Retinal Thickness Irregularities in Preclinical Diabetic Retinopathy

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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University of Toronto

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Abstract

Diabetic retinopathy (DR) is the most common complication of diabetes and the leading cause of vision loss worldwide. Clinical detection relies on the manifestation of sight-threatening microvascular, macrovascular and edematous ocular insults. However, preclinical investigations detected retinal thickness irregularities. We hypothesized that retinal thickness irregularities are localized to specific retinal regions and layers. Training and validation data were collected from participants with diabetes with no/minimal DR and healthy individuals to identify and verify regions of interest. Optical coherence tomography and MATLAB® computing were the primary tools used to measure retinal thickness in each participant. Linear mixed-effects models identified four significant regions of thickness irregularities from the training data; these were not matched by validation data. Nonetheless, retinal thickness irregularities were localized to specific regions and layers. Recognizing that there are retinal regions susceptible to retinal thickness irregularities during preclinical stages of disease is important for early disease detection.
Acknowledgments

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Vision & Imaging Lab (Duke University)

LME Model ‘R’ Scripting ..........................Dr. Thomas Wright, Research Associate
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>amacrine cell</td>
</tr>
<tr>
<td>ACE-I</td>
<td>angiotensin-converting enzyme I</td>
</tr>
<tr>
<td>ACE-II</td>
<td>angiotensin-converting enzyme II</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BC</td>
<td>bipolar cell</td>
</tr>
<tr>
<td>BRB</td>
<td>blood-retina barrier</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CSME</td>
<td>clinically significant macular edema</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>diabetic retinopathy</td>
</tr>
<tr>
<td>ELM</td>
<td>external limiting membrane</td>
</tr>
<tr>
<td>EPC</td>
<td>Electronic Patient Chart</td>
</tr>
<tr>
<td>ERG</td>
<td>electroretinography</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>FAZ</td>
<td>foveal avascular zone</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Association, USA</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>ganglion cell</td>
</tr>
<tr>
<td>GCL</td>
<td>ganglion cell layer</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>HC</td>
<td>horizontal cell</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>AC</td>
<td>I_i – inner inferior</td>
</tr>
<tr>
<td>ACE-I</td>
<td>ILM – inner limiting membrane</td>
</tr>
<tr>
<td>ACE-II</td>
<td>INL – inner nuclear layer</td>
</tr>
<tr>
<td>ADA</td>
<td>I_o – outer inferior</td>
</tr>
<tr>
<td>AGE</td>
<td>IPL – inner plexiform layer</td>
</tr>
<tr>
<td>ANOVA</td>
<td>IRBP – interstitial retinol-binding protein</td>
</tr>
<tr>
<td>ATP</td>
<td>ISL – inner segment layer</td>
</tr>
<tr>
<td>BC</td>
<td>LME – linear mixed-effects</td>
</tr>
<tr>
<td>BRB</td>
<td>logMAR – logarithmic minimum angle of resolution</td>
</tr>
<tr>
<td>cGMP</td>
<td>ME – macular edema</td>
</tr>
<tr>
<td>CSME</td>
<td>mfERG – multifocal electroretinography</td>
</tr>
<tr>
<td>DAG</td>
<td>mfOPs – multifocal oscillatory potentials</td>
</tr>
<tr>
<td>DNA</td>
<td>mGlur6 – metabotropic glutamate receptor 6</td>
</tr>
<tr>
<td>DR</td>
<td>MHC – major histocompatibility complex</td>
</tr>
<tr>
<td>ELM</td>
<td>MSSSII – M&amp;S Smart System II</td>
</tr>
<tr>
<td>EPC</td>
<td>mtDNA – mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>ERG</td>
<td>mtROS – mitochondrial reactive oxygen species</td>
</tr>
<tr>
<td>ETDRS</td>
<td>NAD+ – nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Retinopathy Study</td>
<td>NADPH – nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>FAZ</td>
<td>N_i – inner nasal</td>
</tr>
<tr>
<td>FDA</td>
<td>N_o – outer nasal</td>
</tr>
<tr>
<td>GABA</td>
<td>NPDR – non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>GC</td>
<td>OCT – optical coherence tomography</td>
</tr>
<tr>
<td>GCL</td>
<td>ONL – outer nuclear layer</td>
</tr>
</tbody>
</table>
OPL – outer plexiform layer
OSL – outer segment layer
PDR – proliferative diabetic retinopathy
PEDF – pigment epithelial-derived factor
PERG – pattern electroretinography
PKC – protein kinase C
RAAS – renin-angiotensin-aldosterone system
RAGE – receptor for advanced glycation end-product
REB – Research Ethics Board
RNFL – retinal nerve fiber layer
ROS – reactive oxygen species
RPE – retinal pigment epithelium
SD-OCT – spectral-domain optical coherence tomography
S_i – inner superior
S_o – outer superior
SST – somatostatin
T1D – Type 1 diabetes
T2D – Type 2 diabetes
TD-OCT – time-domain optical coherence tomography
T_i – inner temporal
T_o – outer temporal
VEGF – vascular endothelial growth factor
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1 Literature Review

Well over half a century’s worth of research concerning diabetic retinopathy (DR) has aimed to better understand its development, progression and management. The first studies of DR were published around 1935 and searched for the presence of unique ocular irregularities in individuals with diabetes and the absence of those same irregularities in individuals without diabetes (Waite & Beetham, 1935). For a long time since then, DR was considered a microvascular complication of the retina in individuals with diabetes (L. M. Aiello, 2003). In that time, different therapies were designed to slow, halt and potentially reverse the progression of vision loss in DR. During that same time, however, came new technological developments that allowed us to observe more and understand more. An ophthalmoscope, an instrument that magnifies the surface of the retina, achieved much in terms of defining microangiopathy in DR. But the new technologies allowed for methods to visualize what lay beneath the surface—the retinal tissue. Within the past decade, new insights brought to light an early neurodegenerative component to the pathophysiology of DR. There are non-vascular components, including functional and morphological changes, which precede microvascular insults. It is this new perspective that has motivated this Master of Science research. This thesis focuses on the architectural structure of the retina and its irregularities in early DR. To best appreciate the relevance of this research, this literature review covers the anatomical features of the retinal tissue, the longstanding clinical view of DR and the current research surrounding the early neurodegenerative component of DR, with a particular focus on the architectural irregularities.

1.1 Human Retina

The human retina is the foundation for our sense of vision and the stage for which DR is studied. It is a neurosensory tissue at the inner surface of the posterior ocular hemisphere. Visual input from the external world activates a circuit of retinal cells that process, encode and relay visual information to higher brain centers. The activities of the retina are metabolically demanding and are nourished by two discrete blood supplies. The variety of retinal cells, and even the blood supplies, are organized regionally, both axially (depth-wise through the retina) and laterally (across the retina). This section reviews the cellular and vascular components and their organization in the retina.
1.1.1 Retinal Cells

The cellular component of the retina is composed of neuronal and non-neuronal cell types organized into discrete retinal layers. The neuronal retinal layers are arranged into nuclear and plexiform layers. The three nuclear layers, from outer to inner retina, are the outer nuclear layer, inner nuclear layer and ganglion cell layer. The two plexiform layers are the outer plexiform layer between the outer and inner nuclear layers and inner plexiform layer between the inner nuclear and ganglion cell layers. Adjacent retinal layers communicate by synapses between their projections. The non-neuronal cells are a layer of retinal pigment epithelium, located exteriorly to the outer nuclear layer, and glial cells that are distributed amongst the discrete layers.

Outer Nuclear Layer

The outer nuclear layer (ONL) is composed of photoreceptor cell bodies. Photoreceptors are classified by morphological means as rods, for their cylindrical shape, or cones, for their conical shape. Rods and cones have different sensitivities to light levels and specific wavelengths of light. Rods provide better vision in scotopic (dark) to mesopic (dim) conditions, and cones provide better vision in photopic (light) conditions. In terms of sensitivity to specific wavelengths of light, rods are maximally sensitive to ~495 nm wavelengths; cones are maximally sensitive to ~420, ~530, and ~560 nm wavelengths in the visible light spectrum(Dartnall, Bowmaker, & Mollon, 1983). Cones have three specific sensitivities because there are three subtypes of cones: short wavelength-sensitive cones (S-cones), medium wavelength-sensitive cones (M-cones) and long wavelength-sensitive cones (L-cones).

Beyond the photoreceptor cell bodies are the inner and outer segments of the photoreceptor. In particular, the outer segments are the light-sensitive component of photoreceptors and appropriately render the photoreceptors the first retinal cells in the circuit to respond to light stimulation. Collectively, the inner and outer segments are referred to as the inner segment layer (ISL) and outer segment layer (OSL). In the absence of light, photoreceptors are in a state of depolarization because there is active influx of sodium ions through cGMP-gated sodium channels(Baylor, 1987). When a light stimulus is presented, the cGMP-gated sodium channels close through a process called phototransduction and terminate the inward current, causing the stimulated photoreceptors to hyperpolarize.
Outer Plexiform Layer
The changes in voltage associated with the presence or absence of light are communicated along the photoreceptor processes that synapse with bipolar cells. The photoreceptor processes and synapse give rise to the outer plexiform layer (OPL). During darkness when photoreceptors are depolarized, the neurotransmitter glutamate is continuously released into the synapse(Schmitz & Witkovsky, 1997). With light stimulation, photoreceptors hyperpolarize and glutamate release ceases. The change in rate of glutamate release into the synapse is essential for photoreceptor downstream signaling.

Inner Nuclear Layer
The inner nuclear layer (INL) contains the cell bodies of bipolar cells (BCs), which are second-order retinal neurons that respond to photoreceptor glutamate release and provide downstream signaling to ganglion cells. The two major classes of BCs are OFF and ON BCs, depending on whether the BC hyperpolarizes or depolarizes, respectively, when light stimulus falls within the center of its receptive field. Rod photoreceptors synapse with ON BCs only and cone photoreceptors synapse with OFF or ON BCs. OFF BCs have ionotropic glutamate receptors (iGluRs), AMPA and kainate, which bind glutamate to control non-specific cation channels(DeVries, 2000). In scotopic and mesopic conditions, when glutamate is abundant, these receptors are activated and membrane pores open, allowing an inward current to flow and depolarize the OFF BC. In photopic conditions when glutamate production is reduced, glutamate unbinds from OFF BCs and membrane pores close, halting the inward current and hyperpolarizing the OFF BC. On the other hand, ON BCs express metabotropic glutamate receptors (mGluR6s), which are G-protein-coupled receptors that hyperpolarize and inactivate in scotopic and mesopic conditions when glutamate levels are high(Vardi, Duvoisin, Wu, & Sterling, 2000). In photopic conditions when glutamate levels are reduced, ON BC are activated and depolarized.

