Measuring Retinal Vessel Diameter & Blood Flow Dynamics in Normal and MITF Mutant Mice

Stefán Broddi Danielsson

Ritgerð til B.S. gráðu
Háskóli Íslands
Læknadeild
Heilbrigðisvísindasvið
Ritgerð þessi er til BS gráðu í læknisfræði og er óheimilt að afrita ritgerðina á nokkurn hátt nema með leyfi réthafa.

© Stefán Broddi Danielsson 2018

Printing: Prentsmiðja xxx
Abstract

Purpose

Despite data suggesting an important role in eye development, there is little data on the role of the Mitf gene in vascular development. This study aims to determine both the diameter and blood flow dynamics of retinal vasculature in normal and Mitf mutant mice.

Methods

In measuring the diameter, 6 Mitf\^{mi-vga9+/+} (heterozygote) and 6 Mitf\^{mi-enu22(398)/mi-enu22(398)} (homozygote) mice were compared to 6 wild type. A fundus camera was used to obtain still fundus images after intraperitoneal injection of sodium fluorescence salt. These images were processed with Photoshop and a custom written Matlab program to extract the mean vascular diameter at a specific distance from the optic disc. Each mutation was compared to the wild type. A separate comparison was done for arteries, veins and both combined. The comparison was done with respect to the number of vessels, average diameter and average total diameter (average diameter*number of vessels).

The measuring of blood flow dynamics is still in progress. Therefore, only sample data from wild type, Mitf\^{Mi-Wh/+} and Mitf\^{Mi-Wh/mi/Mitf\^{mi}} mice is provided in the results section.

Results

When comparing mice with the Mitf\^{mi-enu22(398)/mi-enu22(398)} mutation to the wild type we found a significant 24,8% increase in mean total arterial diameter (P=0,0243), a significant 18,8% increase in mean venular diameter (P=0,0259), a significant 38,6% increase in mean total venular diameter (P=0,004) and a significant 31% increase in mean total vessel diameter (P=0,00726). There was also a significant 18,8% increase in the number of arteries (P=0,026), a significant 15,6% increase in the number of veins (P=0,041) and a 17,2% increase in total number of vessels (P=0,026).

When comparing mice with the Mitf\^{mi-vga9/+} mutation to the wild type we found a significant 17,8% increase in mean total venular diameter (P=0,0303).

Only sample data are provided as of now with respect to the blood flow dynamics.

Conclusion

The increase in retinal vasculature in mice with the Mitf\^{mi-enu22(398)/mi-enu22(398)} mutation compared to wild type mice suggests that there is an increased demand of blood flow to the retina in these mutations. The increase in retinal venular vasculature in the Mitf\^{mi-vga9/+} mice without a corresponding increase in retinal arterial vasculature is a surprising finding which may be due to low number of samples.

No conclusions can be drawn so far with respect to the blood flow dynamics.
Acknowledgements

I wish to thank Andrea García Llorca who supplied the images for the diameter measurements. I’d also like to thank Hallur Reynisson who wrote the matlab program for the vessel diameter measurements. Finally, I would like to express my great appreciation toward Pór Eysteinsson who made this project possible and helped me with many important bits of advice.

Contents

Acknowledgements ................................................................................................................................. 1
Contents .................................................................................................................................................. 1
Figures ..................................................................................................................................................... 3
Tables ...................................................................................................................................................... 4
List of Abbreviations ................................................................................................................................ 4
Introduction .............................................................................................................................................. 5
   Discovery of the Mitf Mutation .............................................................. 5
   The Function and Characteristics of MITF ........................................ 5
   The Role of Mitf in Eye Development .................................................. 5
   The Role of Mitf in Hearing Development ........................................... 7
   The Role of Mitf in the Skin and Immune System ................................. 7
   The Role of Mitf in the Central Nervous System ................................... 7
   The Role of Mitf in Kidney Formation .................................................. 8
Human Diseases Associated with Mitf .......................................................... 8
   Waardenburg Syndrome type IIA .......................................................... 8
   Tietz Syndrome ...................................................................................... 8
   Age Dependent Macular Degeneration (AMD) ...................................... 9
   COMMAD syndrome ............................................................................. 9
   Neurofibromatosis type 2 ................................................................. 9
   Melanoma ............................................................................................ 9
   The Mutations Used in this Experiment ............................................. 10
Research Objectives .......................................................................................................................... 11
Methods .............................................................................................................................................. 12
   Animals .............................................................................................. 12
   General Preparation ........................................................................... 12
Figures

**Figure 1.** Shows the major players in eye development of a mouse embryo. Dorsal is to the top while distal is to the left. Red will become RPE, green will become neural retina and yellow will become the optic stalk. Source: [5].......................................................................................................................................................... 6

**Figure 2.** The role of MITF in association to other proteins in human melanoma. Source: [28] .......... 10

**Figure 3.** The result of a measurement. (Left) Wild type. (Right) Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup>. The radius of the outer circle is twice that of the inner circle....................................................................................... 13

**Figure 4.** The vessel bed of a WT after processing. (Left) Image with selection of the vessels. (Right) Image where vessels have been replaced by a high-intensity white background. ................................. 14

**Figure 5.** Average diameter (in pixels) of arteries, veins and both in the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup>, VGA9 = Mitf<sup>mi-vga9/+</sup>. *Significant 18,8% increase (P=0,0259).......................................................................................................................................................... 15

**Figure 6.** Average number (in pixels) of arteries, veins and both in the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup>, VGA9 = Mitf<sup>mi-vga9/+</sup>. *Significant 18,8% increase (P=0,026). **Significant 15,6% increase (P=0,041). ***Significant 17,2% increase (P=0,026). .......................................................................................................................................................... 16

**Figure 7.** Average total diameter (average number of vessels*average diameter, in pixels) for the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup>, VGA9 = Mitf<sup>mi-vga9/+</sup>. *Significant 24,8% increase (P=0,0243). **Significant 38,6% increase (P=0,004). ***Significant 17,8% increase (P=0,0303). ****Significant 31% increase (P=0,00726). ..... 16

