et al., 2012)

CD31 is a marker for blood vessel endothelial cells (Gariano et al., 1996; Penfold et al., 1990) and animal studies have shown retinal endothelial cell dysfunction (labelled by CD31 marker) in AMD and DR (Guo et al., 2014; Wautier et al., 1996) in this study analyses statistically significant distinct CD31 positive labelling of monocytes and neutrophils was used as an inflammation marker corresponding the optical coherence tomography as areas of vascular damage indicative of DR and AMD. The study has the limitation of a lack of donor history sheet for every donor, but combination of post-mortem clinical and histological assessments provides evidence that can overcome this limitation.

CONCLUSION

The findings of this study confirm the presence of lens defects and retinal lesions in donor eyes and suggest that there may be a greater prevalence of ocular disease in the New Zealand population than previously reported. Few limitations of this study are, small sample size, racial representation not necessarily reflective of the overall population, analysing the donor eyes

retrospectively with no prior ophthalmic history available, possible autolysis of the tissues due to post-mortem delay and the complexity of samples and small numbers of unaffected tissues due to the age, the diverse systemic and ocular history of the donor population.

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