Inner Plexiform Layer
Whether the BCs are depolarized or hyperpolarized, the voltage is conducted along the processes of the BCs. These BC processes comprise the inner plexiform layer (IPL).
**Ganglion Cell Layer**

The ganglion cell layer (GCL) consists of ganglion cell (GC) cell bodies that receive inputs directly or indirectly (via amacrine cells) from the BCs. GCs are the first retinal neurons in the circuit thus far to respond to light stimulation with an all-or-nothing action potential (Koch et al., 2004). This response permits long-distance transmission, which is advantageously appropriate for the relatively extensive conduction path between the GCs and optic nerve, where the circuit exits the eye to terminate in higher brain centers. However, the amount of information transmitted by action potentials is limited by the cell’s ability to produce spikes and spike patterns within a given unit of time (Koch et al., 2004). This is referred to as the coding capacity. GCs express ionotropic receptors only, but still follow the ON and OFF dichotomy initiated by the OFF and ON BCs.

In humans, several types of GCs exist; two of these are the parasol and midget GCs, named after their size and dendritic arbor morphologies. Because parasol and midget GCs project to the magnocellular and parvocellular layers of the lateral geniculate nucleus, respectively, they are often referred to as M- and P-cells. In primates, M-cells are not wavelength-selective, have relatively larger receptive fields and respond to low contrast (Shapley & Perry, 1986). P-cells are wavelength-selective, have small receptive fields and are less sensitive to contrast. These characteristics suggest a role for M-cells in motion detection and P-cells in colour vision.

**Retinal Nerve Fiber Layer**

The extensive projections of the GCL form the retinal nerve fiber layer (RNFL), which collects at the optic disc to form the optic nerve. Unlike other retinal layers, which run axially through the retina, the RNFL, for the most part, runs laterally across the retina towards the optic disc.

**Horizontal and Bipolar Cells**

While a primary circuit runs from photoreceptors to BCs to GCs and to higher brain centers, there exist secondary circuits. Horizontal cells (HCs) are second-order retinal neurons that form local feedback circuits with photoreceptors (Byzov, Golubtzov, & Trifonov, 1977; Kamermans et al., 2001). Amacrine cells (ACs), another second-order retinal neuron, form local feedback circuits with BCs (Kaneko & Tachibana, 1987). Both HCs and ACs can be found in the INL. HCs and ACs average the output they receive from photoreceptors and BCs, respectively, and
generate modulatory signals that are fed back to the cell type that generated the original output. These secondary feedback circuits improve the reliability, stability and noise of the signals in the primary circuit (Wu, 1992). Depending on the quality of the original visual stimulation, secondary circuits may be more or less involved. Ultimately, retinal cells in the secondary circuits have a major responsibility to optimize signal transmissions within the primary circuits.

**Retinal Pigment Epithelium**

The non-neuronal cells of the retina maintain and support the activities and architecture of the neuronal cells. The retinal pigment epithelium (RPE) is a layer of pigmented epithelial cells that has a major role in retinal homeostasis. The anterior face of this layer is closely associated with the posterior face of the OSL, to the extent that photoreceptor outer segments are embedded within the RPE. The RPE regenerates photoreceptors, manages waste products generated by photoreceptors and recycles photopigments (Lamb & Pugh, 2004). Since photoreceptors are necessary for vision, the role of the RPE is tremendous. Furthermore, the pigmented nature of the RPE also protects the retina from light damage by absorbing excess or stray photons (Tsacopoulos, Poitry-Yamate, MacLeish, & Poitry, 1998).

**Glial Cells**

Other non-neuronal cells include glial cells. In the human retina, there are three basic types of glial cells: Müller cells, astrocytes and microglia. The principle glial cells are Müller cells. Their cell bodies are found in the INL, but have thick and thin projections that span axially through the retinal layers that wrap around neuronal cell, axon and dendritic bodies (Newman & Reichenbach, 1996). At the interface between the vitreous humour and inner surface of the retina, Müller cell projections reach their inner limit, forming the inner limiting membrane (ILM). At the outer retina, Müller cell projections form adherens junctions between Müller cells and between Müller cells and photoreceptor myoids, forming the external limiting membrane (ELM). The photoreceptor myoid is a region between the inner segment and cell body of the photoreceptor. Both the ILM and ELM are not true membranes—they are a layer formed by junctions. In the mammalian retina, the ELM provides architectural support to the outer retina. ELM mutations alter photoreceptor structure, increase photoreceptor degeneration and induce photoreceptor mislocalization (Mehalow et al., 2003).
In addition to structural integrity, Müller cells have a range of homeostatic functions in the retina. Müller cells remove glucose and neurotransmitters, such as glutamate and GABA, from the extracellular space and recycle them to lactose and glutamine, respectively, which are then returned to the extracellular space to be used by neurons (Newman & Reichenbach, 1996; Tsacopoulos et al., 1998). Müller cells also maintain potassium ion gradients by taking up excess extracellular potassium ions and redistributing it (Tsacopoulos et al., 1998). Müller cells modulate the ionic environment and indirectly, the ionic gradients across cellular membranes. Therefore Müller cells have a major role in regulating neuronal activity on top of providing architectural support.

Astrocytes are found in the RNFL and wrap around the processes and bundles that ultimately form the optic nerve. This type of glial cell is responsible for synaptogenesis and synaptic transmission modulation. Retinal GCs cultured in the absence of astrocytes display little synaptic formation and activity (Pfrieger & Barres, 1997). In the presence of astrocytes, synapses have increased maturity, functionality and stability; excitatory postsynaptic current amplitude and frequency are potentiated; neuron transmissions fail less frequently; and neuronal action potential-independent quantal synaptic release is increased (Pfrieger & Barres, 1997; Ullian, Sapperstein, Christopherson, & Barres, 2001). Astrocytes also modulate GC activity by releasing chemical cofactors, such as glutamate, into the synapse (Parpura et al., 1994). Astrocytes have a range of functions that are concerned with the synaptic quality at the level of the GCL and RNFL.

Microglia are found throughout the retina and are the resident immune cells of the retina. They are involved in innate immune response against pathogens, phagocytosis of endogenous wastes and regenerative processes (Langmann, 2007). While some retinal microglia resemble macrophages, others constitutively express major histocompatibility complex (MHC) II antigen, which is associated with antigen presenting and immune response promotion (Gehrmann, Banati, & Kreutzberg, 1993; Penfold, Provis, & Liew, 1993). Under physiologically normal conditions, microglia remain relatively at rest. However, there is constant immunosurveillance in the retina as a function of resident microglia.
The extensive wiring of different retinal cells in adjacent retinal layers with secondary feedback circuits allow visual signals to be received, processed and encoded for higher neural interpretation. Despite the importance of neuronal cells forming primary and secondary circuits that translate visual signals into optimized signals, non-neuronal cells are also indispensable. In order to maintain proper retinal activity and circuitry, the neuronal cells require the support of the non-neuronal cells. Given the variety, specific functions and organization of retinal cells, dysfunction in any component will compromise retinal physiology. In later sections, we will see that minor changes secondary to diabetes result in major retinal pathophysiology.

1.1.2 Blood Supplies

In order to maintain retinal physiology, a system that supplies nourishment and removes waste is required. These requirements are satisfied by two discrete vascular systems: the retinal and choroidal blood vessels. Both retinal and choroidal blood vessels are derived from the major branches of the ophthalmic artery, which include the central retinal artery, posterior ciliary arteries and muscular branches (Hayreh, 1962). Functionally, there is little overlap despite the presence of two vascular systems in the same tissue. The dual vascular systems also contribute to a blood-retina barrier, which creates a stable environment for retinal cells. Since diabetes is a metabolic disease characterized by hyperglycemia, the vasculature is a key factor that links modified blood supply in diabetes to changes in retinal nourishment and environment that contribute to the onset and progression of DR.

Retinal Blood Supply

The vessels that comprise the retinal blood supply are embedded in the RNFL and GCL and support the inner retinal layers. Before the central retinal artery emerges from the optic nerve, it divides into superior and inferior papillary arteries (Hayreh, 1962). As the superior and inferior arteries branch away from the optic disc and towards the temporal retina, they do not cross the horizontal raphé and therefore supply the superior and inferior hemispheres individually. The retinal capillaries, which arise from the retinal arteries, form a laminar meshwork (Shimizu & Ujiie, 1978). The thickness of the laminar meshwork varies depending on the retinal location. In the peripheral retina, the meshwork appears to be a single layer whereas in the posterior pole, the meshwork exists as three layers. This regional variation ensures adequate perfusion of retinal
tissue. However, it is important to note that the central retinal artery is an end artery, meaning that it has insufficient anastomoses (branches) to other arteries to maintain viability of the tissue supplied if an occlusion occurs. This characteristic renders the inner retinal layers susceptible to ischemia. A retinal venous system follows and is interdigitated with the retinal arterial vasculature.

Retinal capillaries are composed of a layer of endothelial cells and an encapsulating basement membrane. Embedded within the basement membrane are pericytes, contractile cells that regulate endothelial cells. Pericytes control microcirculatory blood flow, endothelial barrier integrity and endothelial cell proliferation (Frank, Turczyn, & Das, 1990; Laties & Jacobowitz, 1966). Though the ophthalmic artery is under autonomic control, there is no central regulation beyond the lamina cribosa, such that retinal capillaries are dependent on pericytes for local autoregulation of blood flow. The ratio of pericytes to endothelial cells is almost one-to-one and comparatively much higher than cerebral arteries (Frank et al., 1990). Relative to the basement membrane surrounding the entire apparatus, the basement membrane between pericytes and endothelial cells is thin. Both the high pericyte/endothelial cell ratio and thinner membrane between pericytes and endothelial cells allow for increased communication.

**Choroidal Blood Supply**

The choroidal blood vessels form a vascular retinal layer referred to as the choroid. It is located between the retina and the sclera and supports the outer retinal layers. The choroid receives an abundant arterial blood supply from the short and long posterior ciliary arteries and the anterior ciliary arteries (derived from the muscular branches) (Ring & Fujino, 1967). Unlike retinal vessels, choroidal vessels are not end arteries; instead, choroidal vessels anastomose with each other and are therefore interconnected. The overall structure of the choroid is segmental, where each branch of the posterior and anterior ciliary arteries supply an independent segment (Weiter & Ernest, 1974). Choroidal blood flow is under the control of the autonomic nervous system; there is no evidence of autoregulation. Drainage occurs in the same segmental fashion by a venous system that mirrors the arterial system.