**Figure 8.** (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds........ 19

**Figure 9.** (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel. ....................................................................................................................................................... 20

**Figure 10.** (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel. ................................. 20

**Figure 11.** (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds. Not much difference can be seen between arteries and veins due to background filling. There are three veins and four arteries. The veins are thicker and have a shared point of origin. The arteries are thinner and have an origin that is to the upper left of the venular point of origin. ................................. 21

**Figure 12.** (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel. ....................................................................................................................................................... 21

**Figure 13.** (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel. ................................. 22

**Figure 14.** (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds. There is a lot of background filling and it is difficult to distinguish veins from arteries in this image. There is also an extra vessel of unknown origin. A possible explanation for this vessel is that this mutation generally lacks a pigment epithelium and thus we may be looking at vasculature that lies posterior to the retina....................................................................................................................................................... 22

**Figure 15.** (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel. ....................................................................................................................................................... 23
Figure 16. (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel. ....................................................... 23

Figure 17. Shows the scores of principal component 1 (intensity) for each of the mutations over time (s). Half rise time and half fall time is the amount of time that has passed when the intensity has risen half way up to it’s maximum value and fallen half way down to it’s end value respectively. Offset is the difference between maximum intensity and the end intensity. .................................................................................. 24

Tables

Table 1. Shows the mutations and their characteristics. Source: [4, 30] .............................................. 10

Table 2. Shows the mean arterial diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ...................................................................................... 16

Table 3. Shows the mean total arterial diameter (mean arterial diameter*average number of arteries, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ... 17

Table 4. Shows the mean venular diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ...................................................................................... 17

Table 5. Shows the mean total venular diameter (mean venular diameter*average number of veins, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ...... 17

Table 6. Shows the mean vessel diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ...................................................................................... 18

Table 7. Shows the mean total vessel diameter (mean vessel diameter*average number of vessels, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation. ...... 18

Table 8. Shows the average number of arteries, the difference from the wild type and the two-tailed P-value for each mutation. ........................................................................................................... 18

Table 9. Shows the average number of veins, the difference from the wild type and the two-tailed P-value for each mutation. ........................................................................................................... 18

Table 10. Shows the average number of vessels, the difference from the wild type and the two-tailed P-value for each mutation. ........................................................................................................... 19

List of Abbreviations

Akt, protein kinase B; AMD, age related macular degeneration; bHLH-Zip, basic helix loop helix-zipper; COMMAD, coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism and deafness; CSF, cerebrospinal fluid; DAPL1, death associated protein like-1; DOPA, dihydroxyphenylalanine; enu22, ethylnitroso-urea 22 mutated (stop codon exon 2A); EP, endocochlear potential; ERG, electroretinography; GCL, glutamate cysteine ligase; GSH, reduced glutathione; H-PGDS, hematopoietic prostaglandin D synthase; L-PGDS, lipocalin-type prostaglandin D synthase; MAPK, mitogen-activated protein kinase; Mitf, microphthalmia transcription factor; NF2, neurofibromin 2; NR, neural retina; PGD2, prostaglandin D2; PGDS, prostaglandin D synthase; PI3K, phosphoinositide 3-kinase; Rdh5, retinol dehydrogenase 5; Rlbp1, retinaldehyde binding protein 1; RPE, retinal pigment epithelium; SOX10, sry-related HMG-Box gene 10; TFE3, transcription factor E3; TFEB, transcription factor EB; TFEC, transcription factor EC; TRP-1, tyrosinase related protein-1; TRP-2, tyrosinase
Introduction

Discovery of the Mitf Mutation

The first mutation of the micropthalmia transcription factor (Mitf) gene was discovered in X-ray irradiated mice by Paula Hertwig in Berlin and was published in 1942. The homozygotes of these mutants had small, unpigmented eyes; lacked upper and lower incisors; and died soon after birth. The heterozygotes showed only reduced eye pigmentation but no coat color [1].

Since then the Mitf has been shown to be involved in the function of many organs, including eye, ear, immune system, bone and skin [1-3].

The Function and Characteristics of MITF

The micropthalmia transcription factor protein (MITF) is a member of the Myc supergene family of basic Helix Loop Helix-Zipper transcription factors. It regulates transcription by binding to DNA as either a homodimer or heterodimer with another related family member. Other closely related bHLH-Zip proteins are TFEB, TFEC and TFE3. Mutations in TFE3 and TFEB have been reported in some types of human cancer. MITF is closely conserved between many animals, including mouse and human [4].

The Role of Mitf in Eye Development

In the optic vesicle (the diencephalic invagination of the mouse embryo that is to become the eye) it seems to have a role in boundary formation between the retinal pigment epithelium (RPE) and the neural retina (NR). Vsx2 and Mitf have an antagonistic relationship in the boundary formation (see figure 1). Mice with loss of function mutations in Mitf show a conversion from RPE to NR [5].
Figure 1. Shows the major players in eye development of a mouse embryo. Dorsal is to the top while distal is to the left. Red will become RPE, green will become neural retina and yellow will become the optic stalk. Source: [5]

One possible mechanism for the lack of RPE in Mitf mutated mice is the reduced expressions of Rlbp1 and Rdh5. Both are controlled by Mitf. The function of Rdh5 is to oxidize 11-cis retinol to 11-cis retinal. The function of Rlbp1 is to bind both 11-cis retinol and 11-cis retinal and release it within the RPE where it is taken up by photoreceptors. Mutations in these genes are associated with retinal dystrophies and other dysfunctions such as retinitis pigmentosa. Mice embryos with Mitf mutations show reduced levels of Rlbp1 and Rdh5 and exogenous addition of 9-cis-retinal partially rescues the RPE degeneration in these mice [6].