The segmented, but interconnected choroidal vessels give rise to the choriocapillaris, an extensive, anastomosing network of capillaries forming the innermost layer of the
choroid (Bernstein & Hollenberg, 1965). The choriocapillaris is closely associated with the posterior surface of the retina. Bruch’s membrane, a compound basement membrane, separates the retina from the choroid. The interface between the choriocapillaris and Bruch’s membrane is an exchange site between the choroid and retina. Studies using electron microscopy observed the diffusion of substances out of the choriocapillaris lumen and towards the retina (Bernstein & Hollenberg, 1965). Furthermore, the capillary walls are polarized—the walls facing the retina are thin and fenestrated, but the walls facing the choroid are thicker and have overlapping endothelium. These characteristics facilitate the movement of fluids and dissolved solutes out of the choriocapillaris and towards the retinal tissue. In comparison, retinal capillaries have continuous endothelium and no fenestrations. In addition to nourishing the outer retinal layers, choroidal blood vessels dissipate heat generated from light focusing on the retina and metabolic processes (Parver, Auker, & Carpenter, 1980). This function maintains a steady thermal environment for retina cells.

**Blood- Retina Barrier**

When a very low rate of penetration of solutes from blood into tissue exists, it is known as a physiological barrier. In humans, this is a feature of the central nervous system and eye (Cumha-Vaz, 1978). In the eye, a blood-retina barrier (BRB) separates the retinal tissue from the blood, such that there is no free diffusion into the retina, with the exception of water and essential gases like oxygen. The BRB exists at two levels—the endothelial cells of the retinal blood vessels and the RPE layer—forming an “inner” and “outer” barrier that encapsulates the retina. At each level, tight junctions (zonulae occludentes) are found between cells, restricting free diffusion of solutes (Cumha-Vaz, 1978; Dunn, Aotaki-Keen, Putkey, & Hjelmeland, 1996). As a result, most substances cannot pass through the intercellular spaces of the endothelial cells lining the retinal blood vessels and the RPE; rather, they must pass through the cell walls by facilitated diffusion or selective active transport. By restricting the movement of molecules into the retina, an appropriate and stable environment for retinal cells is achieved.

Despite having two vascular blood supplies, the retina is very sensitive to oxygen. The retina, particularly the photoreceptors, has a high metabolic demand for oxygen (Braun, Linsenmeier, & Goldstick, 1995). Compared to the brain, the retina has an even higher rate of oxygen consumption (Anderson & Saltzman, 1964; Anderson, 1968). In a healthy individual, an adequate
oxygen supply by the retinal and choroidal blood supplies is necessary for normal retinal function. In conditions where blood supply is insufficient (oxygen demands are unmet), retinal function is impaired and damage may occur (Kaur, Sivakumar, & Foulds, 2006). In many retinal diseases, such as in DR, it is not surprising to find some form of oxygen deprivation secondary to a vascular irregularity.

1.1.3 Regionalization

The retina is extremely exciting in the sense that it has a high degree of geographic variation. In earlier sections, we saw that axially, there are discrete retinal layers composed of different retinal cells and two blood supplies that nourish inner and outer retinal layers. Additionally, the retinal layers and vascularization are not consistent across the retina. This lateral variation permits retinal regionalization by anatomical features, which is both convenient and indicative of the underlying retinal and vascular features. Anatomical regions are based primarily on the visualization of retinal structures, by en face or cross-section imaging, and to a lesser extent retinal function.

Fovea

At the center of the retina is the fovea, the retinal region responsible for high visual acuity. The foveal region has a thin bottom or pit referred to as the foveola, a 22° dip, and a thick margin (Schubert, 2009). The dip is the result of the lateral displacement of second- and third-order retinal neurons. The diameter of the fovea measures 1.5 mm and foveola measures 0.35 mm. The foveola encloses an area of densely packed and elongated central cone photoreceptors. These cone photoreceptors and their projections are enveloped by Müller cell processes. A capillary-free zone 400-500 μm in diameter, known as the foveal avascular zone (FAZ), exists in the fovea (Shimizu & Ujiie, 1978). Both the lateral displacement of retinal neurons and FAZ contribute to the high visual acuity properties of the fovea.

Parafovea

Surrounding the foveal margin is the parafovea, forming a belt that measures 0.5 mm in width (Schubert, 2009). It contains the regular retinal layers with four to six layers of GCs and seven to 11 layers of BCs.
Perifovea
Surrounding the parafovea is the perifovea, forming a belt that measures 1.5 mm in width (Schubert, 2009). It is similar to the parafovea, but the perifovea contains only several layers of GCs and six layers of BCs.

Macula
Collectively, the fovea, parafovea and perifovea form the macula, a pigmented area located temporally to the optic disc. The macular region has a diameter of approximately 5.5 mm and the major vascular arcades course along the macular border (Schubert, 2009). The major structural difference between the macula and the rest of the retina is that the macular region has several layers of GCs. Other regions only have one layer of GCs.

Periphery
The remainder of the retina is referred to as the peripheral region and can be divided into near, middle, far and extreme periphery (Schubert, 2009). The region that is near periphery is a belt 1.5 mm wide and middle periphery or equator is a belt 3 mm wide. Within the middle periphery is another major anatomical landmark, the optic disc. Located nasally to the fovea, the optic disc appears as an orange to pink vertical ellipse. GC projections collect and leave the eye at the optic disc and retinal blood vessels radiate from the optic disc. The far periphery region extends from the equator to the ora serrata, a belt approximately 6 mm wide. The extreme peripheral retina includes the ora serrata and pars plana.

1.2 Clinical Diabetic Retinopathy
The longstanding clinical view of DR has been that it is a microvascular disease of the retina secondary to diabetes. During the latter half of the 20th century, research concerned with preserving vision in individuals with diabetes found that microvascular insults were the hallmark of DR (Early Treatment Diabetic Retinopathy Study Research Group, 1991a). This established microvascular lesions as a dependable biomarker for detecting and monitoring DR (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). To this day, Ophthalmologists and Optometrists define, diagnose and manage DR more or less centered around microvascular lesions (American Diabetes Association, 2014b; Public Health Agency of Canada, 2011).
Although recent research encourages a shift in perspective, the heart of clinical practice regarding DR remains concentrated on microvascular changes in the retina. This section reviews the individuals at risk for developing and worsening DR, pathogenesis of microvascular lesions, classification of clinical DR and current treatments and their limitations. More importantly, this section defines a disease state that puts into perspective the advantage and need for earlier detection.

### 1.2.1 Individuals at Risk

Diabetes mellitus, commonly referred to as diabetes, is a metabolic disorder that sets the stage for DR. As the name suggests, individuals at risk for DR are those living with diabetes. It is defined by hyperglycemia, secondary to inadequate insulin secretion, defective insulin action or a combination (Goldenberg & Punthakee, 2013). Diabetes is associated with organ damage, dysfunction and failure, especially with the long-term development of complications in renal, neural, cardiac, vascular and visual systems (American Diabetes Association, 2014a; Goldenberg & Punthakee, 2013). In particular, chronic hyperglycemia is associated with significant long-term microvascular and macrovascular complications in the eye (Goldenberg & Punthakee, 2013). The control over hyperglycemia determines the rate of progression or worsening of DR. Generally, diabetes is classified as Type 1 diabetes, Type 2 diabetes, gestational diabetes mellitus or other specific types, including pre-diabetes.

#### Type 1 Diabetes

Type 1 diabetes (T1D), previously referred to as insulin-dependent diabetes mellitus or juvenile-onset diabetes, is a chronic, cellular-mediated, autoimmune disorder. A total of 5-10% of individuals with diabetes have T1D (American Diabetes Association, 2014a). Genetic susceptibility in combination with environmental factors are fundamental for developing T1D (Atkinson & Eisenbarth, 2001). T1D may be further sub-classified as immune-mediated diabetes, where there is selective destruction of insulin-producing pancreatic β-cells, or idiopathic diabetes, where the β-cell destruction has unknown etiology (American Diabetes Association, 2014a; Mathis, Vence, & Benoist, 2001; Tisch & McDevitt, 1996). In immune-mediated T1D, the body’s own immune system attacks and destroys the β-cells, leading to sufficient reduction and eventual elimination of insulin production (Tisch & McDevitt, 1996).
Latent autoimmune diabetes in adults is classified under T1D, where individuals present with apparent Type 2 diabetes but have immune-mediated β-cell destruction (Turner et al., 1997).

**Type 2 Diabetes**
Type 2 diabetes (T2D), previously termed non-insulin-dependent diabetes mellitus or adult-onset diabetes, is the dominant type of diabetes, comprising 90-95% of individuals with diagnosed diabetes (American Diabetes Association, 2014a). The risk for developing T2D increases with age, obesity, sedentary lifestyle and body fat in abdominal regions. The specific etiologies of T2D are not well defined, but it is associated with insulin resistance and deficiency. Unlike T1D, T2D is not associated with autoimmune β-cell destruction. Insulin resistance and deficiency associated with T2D develops gradually and may be undetected and undiagnosed for several years before symptoms manifest. In early disease, insulin and blood glucose levels could be within physiological ranges, but may be a result of an elevated basal activity from remaining, undamaged β-cells. Eventually, this compensation mechanism fails, glycemic control becomes dysregulated and hyperglycemia develops. Disease severity may range from predominantly insulin resistance with relative insulin deficiency to predominantly insufficient insulin secretion with insulin resistance (American Diabetes Association, 2014a).

**Gestational Diabetes Mellitus**
Gestational diabetes mellitus (GDM) is glucose intolerance beginning or first recognized during pregnancy (American Diabetes Association, 2014a). Approximately 7% of all pregnancies are complicated by GDM. The human gestational period is characterized by numerous metabolic changes, including a slow progression to insulin resistance (Barbour et al., 2007). Individuals that are unable to produce adequate insulin become hyperglycemic. For most individuals with GDM, glucose levels return to pre-pregnancy levels after delivery, however the risk for developing T2D is increased (Bellamy, Casas, Hingorani, & Williams, 2009). GDM in the mother may lead to perinatal complications, including higher rates of metabolic disorders, macrosomia, morbidity and mortality, especially in undiagnosed or untreated GDM (Langer, Yoge, Most, & Xenakis, 2005). Children of GDM mothers are also at higher risk for long-term complications, such as sustained impaired glucose tolerance, increased insulin resistance and obesity (Silverman, Metzger, Cho, & Loeb, 1995; Wroblewska-Seniuk, Wender-Ozegowska, & Szczapa, 2009).
Proper screening, treatment and management of GDM correct the altered metabolic environment to minimize perinatal complications.

**Other Specific Types**
Other specific types of diabetes include genetic defects involving β-cell function or insulin action, diseases of pancreatic exocrine function, endocrinopathies, drug- or chemical-induced, infections, uncommon forms of immune-mediated diabetes and other genetic syndromes sometimes associated with diabetes(American Diabetes Association, 2014a). Pre-diabetes is a term used to describe individuals at high risk for developing diabetes and associated complications(American Diabetes Association, 2014a). Individuals classified as pre-diabetic have impaired fasting glucose and impaired glucose tolerance.

**Diagnosis**
The diagnosis for diabetes is based on the relationship between glucose levels and presence of complications. Glucose thresholds for diagnosing diabetes were originally determined from associating fasting plasma glucose levels and the presence of DR in a cohort of patients with diabetes(The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Diagnostic tests measure glucose levels associated with time, such as after a period of fasting, after a standardized glucose load or a measurement independent of prandial status(The International Expert Committee, 2009). For example, the diagnosis of T1D and T2D predominantly measures glucose levels from either the fasting plasma glucose, 75 g oral glucose tolerance test or random plasma glucose(The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003; The International Expert Committee, 2009). Family history, clinical indications and lab assays can be employed to differentiate between T1D and T2D.

With wider standardization and uniformity of glycated hemoglobin (HbA1c) assays, its use for diagnosing diabetes is becoming more popular. HbA1c is produced by a non-enzymatic glycosylation process known as glycation, where glucose or another reducing sugar links to the α- and β-chains of hemoglobin A in red blood cells(Rahbar, 2005). The amount of glycation and hence level of HbA1c is dependent on the concentration of blood glucose and duration of hemoglobin exposure to blood glucose. In hyperglycemic states, where glucose concentrations
are elevated, the glycation reaction occurs more frequently and more HbA\(_1c\) products are formed. Since the average life-span of a red blood cell is three months, HbA\(_1c\) represents the average blood glucose concentration during the three months prior to the date it was measured (Bunn, Gabbay, & Gallop, 1978).

Both the American Diabetes Association (ADA) and International Expert Committee, consisting of members appointed by the ADA, the European Association for the Study of Diabetes and the International Diabetes Federation, recommend its use, where an HbA\(_1c\) ≥6.5% is diagnosed as diabetes (American Diabetes Association, 2014a; The International Expert Committee, 2009). The diagnostic thresholds of fasting plasma glucose, oral glucose tolerance test and HbA\(_1c\) are highly correlated.

**Management**

The primary management strategy for diabetes is controlling hyperglycemia. This can be achieved by exogenous insulin supplementation (The Diabetes Control and Complications Trial Research Group, 1993). The hormone insulin is responsible for regulating the metabolism of carbohydrates and fats and promotes glucose absorption from the blood into tissues for storage or energy. Intensive diabetes therapy regimens include the delivery of exogenous insulin three or more times daily by insulin injections or insulin pump. The aim is to promote tight glucose control and achieve blood glucose levels in individuals with diabetes as close to the range of individuals with no diabetes. This strategy reduces the risk of developing and amplifying diabetes-related complications (The Diabetes Control and Complications Trial Research Group, 1993). In a study of individuals with T2D with no or simple diabetes-related complications, improved glycemic control delayed the development and progression of diabetic retinopathy, nephropathy and neuropathy (Ohkubo et al., 1995). However, intensive diabetes therapies are limited by the degree of insulin resistance, but other agents that control hyperglycemia, reduce hepatic gluconeogenesis or increase insulin sensitivity can be substituted (UK Prospective Diabetes Study Group, 1998a, 1998b).

**Glycated Hemoglobin**

In addition to the diagnosis of diabetes, HbA\(_1c\) is also widely used to evaluate glucose control (American Diabetes Association, 2014a). Measuring HbA\(_1c\) is critical in the management
of diabetes, since it is highly correlated with both long-term microvascular and, to a lesser extent, macrovascular complications (Stratton et al., 2000; The Diabetes Control and Complications Trial Research Group, 1995). HbA₁c measurements reflect the adequacy of diabetes treatment and determine if adjustments in therapy are required. The recommended target for HbA₁c in adults with T1D or T2D and adolescents with T1D is ≤7.0% (The Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013a, 2013b).

**Epidemiology**

In 2010, the global prevalence of diabetes among adults aged 20-79 years was 6.4% or 285 million people (Shaw, Sicree, & Zimmet, 2010). By 2030, the global prevalence of diabetes among adults is estimated to reach 7.7% or 439 million people. By the end of 2010 in Canada, 9.2% or 2.9 million Canadians were living with diagnosed diabetes, and projected to reach 11.6% or 4.0 million Canadians by 2030. In 2011, the Public Health Agency of Canada reported an incidence rate of 0.6% or approximately 0.2 million Canadians diagnosed with diabetes for the first time, based on 2008 and 2009 reports (Public Health Agency of Canada, 2011). In the United States of America (USA) in 2010, about 10.3% or 26.8 million Americans were living with diagnosed diabetes and estimated to reach 12.3% or 36.0 million Americans by 2030 (Shaw et al., 2010). In 2012, the Centers for Disease Control and Prevention reported an incidence rate of 0.5% or approximately 1.7 million Americans diagnosed with diabetes for the first time (Centers for Disease Control and Prevention, 2014). Diabetes is a worldwide epidemic that will continue to grow in coming years.

In 2007, the estimated economic burden of diabetes was approximately US$174 billion in the USA alone, accounting for US$116 billion in medical expenditures to care for individuals with diabetes, diabetes-related complications and general medical expenses and US$58 billion as a consequence of reduced national productivity (American Diabetes Association, 2008). Individuals living with diabetes, on average, have annual medical expenditures 2.3 times or US$6600 more than individuals with no diabetes. These costs are an underestimate as they are exclusive of intangible costs or costs unattainable for cost analysis, such as expenses incurred from over-the-counter medications, higher insurance premiums for employers and employees, improving a lower quality of life and non-paid family, friends and caregivers. Diabetes is a
significant economic burden for the individuals affected and the healthcare system. The strain on society will continue to build.

Diabetes is a relatively common, persistent and costly disease that affects individuals, their surrounding community and the whole society. It is important to note that diabetes is an overall systemic shift in metabolism, which renders individuals with diabetes more susceptible to a cascade of complications. With increasingly more individuals diagnosed with diabetes each year and given that diabetes is a prerequisite for developing DR, one cannot ignore the breadth of DR and value in earlier detection and management options.

1.2.2 Pathogenesis

Multiple interconnecting biochemical pathways contribute to the pathogenesis of clinical DR. These include an activated polyol pathway, an accelerated formation of advanced glycation end-products, an activated protein kinase C pathway, hemodynamic changes, an activated renin-angiotensin-aldosterone system, chronic subclinical inflammation and an increased expression of growth factors. Under physiological conditions, these biochemical pathways are well-regulated, but they become detrimental to the retina during chronic hyperglycemia in diabetes. These pathways link chronic hyperglycemia in diabetes with the development of microvascular lesions in clinical DR.

Polyol Pathway

The polyol pathway is hyperactive in diabetes(Gabbay, 1975). Aldose reductase, an enzyme present in retinal cells, reduces glucose to sorbitol, a sugar alcohol that is impermeable to cell membranes and metabolized slowly by retinal cells. For this reaction to occur, the cofactor NADPH is used. Subsequent metabolism of sorbitol to fructose is carried out by sorbitol dehydrogenase in the presence of cofactor NAD+. In hyperglycemic conditions, where glucose is in excess, there is an overproduction of sorbitol and fructose and depletion of NADPH and NAD+. Sorbitol accumulates within the cell because it is metabolized slowly and cannot leave the cell. As a result, the retina experiences cellular osmotic damage(Gabbay, 1973). The elevated fructose levels contribute to the production of toxic advanced glycation end-products(Szwergold, Kappler, & Brown, 1990). The depletion of NADPH decreases the production of reduced glutathione, a major endogenous antioxidant. Low levels of glutathione decrease the capability of
retinal cells to scavenge oxidants and protect against oxidative stress (Barnett, Gómezalez, Chylack, & Cheng, 1986). The depletion of NAD+ stimulates NADH oxidase, which augments oxidative stress within the retina (Lassègue & Clempus, 2003). All these adverse events have been localized to vascular endothelial cells, pericytes, Müller cells and RPE cells in the retina (Chakrabarti, Sima, Nakajima, Yahihashi, & Greene, 1987; Hohman, Nishimura, & Robison, 1989; W. Li, Chan, Khatami, & Rockey, 1986). Consequently, the retinal vasculature weakens, thickens and becomes more permeable and the integrity of the BRB is disrupted (Cheung et al., 2005; Miwa et al., 2003).

**Advanced Glycation End-Products**

In diabetes, the generation of advanced glycation end-products (AGEs) is accelerated. AGEs are the product of reducing sugars reacting non-enzymatically with the free amino groups of proteins, lipids and nucleic acids (Bierhaus, Hofmann, Ziegler, & Nawroth, 1998). When this reaction occurs, a series of intermediate products are generated that ultimately convert to AGEs. In a healthy individual, AGEs form at a constant rate and accumulate over time, but in individuals with diabetes, their formation is accelerated because elevated glucose levels constantly feed the reaction. Each of the intermediate products can be toxic to retinal cells, but AGEs form covalent crosslinks between proteins. The crosslinks alter protein structure and function. Protein targets include cellular matrices, basement membranes and blood vessel wall components. AGEs may also bind to cell surface receptors, such as the receptor for advanced glycation end-products (RAGEs), and induce oxidative stress and inflammation. Accelerated AGEs formation in individuals with diabetes is associated with abnormal endothelial cell proliferation, increased acellular retinal capillaries, capillary closure and formation of microaneurysms (Hammes, Martin, Federlin, Geisen, & Brownlee, 1991).