Recently it has been shown that Mitf plays an important role in regulating anti-oxidant formation in the RPE acting through PGC1α. Mitf deficiency lead to significantly increased ROS species in the RPE and retina. Cells overexpressing Mitf in the human RPE cell line ARPE-19 showed increased levels of reduced glutathione (GSH) and GCL (the rate limiting enzyme in GSH synthesis) [7].

Another study showed that MITF is important for the regulation of the vascular endothelial growth factor (VEGF). Blockage of the transcription factor MITF-Tfe using either pan MITF-Tfe dominant negative or specific small interfering RNA (siRNA) in the human RPE cell line ARPE-19 resulted in a reduction of VEGF expression [8].
The Role of Mitf in Hearing Development

In relation to hearing, Mitf<sup>−/wh/+</sup> mutant mice have been shown to have abnormal stria vascularis and cochlear structure, including an extensive loss of outer hair cells. In addition, they show a reduced number of melanocytes within the cochlea and profound sensorineural hearing deficit resulting from signal defects in both inner and outer hair cells [9].

One study showed that a likely mechanism for hearing loss in Mitf mutant mice is loss of hair cells due to loss of intermediary cells in the stria vascularis which leads to a loss of endocochlear potential (EP) generation. Outer hair cells were much more sensitive to loss of EP (95% loss by postnatal day 18) while inner hair cells were less sensitive [10].

Another study provided evidence that Mitf is necessary for the migration of melanocytes to the stria vascularis. The concentration of Mitf in the region which would later become the stria vascularis was high during embryonal development and became less intense postnatally, after melanocyte migration had occurred [11].

The Role of Mitf in the Skin and Immune System

Concerning the skin, the gene has a complex role in melanocyte survival and differentiation. It has been shown to be of crucial importance, along with BRAF and other genes, in the development of melanomas (see melanoma section) [12].

MITF is a master regulator of melanin production. All signalling to upregulate melanin go through MITF which then upregulates enzymes such as TYR, TRP-1 and TRP-2 which cause melanogenesis. Some of the pathways involved in MITF activation are the α-MSH/cAMP-dependent signalling pathway, the MAPK signalling pathway, the PI3K/Akt signalling pathway, the Wnt/β-catenin signalling pathway and the nitric oxide signalling pathway. This, along with today’s high demand of melanogenesis inhibiting factors to prevent and to treat medical and cosmetic problems, makes MITF a very important drug target [13].

Mitf regulates the production of hematopoietic prostaglandin D synthase (H-PGDS) in mast cells. H-PGDS is responsible for the generation of prostaglandin D2 (PGD<sub>2</sub>) in immune and inflammatory cells including rat mast cells, rat dendritic cells, rat Langerhans cells, rat macrophages and human helper T cells. PGD<sub>2</sub> is also expressed in human Langerhans cells and human keratinocytes [14]. The function of PGD<sub>2</sub> is controversial with some reporting bronchoconstriction and pro-inflammatory effects through induction of immune cell accumulation while others report anti-inflammatory effects by inhibition of dendritic cells and neutrophils [15].

The Role of Mitf in the Central Nervous System

According to one study, Mitf regulates the production of lipocalin-type prostaglandin D synthase (L-PGDS), which is, along with its product PGD<sub>2</sub>, important in many physiologic functions such as sleep and pain responses. L-PGDS binds molecules in the extracellular matrix (such as biliverdin, bilirubin,
retinoic acid and thyroid hormones) and functions as a transporter for the same molecules. It is secreted into cerebrospinal fluid (CSF), plasma and seminal plasma [14].

Melanocytes also secrete DOPA (Dopamine precursor) and neuromelanin which has been hypothesized to have a function in protection against iron radical formation. Selective loss of neurons containing neuromelanin has been implicated in Parkinson’s disease [14].

Mitf<sup>mi-bw</sup> heterozygotes show a have lower breathing frequency and increased tidal volume (slow and deep breathing) compared to wild type mice. This may be due to that both the glomus cells in the carotid body (which serve as peripheral chemoreceptors) and the central respiratory controller (which regulates autonomic ventilation) are derived from neural crest cells (same as melanocytes), the latter being at least in part derived from neural crest cells. Melanocytes also produce β-endorphin from pro-opiomelanocortin. Seeing that opiates play a role in respiratory control, it may be a lack of opiates that causes a change in Mitf<sup>mi-bw</sup> breathing patterns [14].

A recent study found that melanocytes are present in the meninges of mice and are expressed differently depending on age and Mitf mutation. Their functions so far are unknown but there are speculations about a possible role in the immune response due to their anatomic location around the olfactory bulb, in migraine due to the formation of NO in melanocytes and in vasculature formation due to reports of aneurysm formation being linked to meningeal pigment cells [16].

The Role of Mitf in Kidney Formation

It has been shown that mice with an overexpression of the Mitf variant Mitf-A develop significantly larger kidneys with more nephrons than the wild type [17].

Human Diseases Associated with Mitf

Waardenburg Syndrome type IIA

Waardenburg syndrome type IIA is a dominantly inherited disease caused by mutations in Mitf. Waardenburg syndrome type II A accounts for approximately 15% of all patients with WS type II and is diagnosed when an identified mutation in Mitf is found. It is characterized by small eyes, hypopigmentation and hearing loss [9]. Waardenburg syndrome in all its variants has a prevalence of 1/42,000 and accounts for about 1-3% of all cases of congenital deafness [18].

Tietz Syndrome

Tietz syndrome is a rare disorder with an unknown exact prevalence [19]. There are two types of Tietz syndrome. Both have distinct mutations in Mitf and are characterized by deafness and leucism [9]. The syndrome also affects the eyes in that the iris is blue and the RPE lacks normal pigmentation [19]. Tietz syndrome differs from Waardenburg syndrome type IIA in that it has a complete penetrance while in the latter the depigmentation is patchy and the hearing loss is variable [20].
Age Dependent Macular Degeneration (AMD)

Oxidative stress and metabolic dysregulation play important roles in development of age dependent macular degeneration (AMD) [7]. Seeing that Mitf regulates oxidative stress it can be assumed that it plays an important role in the development of AMD. Mitf also regulates death associated protein like-1 (DAPL1) which has been described as a female-specific susceptibility locus for AMD [21].

Cadmium has been shown to directly affect MITF expression. In one study, increased levels of cadmium caused decreased levels of the MITF-M protein, which constitutes more than 80% of MITF protein variants, in both the cochlea and RPE in human melanocyte cell lines. Age associated accumulation of cadmium may therefore be a contributing cause of hair graying, vision loss and loss of hearing in the elderly [22].

COMMAD syndrome

In 2016 a genetic analysis was done on two children born with coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism and deafness (COMMAD). This syndrome is analogous to what is seen in Mitf\textsuperscript{mim/mim} mutant mice and has never before been demonstrated to occur in humans [23].

Neurofibromatosis type 2

Neurofibromatosis type 2 is an autosomal-dominant multiple neoplasia syndrome with a frequency of 1 in 25,000 births and 100% penetrance at age 60. It is caused by a mutation in the neurofibromin 2 (NF2) tumor suppressor gene. It usually presents at age 20-30 years with hearing loss from bilateral vestibular schwannomas. Other symptoms include spinal tumors, meningiomas, cataract, and cutaneous lesions [24].

It has been shown that MITF activation of NF2 is necessary for the differentiation of cell lines in eye development. It seems to regulate the expression of NF2 by binding to promoter sequences [25]. Another study found that reduction of neurofibromin caused an increase in melanogenic gene expression [26]. Another interesting fact is that both cutaneous lesions and retinal hamartomas in neurofibromatosis type 2 show slightly increased pigmentation [27]. Whether MITF plays a role in this disease has not been examined.

Melanoma

Melanoma is a lethal skin cancer with a 5 years survival rate of about 9%. Its incidence has been rising the last 30 years and in 2015 there were, according to the World Health Organization, around 132,000 new cases diagnosed. The primary risk factor is sun-exposure but moderate alcohol consumption has also been shown to increase the risk of developing melanoma by about 20% [28].

The SOX10-MITF pathway is of crucial development in developing melanoma. SOX10 directly regulates MITF and MITF mutations are common in melanomas. Mutations in MITF affect melanocyte survival by hindering the cells ability to activate the p21 promoter, which is necessary to promote cell cycle arrest. MITF also influences and is influenced by other factors (see figure 2) [28].
Figure 2. The role of MITF in association to other proteins in human melanoma. Source: [28]

The discovery of BRAF inhibitors has made the prognosis of metastatic melanoma much better. Unfortunately, resistance is a major problem. High MITF levels, which are found in around 80% of melanomas during initial treatment, have been shown to make melanoma cells able to avoid cell death triggered by BRAF inhibitors [29].

MITF also regulates the production of L-PGDS which is a potential tumor suppressor of human melanoma cells [14].

The Mutations Used in this Experiment

The mutations used for the vessel diameter measurements are Mitf<sup>mi-vga9/+</sup> (heterozygotes) and Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup> (homozygotes). These have normal vision. The mutations used for the blood flow measurements so far are Mitf<sup>Mi-Wh/+</sup> (heterozygotes) and Mitf<sup>Mi-Wh/mi/Mitf<sup>mi</sup></sup> (compound heterozygotes) which are white and have normal sized eyes. (See Table 1).

Table 1. Shows the mutations and their characteristics. Source: [4, 30]

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Mode of induction</th>
<th>Heterozygote</th>
<th>Homozygote</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitf&lt;sup&gt;mi-vga9&lt;/sup&gt;</td>
<td>Transgene insertion</td>
<td>Normal</td>
<td>White coat, eyes red and small; inner ear defects</td>
<td>Transgene insertion and 882-bp deletion</td>
</tr>
</tbody>
</table>
We have noticed in a previous study that the RPE is thinner in the \textit{Mitf}\textsuperscript{mi-enu22(398)/mi-enu22(398)} mutation compared to the \textit{Mitf}\textsuperscript{mi-vga9/+} mutation [31]. Furthermore, these mutants have normal vision according to ERG (electroretinography) measurements.

Another study showed that the RPE in \textit{Mitf}\textsuperscript{Mi-Wh/+} is profoundly degenerated and missing in the \textit{Mitf}\textsuperscript{Mi-Wh/mi\textbackslash{}Mi}\textsuperscript{fns} mutation. ERG measurements performed in the same study showed that these mutations had significantly lower mean amplitudes of photopic a-waves and scotopic oscillatory potentials compared to wild type. Both mutations were blind, having immense retinal degeneration and lacking both the photoreceptor and outer plexiform layers [32].

**Research Objectives**

Despite much research on the \textit{Mitf} gene, very little is known about its effect on vasculature development and character. The goal of this study is to analyse the retinal vasculature in some of these mutations with respect to diameter and blood flow dynamics. This may prove important in understanding the effects of \textit{Mitf} on eye development and the pathology of the microphthalmia that occurs in Waardenburg and Tietz syndrome. To analyse the vessel diameter, we developed a software that determines the diameter of fluorescence filled retinal vessels. Analysis of the blood flow dynamics has been described in another study [33]. We will use the same method here.
Methods

Animals
All experiments were approved by the Icelandic Food and Veterinary Authority (License No. 2017-04-03). Animals were kept in a 12-hour light, 12-hour dark cycle. The mice were 3 months of age at the time of experiments.

General Preparation
Mice were anaesthetized by an intraperitoneal injection of 40 mg/kg ketamine and 4 mg/kg xylazine prior to surgery and/or imaging. Corneal anesthesia and mydriasis were produced using tetracaine (1% MINIMS, Bausch&Lomb) and tropicamide (10 mg/ml Mydriacyl, Alcon Laboratories) respectively. Methylcellulose (2% Methocel, OmniVision) along with contact lenses were applied to the cornea afterwards, to make sure the cornea remained moist and prevent the formation of cataract [34].