**Protein Kinase C**

Activation of the protein kinase C (PKC) pathway in the retina has several adverse effects. PKC is a family of protein kinase enzymes involved in many transduction events. The PKC family consists of 10 isoforms, but the PKC-β isoform is particularly related to the pathogenesis of DR (L. Aiello et al., 2006; Ishii et al., 1996; Nonaka et al., 2000). The key activator of PKC is diacylglycerol (DAG), which is synthesized de novo through the glycolysis pathway. In diabetes, hyperglycemia drives the glycolysis pathway and synthesis of DAG, which leads to increased
transduction events via PKC (Go, Sekiguchi, Nomura, Kikkawa, & Nishizuka, 1987). This DAG-PKC interaction has been shown to occur in the retina, where it regulates vascular functions, including endothelial permeability, contractility, extracellular matrix synthesis and turnover, cell growth, angiogenesis, cytokine actions and leukocyte adhesion (Shiba et al., 1993; Xia et al., 1994). However, under hyperglycemic conditions, PKC events are associated with hemodynamic changes, capillary occlusion and inflammation.

**Hemodynamic Changes**

Individuals with diabetes with poorly controlled hyperglycemia experience unfavourable hemodynamic changes. Hemodynamics refer to the fluid dynamics of blood flow in the circulatory system and is influenced by blood pressure and viscosity. Healthy retinal blood vessels, which have no sympathetic control and are dependent on local autoregulation for blood flow regulation, constrict to maintain constant blood flow in response to higher blood pressure. In individuals with diabetes, hyperglycemia increases blood pressure by altering blood flow, viscosity and shear stress (Kohner, 1993). Initially, local autoregulation of blood flow is sufficient, but eventually, it becomes impaired and retinal vessels suffer damage (Clermont, Aiello, Mori, Aiello, & Bursell, 1997; Grunwald, Riva, Sinclair, Brucker, & Petrig, 1986). Individuals with diabetes with poorly controlled blood pressure have accelerated progression and worsening of DR compared to individuals with tighter control. Those with poorly controlled blood pressure develop more microaneurysms, hard exudates and cotton wool spots and have increased rates of progression to advanced stages of DR (Matthews, Stratton, Aldington, Holman, & Kohner, 2004).

**Renin-Angiotensin-Aldosterone System**

The elevated blood pressure in diabetes may be a result of chronic activation of the renin-angiotensin-aldosterone system (RAAS). As part of the endocrine system, RAAS has a major role in regulating blood pressure and fluid balance. It is an enzymatic cascade that begins with the conversion of inactive enzyme pro-renin to the active enzyme renin, which facilitates the production of angiotensin I from angiotensinogen (Wilkinson-Berka, 2006). The subsequent actions of angiotensin-converting enzyme I (ACE-I) liberate angiotensin II from angiotensin I. Angiotensin II can act on adrenal glands to increase secretion of aldosterone, a steroid hormone that increases water retention in the kidneys by actively reabsorbing sodium ions. Angiotensin-
converting enzyme II (ACE-II), a homologue of ACE-I, acts on angiotensin II to form other angiotensin signaling molecules. In diabetes, there is an upregulation of renin production and angiotensin signaling molecules, which drives the retention of fluids and increases blood pressure (Franken et al., 1988; Luetscher, Kraemer, Wilson, Schwartz, & Bryer-Ash, 1985).

**Subclinical Inflammation**

A major component of DR pathogenesis is subclinical inflammation, which refers to a low-grade inflammation in the retina that in other tissues would not be considered clinically relevant. While many of the biochemical pathways involved in DR pathogenesis lead to inflammation, the inflammatory response itself exaggerates these pathways. In the retinas of individuals with diabetes, there is increased expression and activation of pro-inflammatory factors, such as cytokines, adhesion molecules and immune cells (Doganay et al., 2002; Hernandez et al., 2005). The levels of these pro-inflammatory factors are correlated with the progression of DR. Not surprisingly, the levels of anti-inflammatory factors, such as anti-inflammatory cytokines, remain unresponsive (Hernandez et al., 2005). Crosstalk between pro-inflammatory factors also contributes to leukostasis, a process by which leukocytes aggregate within blood vessels. In diabetes, there is increased endothelial cell-surface expression of adhesion molecules that promote the binding of leukocytes to vessel walls (Takami, Yamashita, Kihara, Kameda-Takemura, & Matsuzawa, 1998). This aggregation increases blood viscosity, which contributes to capillary occlusion, reactive oxygen species-mediated retinal cell death and amplifies the existing local inflammatory response (Larson & Springer, 1990).

Subclinical inflammation also activates microglia, the resident immune cells of the retina, and other retinal macrophages and immune cells. When there are disturbances to the retinal environment, microglia are activated and secrete cytokines, chemokines and neurotoxins (Langmann, 2007). Typically, these events promote retinal neuroprotection, tissue regeneration and acute inflammation. However in diabetes, their chronic activation amplifies the immunological response and promotes retinal cell damage and apoptosis. In diabetes, microglia activation in the retina boost the production of pro-inflammatory cytokines, reactive oxygen species, growth factors, matrix metalloproteinases and nitric oxides, all of which encourage subclinical inflammation.
**Growth Factors**

Important contributors to the pathogenesis of clinical DR are growth factors. Normally, growth factors are mediators of cell growth, differentiation and transformation. However, in diabetes the expression of growth factors is elevated, which leads to adverse microvascular changes and worsening of DR. In particular, the growth factors insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) are elevated in individuals with diabetes (Boulton et al., 1997; Meyer-Schwickerath et al., 1993). Retinal ischemia is a strong stimulus for the production of IGF-1 and results from other biochemical pathways involved in DR (Meyer-Schwickerath et al., 1993). In animal models, intravitreal injection of IGF-1 causes vascular tortuosity, hemorrhage and hyperemia in early stages and retinal capillary basement membrane thickening, BRB disruption and neovascularization in later stages (Grant et al., 1993). For maximal neovascularization, IGF-1 interacts with VEGF, which promotes the growth of new blood vessels (Smith et al., 1999). In the presence of retinal hypoxia, VEGF expression promotes the growth of new blood vessels, such that retinal hypoxia is reduced and tissue oxygenation is sufficient (Shweiki, Itin, Soffer, & Keshet, 1992). However, atypical elevated expression of VEGF promotes endothelial cell growth, neovascularization, vascular permeability and BRB disruption (Bates & Curry, 1997).

**1.2.3 Classification by Pathophysiology**

The classification of DR is based on vascular lesions in the retina. In 1968, an international group of clinicians and researchers gathered at the Airlie House Convention to determine methods to preserve vision in individuals with diabetes. A set of standard photographs illustrating the degrees of DR was established and became known as the Airlie House Classification of Diabetic Retinopathy, which in 1981, was modified by the Diabetic Retinopathy Study (Diabetic Retinopathy Study, 1981). In 1991, it was extended by the Early Treatment Diabetic Retinopathy Study (ETDRS) to improve DR grading (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). Today, DR is divided into two major forms, non-proliferative and proliferative, based on the absence or presence of irregular neovascularization in the retina (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). Each form is further classified by severity.
Non-Proliferative Diabetic Retinopathy

In non-proliferative diabetic retinopathy (NPDR), there is a variable display of microvascular lesions, including dilated or tortuous vessels, occluded vessels, microaneurysms, intraretinal hemorrhages, hard exudates and cotton wool spots. These findings manifest primarily in the macula and posterior retina. NPDR can be further classified as mild, moderate, severe and very severe based on the presence and combination of microvascular lesions (L. M. Aiello, 2003). It is important to note that the rate of progression of DR severity is not linear. The one-year risk of progression to proliferative DR from mild and moderate NPDR are 5 and 20%, respectively, whereas from severe and very severe are 52 and 75%, respectively (Early Treatment Diabetic Retinopathy Study Research Group, 1991). The severity of NPDR is an indicator of the rate of progression of DR and impacts the frequency of clinic visits and eligibility for different forms of treatment strategies.

Proliferative Diabetic Retinopathy

Proliferative diabetic retinopathy (PDR) is marked by neovascularization arising from the optic disc and/or retinal blood vessels (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). Neovascularization may lead to preretinal and vitreous hemorrhage, subsequent fibrosis and traction retinal detachment. PDR can exist with prior or coexisting severe NPDR or without substantial NPDR. PDR is further classified as early, high-risk and severe based on the severity of vessel proliferation. In early PDR, there are new vessels in the form of fine loops or networks. In high-risk PDR, there is neovascularization of the optic disc greater than one-third to one-half the optic disc area; neovascularization of the optic disc and preretinal and vitreous hemorrhage; or neovascularization elsewhere greater than half the optic disc area with vitreous or preretinal hemorrhage. The five-year risk of progression from early to high-risk PDR is 75% (Early Treatment Diabetic Retinopathy Study Research Group, 1991a). Severe PDR includes posterior fundus obscuring by preretinal or vitreous hemorrhage, or macular detachment. Acute vision loss in PDR may occur if blood leaks from the new blood vessels into the vitreous because the blood blocks light from reaching the retina. More permanent vision loss may occur by retinal detachment, macular ischemia or a combination.
**Macular Edema**

Macular edema (ME) is defined as retinal thickening and edema involving the macula, resulting from capillary leakage (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). Unfortunately, ME can develop at any stage of DR and is the primary cause of vision loss in NPDR, presenting as a gradual blurring of vision. ME can be observed using stereoscopic fundus examination, fluorescein angiography and most directly by optical coherence tomography. The term clinically significant macular edema (CSME) refers to retinal thickening within 500 µm of the fovea; hard exudates within 500 µm of the fovea if associated with adjacent retinal thickening; or one or more areas of retinal thickening at least 1500 µm in diameter and within 1500 µm (one optic disc diameter) of the fovea.

### 1.2.4 Current Therapies & Limitations

Currently, there are limited therapies for clinical DR. These include laser photocoagulation and anti-VEGF medication. Laser photocoagulation is available for individuals with high-risk PDR, CSME and some cases of NPDR. An argon laser is used to heat seal irregular, leaking blood vessels. In the Diabetic Retinopathy Study, scatter (pan-retinal) photocoagulation reduced the risk of severe vision loss from PDR by 9.5% (The Diabetic Retinopathy Study Research Group, 1976). In the ETDRS, focal laser photocoagulation therapy was beneficial for eyes with CSME (Early Treatment Diabetic Retinopathy Study Research Group, 1985). The ETDRS also reported that scatter photocoagulation was beneficial for individuals with high-risk PDR or older-onset individuals with severe NPDR or less than high-risk PDR. Both studies established laser photocoagulation as beneficial for certain patients. However, laser photocoagulation is only useful for a subset of individuals with advanced DR. Furthermore, it only reduces the risk of further vision loss and does not restore vision already lost.

Anti-VEGF medication reduces the risk and reverses the progression of DR. Anti-VEGF medications are recombinant monoclonal neutralizing antibodies to VEGF. Since VEGF has major roles in endothelial cell growth, neovascularization and vascular permeability, it is an appropriate target for inhibition. More information regarding anti-VEGF medications can be found in a 2010 review by Nicholson and Schachat (Nicholson & Schachat, 2010). So far, the neutralizing effects of anti-VEGF medications have short-term and temporary ameliorations to vision loss and CSME (Nguyen et al., 2012; Nicholson & Schachat, 2010). Furthermore, anti-
VEGF medications are not currently approved by Food and Drug Association (FDA) for full use in DR. Other potential drug therapies include sustained intravitreal fluocinolone acetonide (steroid agent) and fenofibrate (lipid-lowering agent) to reduce the progression of DR (Keech et al., 2007; Pearson et al., 2011).

The complexity of DR has required several decades of joint investigation in order to understand the etiology of clinical DR. In diabetes, chronic hyperglycemia disturbs the physiology of many interconnected biochemical pathways. From a medical research perspective, research is intended to generate knowledge and impact clinical practice. In the story of clinical DR, the observations of microvascular lesions led to the association of hyperglycemia and affected biochemical pathways to DR and the development of treatments to minimize the impact of DR on vision loss. The core of this research project has a similar story to the elucidation of clinical DR, but it begins at an earlier time point: the observation of retinal neurodegeneration preceding microvascular lesions.

1.3 Preclinical Diabetic Retinopathy

Preclinical DR is an active area of DR research that evolved with new insights and technology. Preclinical DR embodies the effect of diabetes and hyperglycemia on the retina, before the development of microvascular complications. In other words, preclinical DR is concerned with an earlier neurodegenerative stage of DR—after the onset of diabetes, but before clinically-relevant DR. This section reviews the novel concept of retinal neurodegeneration in preclinical DR and the supporting functional and morphological evidence.

1.3.1 Neurodegeneration

Retinal neurodegeneration is a progressive degenerative process in the retina induced by diabetes. It is different than retinal microvascular complications in that neurodegenerative changes occur in early NPDR or even earlier, during which microvascular complications are undetected. Major pathways involved with retinal neurodegeneration include oxidative stress, glutamate homeostasis and expression of neuroprotective factors. There are likely additional pathways involved, but are yet to be associated with preclinical DR. It is important to note that these neurodegenerative pathways develop into or contribute to the pathogenic pathways in
clinical DR. But like clinical DR, preclinical DR arises from a diabetic and hyperglycemic environment.

**Oxidative Stress**

Oxidative stress is a major contributor to neurodegeneration in preclinical DR. It is defined as an imbalance between the abundance of reactive oxygen species (ROS) or oxygen radicals and antioxidant defenses. ROS such as superoxide anion and hydrogen peroxide are toxic to cells because of their high reactivity. ROS can be generated from enzymatic processes, such as oxidation, oxygenation and metal catalysis. Under physiological conditions, ROS are eliminated by endogenous reducing and sequestering agents, such as glutathione, or specific enzymes, such as manganese superoxide dismutase, that exert compensative actions. The extent to which this occurs is surpassed in diabetes, where hyperglycemia renders the compensative efforts insufficient. The overproduction of ROS precursors or insufficient transformation, inhibition or scavenging of ROS are responsible for elevated ROS levels (Cui et al., 2006). ROS target macromolecules such as lipids, proteins, nucleic acids and glycoconjungates and precipitate chemical modifications such as oxidation, fragmentation, crosslinking and fluorescence. These modifications damage and disrupt retinal cell function and morphology. In some cases, these modifications may even grant reversal-resistant properties to DR after establishing glycemic control (Kowluru, 2003).

The primary source of ROS in the retina is produced by the mitochondria (Cui et al., 2006). In diabetes, hyperglycemia stimulates an overproduction of mitochondrial ROS (mtROS). The electron transport chain at the site of the inner mitochondrial membrane is essential to the production of ATP energy (Du et al., 2000). Normally, manageable amounts of mtROS are generated. However, the elevated glucose levels in diabetes drive glycolysis and the citric acid cycle, producing an overabundance of reactants that feed into the electron transport chain. As a result, increased levels of mtROS are produced. The production of mtROS may be reduced by mitochondrial transporters, such as uncoupling proteins, at the inner mitochondrial membrane.

Due to the high reactivity of ROS and elevated local production within the mitochondria, a major target for mtROS-inflicted damage is mitochondrial DNA (mtDNA). mtDNA are the first to be exposed and damaged by mtROS, and may cause mitochondrial ATP production to drop below
physiological cellular requirements (Jarrett, Lin, Godley, & Boulton, 2008). For example, the RPE is a highly metabolically active retinal layer responsible for retinal homeostasis. Reasonably, the RPE has a high density of mitochondria. In diabetes, the chronic exposure to elevated mtROS damages mtDNA, mitochondria become dysfunctional and the RPE does not have a sufficient source of ATP energy. As a result, the activities of the RPE and retinal cells that it supports deteriorate. Overall, the chronic damage by ROS on the retina leads to neurodegenerative loss of both retinal cells and their functions. ROS breakage of DNA activates a chain of events that accumulate glycolytic metabolites, which go on to activate the polyol pathway, form AGEs, activate the PKC pathway, induce subclinical inflammation and other biochemical pathways, which all contribute to clinical DR (Brownlee, 2005). Oxidative stress is an important factor to consider in terms of retinal neurodegeneration in preclinical DR.

Glutamate

In diabetes, glutamate homeostasis in the retina is disrupted. Glutamate is the major excitatory neurotransmitter used for signaling between photoreceptors and BCs. In diabetes, there is an accumulation of extracellular glutamate, referred to as glutamate excitotoxicity (Ambati et al., 1997; Kowluru, Engerman, Case, & Kern, 2001; E Lieth et al., 1998; Erich Lieth, LaNoue, Antonetti, & Ratz, 2000; Pulido et al., 2007). The higher concentration of extracellular glutamate is due to a decrease in glutamine synthetase, a Müller cell-specific enzyme that recycles glutamate to glutamine (E Lieth et al., 1998; Erich Lieth et al., 2000); a reduction in retinal glutamate oxidation to alpha-ketoglutarate (Kowluru et al., 2001); and a decrease in Müller cell function, which typically contributes to glutamate uptake (Q. Li & Puro, 2002). Excess glutamate in the extracellular and synaptic spaces leads to chronic activation of iGluRs and uncontrolled intracellular calcium signaling in retinal cells. Taken together, in preclinical DR, the effect of glutamate excitotoxicity in the retina leads to uncontrolled cell death.

Neuroprotective Factors

Another pathway for retinal neurodegeneration is the loss of neuroprotective factors synthetized by the retina. Neuroprotective factors protect the retina against neurotoxic elements and repair damage. Pigment epithelial-derived factor (PEDF), somatostatin (SST) and interstitial retinol-binding protein (IRBP) are important neuroprotective factors in the retina. PEDF and SST are both synthesized by the RPE (Barnstable, 2004; Carrasco et al., 2007). PEDF protects retinal
neurons from light damage, oxidative stress and angiogenesis (Barnstable, 2004). It also protects against glutamate excitotoxicity because it controls Müller cell glutamine synthetase expression (Shen, Xie, Cheng, Jiao, & Zhong, 2011). SST acts as a neuromodulator and anti-angiogenic factor in the retina (Carrasco et al., 2007). IRBP is a glycoprotein that is synthesized by photoreceptors and is important for the visual cycle and photoreceptor maintenance (García-Ramírez et al., 2009). In preclinical DR, all of these neuroprotective factors are down-regulated and their neuroprotective and anti-angiogenic properties absent (Barnstable, 2004; Carrasco et al., 2007; García-Ramírez et al., 2009). Down-regulation of neuroprotective factors in the retina compromise neuroprotection in favour of neurodegeneration.

1.3.2 Manifestation

Neurodegenerative pathways in preclinical DR may manifest as functional or morphological irregularities in the retina. In every case of human DR, some form of retinal neurodegeneration is present. This applies to when vision loss in diabetes was first studied in 1935 and even when microvascular lesions were analyzed at the Airlie House Convention in 1968. Neurodegeneration has always been associated with DR, but for a long time was undetected and unknown. Neurodegenerative manifestations are not detectable by ophthalmoscopy. With the development of new technologies, improvement of existing technologies or use of pre-existing technologies in novel applications, functional and morphological irregularities in the retina in preclinical DR were exposed.

1.3.2.1 Functional Irregularities

Retinal functional irregularities in preclinical DR have been studied using measures of retinal activity. As discussed in earlier sections, neuronal retinal cells process, encode and relay visual input to higher brain centers and non-neuronal retinal cells maintain retinal physiology and support other activities. Therefore, measuring retinal cell function in light of diabetes may provide insights to functional changes. This section covers the application of electroretinography and neurovascular coupling to the study of functional loss in the retina during preclinical DR.

**Electroretinography**

Electroretinography (ERG) is an electrophysiological technique used to measure the electrical responses of retinal cells. Different retinal cells are studied depending on the ERG stimulus. In
pattern-ERG (PERG), electrical responses are correlated with GC activity. This correlation provides a direct index of inner retinal function. In preclinical DR, the PERG is irregular and suggests early sensory deficit in the inner retinal layers(MAS Di Leo et al., 1990). A longitudinal study of preclinical DR found irregular photoreceptor and GC activity using flicker-ERG and PERG, respectively(M Di Leo et al., 1994). Initially, there was a loss of photoreceptor and GC activity, but after 3 years, dysfunction was significantly greater in photoreceptors.

More recent studies looking at delayed implicit time using multifocal-ERG (mfERG), a technique used to simultaneously record local ERG responses from different regions of the retina, associated local functional irregularities in preclinical DR with predicting the development of microvascular lesions in clinical DR(Bearse et al., 2006; Harrison et al., 2011; Tan, Wright, Dupuis, Lakhani, & Westall, 2014). Local reduction of multifocal oscillatory potentials (mfOPs) using slow-flash mfERG, a measure of AC activity, suggested early AC dysfunction and implicates the functional loss of photoreceptors and BCs(Bearse et al., 2004).

**Neurovascular Coupling**

Another useful measure of retinal function is neurovascular coupling induced by flicker-light stimulation. Neurovascular coupling is the physiological association between neural activity and retinal blood flow. As retinal activity increases, metabolic demands rise and retinal blood vessels dilate to increase the delivery of nutrients and removal of wastes. The retinal blood vessels are able to respond to metabolic demands because of local autoregulation of blood flow. Conveniently, flicker-light stimulation, which consist of intermittent flashes, is a strong inducer of retinal activity and blood vessel dilation(Formaz, Riva, & Geiser, 1997). Using this technique, it was shown that in preclinical DR, neurovascular coupling is reduced even when blood pressure is controlled(Lecleire-Collet et al., 2011).