Measuring the Diameter
A total of 6 WT, 6 Mitf mi-vga9/+ and 6 Mitf mi-enu22(398)/mi-enu22(398) fundus images were used in this study. It varies whether the left or right eye was imaged because the mice tend to develop a cataract, often just in one eye but sometimes in neither or both.

Fluorescein Angiography
Fluorescence sodium salt 98,5-100,5% (100mg/mL) diluted with distilled water (final concentration 50mg/mL) was administered in one bolus (150μL) by intraperitoneal injection. Fundus images were taken with a Phoenix Micron IV fundus camera.

The Software
A Matlab program was created to measure the diameter of the vessels. In short it measures the intensity of pixels in a circular, clockwise direction from the center of the optic disc at a radius that is twice that of the optic disc. When it encounters pixels above and below a user specified threshold it marks those pixels with a start- or endpoint respectively. It does this 30 times, each time moving a little further from the optic disc center. After this the program goes through each of the start points and finds the shortest distance to one of its 30 corresponding end points. The result is a figure containing the points and a white line drawn between them that represents the measurements (see figure 3). It also provides an excel document containing the measurement values for each vessel and a calculation of the mean, median and standard deviation.
Figure 3. The result of a measurement. (Left) Wild type. (Right) Mitf\(^{\text{mi-enu22(398)/mi-enu22(398)}}\). The radius of the outer circle is twice that of the inner circle.

Processing of Images Prior to Analysis

Before the images could be analysed by the software, they had to be processed. This was due to the fact that the fundus images from mice with the Mitf\(^{\text{mi-enu22(398)/mi-enu22(398)}}\) mutation had a lot of background which had a similar intensity with that of the vessels. The selection tool in Adobe Photoshop CC 2017 was used to select the vessels and they were subsequently removed and replaced by a maximum-intensity white background (RGB=255, 255, 255). It was not necessary to select the whole vessel bed as shown in figure 4, only the part which was within the measurement range. To make the program described above work with processed images, a line was added which allowed the program to read both the original image and the processed image. Although the program made points on the processed image, they were displayed to the user as if they were on the original image. This meant that the user could determine if the measurements are accurate and if not, go back and make changes to the selection. The software intensity threshold was set to 80% on all the images analysed.

After the vessels had been carefully selected they were run through the Matlab program. Based on the program output the selections were improved if a point did not fall exactly on the margin of a vessel. This was done the same way for all the images.
Determining Vessel Type

An alternating vascular pattern was assumed when determining which vessels were arteries or veins. This alternating pattern has been confirmed in WT by our blood flow experiments in which fluorescence is injected into the femoral vein and a video is acquired of the retinal vasculature filling. In addition, a difference can be seen in the shape of the vessels: the veins are usually wider, with fewer curves and follow a straighter path than the arteries.

Statistical Analysis

All data was managed in Excel. The bar-charts were made in excel. Statistical analysis was done in SigmaPlot 13.0. A threshold of $\alpha = 0.05$ was used to reject the null-hypothesis. We used a simple t-test to compare the means between groups, with an exception of 4 comparisons where the normality test failed. In those instances, we used the Mann-Whitney Rank Sum Test. All P-values given are two-tailed.

The raw data used were the average values for each vessel extracted from the Matlab program.

When calculating the average vessel diameter, the average diameter for arteries, veins and both, were calculated using the mean diameter of each vessel (given by the 29 measurement values supplied by the Matlab program). All subsequent data analysis was done using this average (thus one value for the arterial, venular and total diameter respectively for each image analysed).

Measuring Blood Flow

Fluorescein Angiography

A femoral vein cannulation was performed using polyethylene tubing. Temperature was continuously monitored during the experiment. In each experiment, 20 µl of fluorescence salt solution was injected in one bolus through the cannulation at a rate of 0.3 ml/min with a NE-1000 syringe pump (Serial NO.
A 30 second video recording (15 frames per second) of the fundus was taken starting at 5 seconds prior to the injection of fluorescence.

The Software

We used the same software as is described in [33]. In short it uses an built-in Matlab function to perform Principal Component Analysis, after correcting for any animal movement, to measure the change in intensity over time.

Results

Diameter Measurement

The results showed an increase in both venular and arterial vessel diameter for the Mitf^{mi-enu22(398)/mi-enu22(398)} mutation and a significant increase in total venular diameter in the Mitf^{mi-vga9/+} mutation (see figure 6-8). The arteries, veins and both combined are compared separately below. To account for the fact that there sometimes was a difference in the number of vessels, we also compared the average total vessel diameter, which is calculated by multiplying the average diameter of the respective vessel type with the average number of vessels. This number sums up the amount of vasculature for each vessel type in each mutation (see figure 8). The difference in the number of vessels was also compared below. In 4 comparisons concerning the Mitf^{mi-enu22(398)/mi-enu22(398)} mutation the normality test failed which lead to the use of the Mann-Whitney Rank Sum Test.

*Figure 5. Average diameter (in pixels) of arteries, veins and both in the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf^{mi-enu22(398)/mi-enu22(398)}, VGA9 = Mitf^{mi-vga9/+}. *Significant 18.8% increase (P=0.0259).*
Figure 6. Average number (in pixels) of arteries, veins and both in the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf^{mi-enu22(398)/mi-enu22(398)}, VGA9 = Mitf^{mi-vga9/+}. *Significant 18.8% increase (P=0.026). **Significant 15.6% increase (P=0.041). ***Significant 17.2% increase (P=0.026).

Figure 7. Average total diameter (average number of vessels*average diameter, in pixels) for the three mutations. Error bars denote one standard error around the mean. ENU22 = Mitf^{mi-enu22(398)/mi-enu22(398)}, VGA9 = Mitf^{mi-vga9/+}. *Significant 24.8% increase (P=0.0243). **Significant 38.6% increase (P=0.004). ***Significant 17.8% increase (P=0.0303). ****Significant 31% increase (P=0.00726).