Investigating retinal function by ERG and neurovascular coupling provides sensitive measurement of functional irregularities in progressive diabetes. Although these techniques are not novel, their improvement and application to preclinical DR have revealed functional loss in corresponding retinal cells.
1.3.2.2  **Morphological Irregularities**

In preclinical DR, morphological irregularities have been observed as a manifestation of retinal neurodegeneration. As discussed earlier, retinal cells are organized into discrete retinal layers. In preclinical DR, the thicknesses of the retinal layers may experience irregular thinning or thickening. Retinal thicknesses can be measured from cross-section scans of the retina using optical coherence tomography imaging. Because of the variation across the retinal landscape, retinal thicknesses are measured from different regions. This section covers the application of optical coherence tomography to visualize retinal layers, an ETDRS approach to regionalizing the retina and retinal thickness findings from recent investigations.

**Optical Coherence Tomography**

Optical coherence tomography (OCT) is used to study ocular morphology. Its popularity arose from its ability to visualize ocular structures instantaneously with high-resolution *in vivo*. In the case of preclinical DR, OCT is especially useful for visualizing retinal thickness morphology. The basic principle of OCT extends from low-coherence interferometry, a technique originally used in Ophthalmology for measuring the axial length of the eye (Fercher, Mengedoht, & Werner, 1988). Low-coherence interferometry requires an initial light beam to be split and sent to a reference arm with a mirror and a tissue sample. If both reflected beams recombine with equal path length within the coherence length of the light source, then a strong interference occurs. The interference pattern is important for creating a reflectivity profile, which gives intensity information. The intensity information is used to construct the OCT images (Figure 1).
The practicality of OCT for clinical use has advanced tremendously. The first commercial OCT system available for clinical use was time-domain OCT (TD-OCT). The TD-OCT has a reference mirror that shifts locations in order to detect intensity information at different axial depths of the tissue sample. An axial scan, or A-scan, is the collection of depth scans at a single location. However, the shifting reference mirror was a physical constraint of the TD-OCT system in that it limited the scanning speed. Today, the spectral-domain OCT (SD-OCT) is the preferred OCT system. SD-OCT uses broadband light sources and frequency-domain signal detection to significantly enhance axial resolution and scanning speeds, respectively (Drexler et al., 2001). The improved sensitivity and speed in SD-OCT allow for 3D volumetric scans of the retina by combining adjacent B-scans, which are a linear series of A-scans (Figure 2). Altogether, OCT has become a prevailing imaging technique to obtain high-resolution cross-section or 3D volumetric scans of the retina with a resolution of 2 μm axially or 47 μm laterally.
ETDRS Regions

The use of ETDRS regions to describe different areas of the retina has emerged in studies of retinal thickness irregularities in preclinical DR. The ETDRS regions were first designed for grading fluorescein angiograms and subsequently applied to grading retinal thickness and hard exudates in field 2 in 7-field stereoscopic color fundus photographs by the ETDRS research group (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). This grid design consists of a central circular foveal region with a 1 mm diameter, a pericentral region with an inner and outer diameter of 1 and 3 mm, respectively, and a peripheral region with an inner and outer diameter of 3 and 6 mm, respectively (Figure 3). The pericentral and peripheral regions are subdivided into four regions: superior, temporal, inferior and nasal regions. The pericentral and peripheral regions may be referred to as “inner” and “outer” regions, respectively. Altogether, there are nine ETDRS regions: inner superior (S_i), inner temporal (T_i), inner inferior (I_i), inner nasal (N_i), outer superior (S_o), outer temporal (T_o), outer inferior (I_o), outer nasal (N_o) and foveal regions.

Figure 2. Relationship between a volumetric scan, B-scan and A-scan. A volumetric scan is composed of laterally adjacent B-scans, which are a linear series of axial A-scans.
Figure 3. The Early Treatment Diabetic Retinopathy Study grid design. The foveal area is a central circular zone 1 mm in diameter, an inner ring area with an inner and outer diameter of 1 and 3 mm, respectively, and an outer ring area with an inner and outer diameter of 3 and 6 mm, respectively. Diagonals that transect the inner and outer ring areas form superior (S), temporal (T), inferior (I), and nasal (N) quadrants, differentiated by “i” and “o” subscripts for inner and outer ring areas, respectively.

It is important to note that ETDRS regions do not superimpose exactly with anatomical regions. Additionally, the anatomical regions are variable across individuals. Therefore, the central foveal region approximates the fovea, the pericentral region approximates the parafovea and the peripheral region approximates the perifovea. The anatomical peripheral retinal areas are not covered by the ETDRS regions. Despite it only being an approximation, the ETDRS regions are currently our best tool to describe different retinal regions without compromising practicality, standardization and consistency in research investigations.

**Retinal Thickness**
Retinal thickness irregularities are irregular thinning and/or thickening within a retinal layer. On a cellular level, retinal cells change shape and form in response to a chronic change in environment. As retinal cells undergo degeneration in preclinical DR, they may become atrophic or hypertrophic. On a grosser level, these cellular changes can be observed as retinal layer
thinning and/or thickening using OCT imaging. Depending on how it is defined, retinal thickness is the thickness of individual retinal layers, two or three retinal layers combined or all the retinal layers combined as one total retinal thickness. The retinal thickness may be measured from a single location or an average of measurements from multiple locations. This section covers the approaches of different research groups and their findings. A summary can be seen in Table 1.

One of the first investigations of retinal thickness irregularities in preclinical DR was in 2007 by Biallosterski et al. (Biallosterski et al., 2007). This observational cross-sectional study compared total retinal thickness between middle-aged adults with T1D with no DR, T1D with minimal DR, and healthy controls. Minimal DR was defined as no more than the presence of two or more microaneurysms, minor hemorrhages or a combination, in the central retina. Using TD-OCT, six B-scans were collected from each individual. The B-scans were 6 mm in length, radially spaced at 30° orientations and centered on the fovea. The mean total retinal thickness was measured in four regions: the foveal, pericentral and peripheral ETDRS regions and a central foveal point where the B-scans intersected. Unpaired Student’s t-test comparisons found total retinal thinning in the pericentral region in individuals with T1D with minimal DR. These findings were replicated with the same participants four months later.

A few years later, van Dijk et al. conducted a series of similar investigation with the objective of identifying which set of retinal layers contributed to the total retinal thinning observed by Biallosterski et al. Their 2009 investigation followed the same protocol as Biallosterski et al. with minor differences (van Dijk et al., 2009). Minimal DR was defined as the presence of at least one microaneurysm or hemorrhage in the central retina. Rather than measuring only total retinal thickness, six additional retinal layers were defined, which included the RNFL, GCL/IPL, INL, OPL, ONL/ISL and OSL. The same foveal, pericentral and peripheral ETDRS regions were studied but the central foveal point was omitted. Using analysis of variance (ANOVA) with Bonferroni correction, total retinal thinning was observed in the pericentral region of individuals with T1D with minimal DR. In the same region and participant group, the GCL/IPL and INL were thinner. However, the GCL/IPL was also thinner in the peripheral region, even though there were no total retinal thickness differences in the same region. The primary conclusion was that the selective thinning of total retinal thickness in the pericentral region was attributed to thinning of the inner retinal layers (GCL, IPL and INL).
In 2010, van Dijk et al. continued their investigations and identified which specific retinal layer contributed the most to the inner retinal layer thinning (van Dijk et al., 2010). The only considerable distinction from their previous investigation was the use of SD-OCT imaging instead of TD-OCT. Because of the improved speed and sensitivity in SD-OCT imaging, their scan area and retinal layers were modified. Instead of six radial B-scans, a 3D volumetric retinal scan centered on the fovea and measuring 6 x 6 x 2.2 mm was collected from each participant. A 3D graph-search algorithm was used to define the total retinal thickness, along with 8 retinal layers, which included the RNFL, GCL, IPL, INL, OPL, ONL/ISL, OSL and RPE. Rather than measuring the mean retinal thickness in three regions, only the pericentral and peripheral regions were analyzed. With the same statistical analysis, there was thinning in the GCL and RNFL in the pericentral and peripheral regions, respectively, in individuals with T1D with minimal DR. The primary conclusion was that GCL thinning contributed the most to inner retinal layer thinning in the pericentral region and that peripheral RNFL thinning was an extension of GCL degeneration.

Another group, Vujosevic et al., investigated retinal thickness with greater regional resolution than either Biallosterski et al. or van Dijk et al. (Vujosevic & Midena, 2013). Unfortunately, components of their publication are unclear and inconsistent. Using SD-OCT, individuals with diabetes with no DR, diabetes with early DR and controls were compared. Early DR was defined as mild to moderate NPDR. The diabetes groups were non-homogeneous; approximately ¼ were individuals with T1D and ¾ with T2D. This investigation used macular and peripapillary (optic disc) protocols to image a macular area measuring 6 x 6 mm centered on the fovea and a circular area with a 3.46 mm diameter centered on the optic disc. An automatic segmentation algorithm (publication does not describe or reference the protocol) measured total retinal thickness, along with 5 retinal layers, which included ILM/RNFL, GCL/IPL, INL/OPL, ONL/ELM and ISL/OSL/RPE. In the macular and peripapillary areas, mean retinal thicknesses were calculated in all nine ETDRS regions and four regions (superior, temporal, inferior and nasal), respectively. Using ANOVA with repeated measures and Bonferroni correction, there was retinal thinning and thickening at specific macular regions in diabetes with and without DR and no differences in retinal thickness at peripapillary regions. The limitation of this study is an inconsistency of reporting between results and conclusions.
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<tr>
<td><strong>Participant Groups (mean age ± SD)</strong></td>
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<tr>
<td></td>
<td>T1D with no DR (28 ± 10); T1D with min. DR (36 ± 8); Control (34 ± 11).</td>
<td>T1D with no DR (30 ± 11); T1D with min. DR (37 ± 10); Control (33 ± 11).</td>
<td>T1D with no DR (30 ± 11); T1D with min. DR (37 ± 10); Control (33 ± 9).</td>
<td>T1D/T2D with no DR (56 ± 13); T1D/T2D with early DR (56 ± 13); Control (56 ± 13).</td>
</tr>
<tr>
<td><strong>Imaging Protocol</strong></td>
<td>TD-OCT; 6 radial B-scans (128 A-scans each)</td>
<td>TD-OCT; 6 radial B-scans (128 A-scans each)</td>
<td>SD-OCT; 6 x 6 x 2.2 mm volume (512 A-scans x 128 B-scans)</td>
<td>SD-OCT; &quot;Macula Map Protocol&quot; (1024 A-scans x 64 B-scans)</td>
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<td><strong>Retinal Layers Studied</strong></td>
<td>Total retinal thickness</td>
<td>Total retinal thickness</td>
<td>Total retinal thickness</td>
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<td></td>
<td>RNFL</td>
<td>GCL/IPL</td>
<td>RNFL</td>
<td>ILM/RNFL</td>
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<td>GCL</td>
<td>IPL</td>
<td>GCL</td>
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<td>INL</td>
<td>IPL</td>
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<td>OPL</td>
<td>OPL</td>
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<td>ONL/ISL</td>
<td>ONL/ISL</td>
<td>ONL/ISL</td>
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<td>Foveal</td>
<td>Pericentral</td>
<td>Nine ETDRS Regions</td>
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<td>Pericentral</td>
<td>Central Point</td>
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<td></td>
<td>Peripheral</td>
<td>Peripheral</td>
<td>Peripheral</td>
<td></td>
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<tr>
<td><strong>Primary Findings</strong></td>
<td>Total retinal thickness thinning in pericentral area in T1D with minimal DR.</td>
<td>Total retinal thickness thinning in pericentral area in T1D with minimal DR; GCL/IPL and INL thinning in pericentral area in T1D with minimal DR;</td>
<td>GCL thinning in pericentral area in T1D with minimal DR; RNFL thinning in peripheral area in T1D with minimal DR.</td>
<td>Thinning and thickening in specific subfields in T1D/T2D with no DR and with early DR.</td>
</tr>
</tbody>
</table>