Arterial Comparison

There was not a statistically significant difference in the mean arterial diameter between WT and Mitf^{mi-enu22(398)/mi-enu22(398)} (P=0.598). The same applies to the difference between WT and Mitf^{mi-vga9/+} (P=0.579). See Table 2.

Table 2. Shows the mean arterial diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.
There was a statistically significant difference in the mean total arterial diameter (average diameter*number of arteries) between WT and Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup> (P=0.0243). There was not a statistically significant difference in corresponding measurements between WT and Mitf<sup>mi-vga9/+</sup> (P=0.236).

*Table 3.* Shows the mean total arterial diameter (mean arterial diameter*average number of arteries, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Total Arterial Diameter (Pixels)</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>70,957</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-enu22(398)/mi-enu22(398)&lt;/sup&gt;</td>
<td>88,577</td>
<td>124,8327</td>
<td>0.0243</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-vga9/+&lt;/sup&gt;</td>
<td>79,365</td>
<td>111,8506</td>
<td>0.236</td>
</tr>
</tbody>
</table>

Venular Comparison

There was a statistically significant difference in the venular diameter between WT and Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup> (P=0.0259). There was not a statistically significant difference in the venular diameter between WT and Mitf<sup>mi-vga9/+</sup> (P=0.101). See table 4.

*Table 4.* Shows the mean venular diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Venular Diameter (Pixels)</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>13,418</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-enu22(398)/mi-enu22(398)&lt;/sup&gt;</td>
<td>15,9425</td>
<td>118,7982</td>
<td>0.0259</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-vga9/+&lt;/sup&gt;</td>
<td>14,8438</td>
<td>110,6111</td>
<td>0.101</td>
</tr>
</tbody>
</table>

The normality test failed when using the t-test to compare the difference in total venular diameter between WT and Mitf<sup>mi-enu22(398)/mi-enu22(398)</sup>. Therefore, we used the Mann-Whitney Rank Sum Test, which yielded a statistically significant difference (P=0.004). There was a statistically significant difference in the mean total venular diameter between WT and Mitf<sup>mi-vga9/+</sup> (P=0.0303).

*Table 5.* Shows the mean total venular diameter (mean venular diameter*average number of veins, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Total Venular Diameter (Pixels)</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>71,2680</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-enu22(398)/mi-enu22(398)&lt;/sup&gt;</td>
<td>98,7619</td>
<td>138,5781</td>
<td>0.004</td>
</tr>
<tr>
<td>Mitf&lt;sup&gt;mi-vga9/+&lt;/sup&gt;</td>
<td>83,9785</td>
<td>117,8347</td>
<td>0.0303</td>
</tr>
</tbody>
</table>
Total Comparison

There was not a statistically significant difference in the mean total vessel diameter between WT and Mitf^{mi-enu22(398)/mi-enu22(398)} (P=0.152). Same applies for the difference between WT and Mitf^{mi-vga9/+} (P=0.288).

Table 6. Shows the mean vessel diameter (in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Vessel Diameter (Pixels)</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>13,3941</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf^{mi-enu22(398)/mi-enu22(398)}</td>
<td>14,9683</td>
<td>111,752</td>
<td>0.152</td>
</tr>
<tr>
<td>Mitf^{mi-vga9/+}</td>
<td>14,4273</td>
<td>107,7142</td>
<td>0.288</td>
</tr>
</tbody>
</table>

There was a statistically significant difference in the total vessel diameter*number of vessels between WT and Mitf^{mi-enu22(398)/mi-enu22(398)} (P=0.00726). The same did not apply between WT and Mitf^{mi-vga9/+} (P=0.0931).

Table 7. Shows the mean total vessel diameter (mean vessel diameter*average number of vessels, in pixels), the diameter difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Total Vessel Diameter (Pixels)</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>142,8704</td>
<td>100,000</td>
<td></td>
</tr>
<tr>
<td>Mitf^{mi-enu22(398)/mi-enu22(398)}</td>
<td>187,1037</td>
<td>130,9605</td>
<td>0.00726</td>
</tr>
<tr>
<td>Mitf^{mi-vga9/+}</td>
<td>163,5099</td>
<td>114,4463</td>
<td>0.0931</td>
</tr>
</tbody>
</table>

Number of Vessels Comparison

The normality test failed for comparison of the average number of arteries between WT and Mitf^{mi-enu22(398)/mi-enu22(398)}. Therefore, we used the Mann-Whitney Rank Sum Test, which yielded a statistically significant difference (P=0.026). There was not a statistically significant difference in the number of arteries between WT and Mitf^{mi-vga9/+} (P=0.290).

Table 8. Shows the average number of arteries, the difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Number of Arteries</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>5,3333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf^{mi-enu22(398)/mi-enu22(398)}</td>
<td>6,3333</td>
<td>118,7500</td>
<td>0.026</td>
</tr>
<tr>
<td>Mitf^{mi-vga9/+}</td>
<td>5,6667</td>
<td>106,2500</td>
<td>0.290</td>
</tr>
</tbody>
</table>

The normality test failed for comparison of the average number of veins between WT and Mitf^{mi-enu22(398)/mi-enu22(398)}. Therefore, we used the Mann-Whitney Rank Sum Test, which yielded a statistically significant difference (P=0.041). There was not a statistically significant difference in the number of veins between WT and Mitf^{mi-vga9/+} (P=0.290).
Table 9. Shows the average number of veins, the difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Number of Veins</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>5,3333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf^mi-enu22(398)/mi-enu22(398)</td>
<td>6,1667</td>
<td>115,6250</td>
<td>0,041</td>
</tr>
<tr>
<td>Mitf^mi-vga9/+</td>
<td>5,6667</td>
<td>106,2500</td>
<td>0,290</td>
</tr>
</tbody>
</table>

The normality test failed for comparison of the average number of vessels between WT and Mitf^mi-enu22(398)/mi-enu22(398). Therefore, we used the Mann-Whitney Rank Sum Test, which yielded a statistically significant difference (P=0,026). There was not a statistically significant difference in the number of vessels between WT and Mitf^mi-vga9/+ (P=0,290).

Table 10. Shows the average number of vessels, the difference from the wild type and the two-tailed P-value for each mutation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mean Number of Vessels</th>
<th>% Of Wild Type</th>
<th>Two-tailed P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>10,6667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitf^mi-enu22(398)/mi-enu22(398)</td>
<td>12,5000</td>
<td>117,1875</td>
<td>0,026</td>
</tr>
<tr>
<td>Mitf^mi-vga9/+</td>
<td>11,3333</td>
<td>106,2500</td>
<td>0,290</td>
</tr>
</tbody>
</table>

Samples of Blood Flow Measurements

Wild Type

2017-07-06, Video 2

Figure 8. (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds.
**Figure 9.** (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel.

**Figure 10.** (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel.
Figure 11. (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds. Not much difference can be seen between arteries and veins due to background filling. There are three veins and four arteries. The veins are thicker and have a shared point of origin. The arteries are thinner and have an origin that is to the upper left of the venular point of origin.

Figure 12. (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel.
Figure 13. (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel.

Mitf^Mi-Wh/mi /Mitf^mi

2017-08-09, Video 6

Figure 14. (Left) Fundus at maximum filling. (Right) Relative offset to peak intensity in seconds. There is a lot of background filling and it is difficult to distinguish veins from arteries in this image. There is also an extra vessel of unknown origin. A possible explanation for this vessel is that this mutation generally lacks a pigment epithelium and thus we may be looking at vasculature that lies posterior to the retina.
Figure 15. (Left) Principal component 1. Y-axis shows average intensity. X-axis shows time in seconds. (Right) Shows the maximum intensity for each pixel.

Figure 16. (Left) Time (in seconds) to reach half of maximum intensity for each pixel. (Right) Time (in seconds) to fall down to half of maximum intensity for each pixel.
Comparison Between the Three Mutations

Figure 17. Shows the scores of principal component 1 (intensity) for each of the mutations over time (s). Half rise time and half fall time is the amount of time that has passed when the intensity has risen half way up to it’s maximum value and fallen half way down to it’s end value respectively. Offset is the difference between maximum intensity and the end intensity.

Discussion

Diameter

As mentioned earlier, previous studies have shown that the Mitf gene plays a crucial role in eye development [35]. Despite this there has been no previous analysis of vessel diameter in Mitf mutant mice. The main findings of this study are that there are significant differences in the vasculature between WT and the two mutations, Mitf\textsuperscript{mi-vga9/+} and Mitf\textsuperscript{mi-enu22(398)/mi-enu22(398)}. Possible causes for these differences include increased formation of ROS in mice that lack Mitf [7] and reduction in VEGF expression [8] (see The role of Mitf in eye development). It may also be explained by the difference in RPE integrity (see The Mutations Used in this Experiment). The RPE has important functions such as absorbing light which reduces stress on the photoreceptors, transportation of metabolic end products from the subretinal space to the blood, delivery of nutrients to photoreceptors, digestion and recycling of photoreceptors, secretion of growth factors such as VEGF, secretion of immunosuppressive factors, formation of blood-retina barrier and finally reisomerization of all-\textit{trans}-retinal to 11-\textit{cis}-retinal after absorption of light by the photoreceptors [36]. This makes it reasonable to assume that changes in RPE integrity can have profound influences on visual structure and function. This would also explain why there was more of a difference in the vasculature diameter compared to WT in the Mitf\textsuperscript{mi-enu22(398)/mi-enu22(398)} mutation than the Mitf\textsuperscript{mi-vga9/+} mutation, because the RPE is more degraded in Mitf\textsuperscript{mi-enu22(398)/mi-enu22(398)} than Mitf\textsuperscript{mi-vga9/+} mutants. The results are discussed separately for each mutation below.
The normality test failed when using the t-test to compare the average number of vessels between the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation and WT (see Diameter Measurement). This suggests that the distribution was not normalized and may be due to the low number of samples. Another possible cause is that one mouse with the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation differed from the other retinas in that it had an uneven number of vessels (it had one extra artery). Using the Mann-Whitney Rank Sum Test we could demonstrate that the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation did indeed have a significantly higher number of arteries, veins and both combined compared to the WT.

In mice with the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation there was no difference in the mean arterial diameter when compared to WT (see Arterial Comparison). There was however a significant 24.8% increase in mean total arterial diameter in mice with the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation compared to WT. This means that while each artery was similar in size compared to the WT, there was more total retinal arterial vasculature in the mutation. One might assume from this that there is an increased arterial blood supply to the retinas of the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutants. This was an unexpected result and suggests that there may be a change in metabolism or oxygen demand of the retina in these mutations. Furthermore, it suggests that the retinal arterial development is normal in this mutation.

There was also a significant 18.8% increase in the mean venular diameter in mice with the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mutation compared to the WT mice (see Venular Comparison). Interestingly the normality test failed when using the t-test to analyse the 39% increase in mean total venular diameter in the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mice compared to WT (see Total Comparison). This may be linked to the above failure in comparing the number of vessels due to lack of normality in the data. Using the Mann-Whitney Rank Sum Test we could verify that this increase is statistically significant. This increase in venular diameter indicates that there is need to move extra blood from the eye. This was expected seeing that there was an increase in mean total arterial diameter. If there is more afferent blood flow, there also must be a corresponding increase in efferent blood flow.

There was not a significant difference in mean vessel diameter between the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mice and WT. However, there was a significant 31% increase in the mean total retinal vessel diameter in the $\text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}}$ mice compared to the WT (see Total Comparison). This again suggests that there is an increase demand for retinal blood flow in these mutants.

$\text{Mitf}^{\text{mi-vga9/+}}$

No significant difference was seen in the number of retinal arteries, veins or both combined between the WT mice and mice with the $\text{Mitf}^{\text{mi-vga9/+}}$ mutation.

There was no significant difference in the mean arterial diameter nor was there a difference in the total mean arterial diameter between mice with the WT and the $\text{Mitf}^{\text{mi-vga9/+}}$ mice. This indicates that there is no change in retinal blood supply demand in the mutant mice.

There was not a significant difference in the mean venular diameter, while there was a statistically significant 17.8% increase in the mean total venular diameter in the $\text{Mitf}^{\text{mi-vga9/+}}$ mice compared to the
WT (see Arterial Comparison). Considering that there was not a significant change in the retinal arterial vasculature compared to the WT mice, this seems strange since an increase in afferent blood flow requires a corresponding increase in efferent blood flow. This may be explained by the low number of samples.

There was not a significant difference in the mean vessel diameter between the Mitf \textsuperscript{vga9/+} mice and WT nor was there a significant difference in the mean total vessel diameter.

Comments on Further Improvements of the Analysis and Software

The Matlab program used for analysis requires that there is no vessel in a vertical line straight above the center of the optic disc. This was usually the case but in one of the images it was not, and the image had to be rotated. This had no effect on the analysis as such but is a potential area for improvement in the software.

In some cases, the vessels branched within the range that the program was measuring (a good example of this is the right image in figure 3). In those cases, we chose to alter the diameter of the circle that the program uses to measure. This did not happen often and when it did the change was minimal so that it started to measure just barely past the branch-point. Nevertheless, branching is a factor that influences the measurements and sometimes it was difficult to determine if a smaller branch should be included or not.

The images were of varying quality and taken at varying times of vascular filling. This may influence the visually determined diameter. Intraperitoneal injection is believed to be a cause of varying quality. Hopefully the images obtained during femoral vein injection will have a more consistent quality. We also make no distinction between the right and left eye, and it was not always the same eye being imaged, that is sometimes the left eye was used and sometimes the right. This was mainly due to the fact that the mice had a tendency to develop a cataract, which is a known effect of anaesthetics in mice [34], but most often only on one eye.

The Mitf \textsuperscript{enu22(398)/enu22(398)} mutation had an increase in background intensity in the fundus images. This may have been caused by leakage of the vessels which may in turn affect vascular diameter. It is also possible that the background in the fundi of some of the Mitf \textsuperscript{enu22(398)/enu22(398)} mice reach a higher level than the 80% threshold, but this would have been seen in the software and is thus also a negligible error. The possibility of making the background black was considered but not deemed necessary.

Determining the vessel type proved to be a difficult task because we did not have a recording of the retinal vessels filling. It was however made simpler by the fact that the vessel types always alternate, the veins are generally larger and the arteries are generally more curved than the veins. This may be a source of error, but we find it unlikely given the vessels relatively clear characteristics.

The distance from the camera to the retinal vasculature could vary. This should however be minimal given that the camera was always in contact with the eye and eye size is assumed to be approximately the same among mice.
It would have been possible to use each of the 29 measurement values for each vessel instead of the mean when calculating the P values (thus approximately 300 measurement values per mouse) but this was not deemed necessary for our purpose.

In the future we’d like to make the process of determining vascular diameter completely automatic, but that has proved to be a difficult task due to the differences in fluorescence intensities, potential vasculature leakage and vascular differences between mutations.

**Blood Flow**

**Status**

So far, we’ve only analysed the results from blood flow measurements from a couple of mice due to difficulties in developing this technique. We’ve managed to perform the surgery by using specifically ordered catheters, but it still takes too long to perform. The time factor resulted in a problem in keeping the mice warm during the surgery. We seem to have corrected that problem using a heating pad and by preheating the platform used in the imaging procedure.

Unfortunately, we were not able to monitor heartrate due to problems with the heart rate monitor. This is a problem given that the heart rate should theoretically significantly affect the rate at which the fluorescence salt solution is moved from the site of injection to the retinal vasculature.

During this procedure it may be that the dose of anaesthesia used for intraperitoneal injections needs to be higher, which may influence the physiology of the mouse. It does increase the risk of cataract, which has proven to be a significant limitation in these analyses. If the mouse has cataract one can often not see the fundus at all, or just parts of the fundus.

**Sample Images**

A few sample images are supplied within the result section for the blood flow measurements. They show the preliminary processing. The Mitf^{Mi-Wh/mi/Mitf^{mi}} mutation shown (see Mitf^{Mi-Wh/mi/Mitf^{mi}}) had different characteristics compared to the others in that it had a large vessel of unknown origin. A possible explanation for this is that these mice are blind and have profound RPE degeneration (see The Mutations Used in this Experiment).

The images that show the offset in filling time do not show an obvious difference between arteries and veins in the case of the mutations. This is probably due to extensive background filling.

Note that there are differences in the time it took to complete the surgical procedure, amount of fluorescence injected before a good quality video could be obtained, heart rate and body temperature that are not accounted for in the blood flow results section.

In figure 17 (see Comparison Between the Three Mutations), note that the baseline does not always start from 0. This is due to remnant fluorescence from previous infusions.

A future goal is to have many measurements from each mutation and combine them into a similar graph like this so that one can find if there are differences in filling patterns between mutations.
Conclusions

In short, we found that there is a statistically significant difference between the WT mice and the two mutations, \( \text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}} \) and \( \text{Mitf}^{\text{mi-vga9/+}} \). The difference is more profound between the \( \text{Mitf}^{\text{mi-enu22(398)/mi-enu22(398)}} \) mice and the WT, showing an increase in arterial and venular retinal vasculature. The difference is less between the mice with the \( \text{Mitf}^{\text{mi-vga9/+}} \) mutation and WT mice, in which there is an increase in venular, but not arterial, retinal vasculature diameter. This indicates that there is an increase in vasculature in both these mutations, possibly due to an increase in metabolic needs, which needs to be investigated further.

No conclusions can be made so far regarding the blood flow experiments.

References