Table 1. Summary of studies investigating retinal thickness irregularities in preclinical DR.
Morphological irregularities in preclinical DR are concerned with architectural degeneration that culminates as differences in retinal thickness. As illustrated by this section, there are many approaches to investigating retinal thickness irregularities. However, the general direction of research is moving towards greater spatial resolution, both axially and laterally. Rather than measuring mean total retinal thicknesses in large foveal, pericentral and peripheral regions, subsequent investigations measure mean retinal thicknesses for individual retinal layers or two or three layers combined and in smaller regions. This movement is partly due to the improved speed and sensitivity of SD-OCT imaging.

2 Rationale and Purpose

Rationale

The time at which clinical manifestations of DR develop is variable. The Canadian Ophthalmological Society recommends that 1) individuals with T1D diagnosed after puberty be screened for DR five years after the diagnosis of T1D, 2) individuals with T1D diagnosed before puberty be screened for DR at puberty and 3) individuals with T2D be screened for DR at the time of diagnosis of T2D (Hooper et al., 2012). If DR is present, then the frequency of screening depends on the severity of DR. If DR is not detected, then individuals with T1D are screened annually and individuals with T2D are screened every one to two years. Factors such as the duration of diabetes, onset and control of hyperglycemia, use of medications and other medical components, can greatly impact when microvascular lesions develop and the rate at which DR progresses. Furthermore, individuals are often initially asymptomatic for microvascular lesions. Depending on when the next scheduled clinic visit is, vision loss may be a risk.

Preclinical DR, the duration after the onset of diabetes and before clinically-detectable DR, is a period of retinal neurodegeneration characterized by functional and morphological irregularities. These retinal irregularities are an early response to hyperglycemia during diabetes and pose a minor threat to vision compared to microvascular lesions. Given that treatment options for clinical DR are limited and that associated vision loss may be irreversible and permanent, the ability to detect retinal irregularities during preclinical DR is undoubtedly advantageous.
Purpose
Retinal thickness irregularities are observed before clinically detectable DR and may serve as a biomarker for preclinical DR. However, retinal thickness irregularities are currently poorly defined and localized. For clinical purposes, it would be impractical to look for regions of retinal thickness irregularities across the entire retina in every retinal layer. Therefore, the purpose of this study is to identify specific ETDRS regions in individual retinal layers where there are significant retinal thickness irregularities in preclinical DR. In order to use retinal thickness as an early biomarker for DR, an Ophthalmologist or Optometrist must know which areas are susceptible to developing retinal thickness irregularities.

3 Hypothesis
We hypothesized that in preclinical DR, retinal thickness irregularities are localized to specific retinal regions and layers.

4 Methods
4.1 Study Design
This was an observational cross-sectional study composed of a training set and a validation set. The training set was used to establish protocol and feasibility and the validation set was used to verify training set findings. In addition, the training and validation sets were used to address a multiple comparisons issue. In either dataset, we did not adjust for multiple comparisons. However, the training set was used to identify a smaller subset of regions that presented with retinal thickness irregularities, which were verified using the validation set. This limited and reduced the number of comparisons.

4.2 Research Ethics Board Approval
This study and all procedures were approved by the Research Ethics Board (REB) at The Hospital for Sick Children (see Appendix A. Research Ethics Board (REB) Approval (2013-2014) and Appendix B. Research Ethics Board (REB) Approval (2014-2015)). To maintain compliance with REB guidelines, documentation was written for each testing session and reviewed by the Principal Investigator (see Appendix C. Note to File Form and Appendix D. Quality Improvement Sign-Off by Principal Investigator Form).
4.3 Recruitment

Individuals with T1D were recruited from the Endocrinology Clinic at The Hospital for Sick Children, Juvenile Diabetes Research Foundation and Canadian Diabetes Association University of Toronto Chapter, in Toronto, ON, Canada. Healthy individuals without diabetes, who were recruited from the community and enrolled based on age-similarity to participants with T1D, acted as control participants. Other forms of recruitment included information pamphlets in clinic rooms at The Hospital for Sick Children, web advertisements on the Research4kids research database and recruitment posters on bulletin boards across the University of Toronto campus (see Appendix E. Research Study Information Pamphlet and Appendix F. Research Study Advertisement). Siblings and friends of all participants were invited to participate too. All forms of recruitment were approved by the REB at The Hospital for Sick Children and University of Toronto.

Individuals interested in participating in the research study were contacted by telephone or email by the Research Coordinator and a testing session was scheduled.

4.4 Participant Criteria

Two groups of participants were recruited: 1) individuals with T1D with no or minimal DR and 2) individuals with no diabetes. Detailed inclusion and exclusion criteria are summarized in Table 2.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>T1D</td>
<td>Duration of T1D &gt; 5 years&lt;br&gt;No or minimal DR&lt;br&gt;Normal visual development prior to T1D diagnosis</td>
<td>Duration of T1D ≤ 5 years&lt;br&gt;DR more severe than minimal DR</td>
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<tr>
<td>Control</td>
<td>Normal visual development</td>
<td>Abnormal visual development</td>
</tr>
<tr>
<td>All</td>
<td>Age at testing between 12-35 years&lt;br&gt;Have best-corrected visual acuity of 0.3 logMAR or better&lt;br&gt;Refractive error &lt; ±5 dipters</td>
<td>Presence of ocular/systemic disease or medication affecting the eye</td>
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Table 2. Summary of participant inclusion and exclusion criteria.
Inclusion Criteria
Inclusion criteria for individuals with T1D were a minimum duration of T1D of 5 years, normal visual development prior to T1D diagnosis and no or minimal DR. Minimal DR was defined as the presence of at least one microaneurysm or hemorrhage in the central retina only. Inclusion criterion for controls was a history of normal visual development. All participants included were adolescents and young adults between the ages of 12-35 years of age, had best-corrected visual acuity of 0.3 logarithmic minimum angle of resolution (logMAR) or better and distance refractive error not exceeding a spherical equivalent of ±5.0 diopters.

Exclusion Criteria
Any participants that did not meet inclusion criteria were excluded. Other exclusion criteria were the presence of ocular or systemic disease other than T1D or use of medications known to affect eye structure or function, acutely or longitudinally.

4.5 Participant Information
The Hospital for Sick Children Electronic Patient Chart (EPC) system was accessed to retrieve information regarding participant medications, diagnosed diseases and HbA1c levels. Equivalent information was collected directly from participants and/or their family if EPC files did not provide sufficient information or were not available. Written authorization to collect HbA1c levels was obtained from participants with T1D if their HbA1c levels were not measured at The Hospital for Sick Children (see Appendix G. Authorization Letter for HbA1c). Other data collected directly from participants and their family included personal and family medical and drug history (see Appendix H. Information Intake Form). All participant information was kept private and secure with dual-lock policy or on encrypted and password-protected hospital servers. Non-identifying participant identifiers were used where appropriate.

4.6 Consent
This study and all procedures were conducted in compliance with the tenets of the Declaration of Helsinki. Consent forms for individuals with T1D and controls contained the names of study investigators and their roles, contact information, purpose of the research, description of research
and procedures involved, possible harms/discomforts/inconveniences, potential benefits, confidentiality measures, reimbursement policies, participation agreement and sponsorship and conflict of interest information (see Appendix I. Research Consent Form (T1D) and Appendix J. Research Consent Form (Control)).

All participants had the capacity for consent. The study and consent form were formally explained to participants and family members present by an investigator. Sufficient time was also provided for participants to read the consent form and ask any questions. All questions were addressed by the investigator before written informed consent was obtained. The study did not proceed if consent was not provided.

4.7 Study Protocol
4.7.1 Blood Glucose Levels
Participant blood glucose levels were measured throughout the testing session to monitor retinal function. Acute changes in blood glucose levels can affect retinal function and alter data collected (Khan, Barlow, & Weinstock, 2011; Niemeyer, 1997). Blood glucose levels were maintained between 4 and 10 mmol/L to minimize fluctuations in retinal function.

The fingertips of participants were cleaned with 70% v/v isopropyl alcohol swabs (Canada Custom Packaging Company; Toronto, ON, Canada) and air-dried before using a BD Microtainer® contact activated lancet (Becton, Dickson and Company Limited; Dun Langhair, County Dublin, Ireland) to produce a single bead of blood on the fingertip. OneTouch Ultra® test strips (LifeScan Europe; Zug, Switzerland) were inserted into a calibrated OneTouch® Ultra® 2 blood glucose meter (LifeScan Europe; Zug, Switzerland) and used to analyze the bead of blood. Glucose meter readings were recorded to monitor blood glucose levels over the duration of the testing session. Blood glucose levels were measured after consent prior to clinical assessment, after clinical assessment prior to imaging and after imaging prior to the end of the testing session. On average, blood glucose was measured every 30 minutes.

All blood glucose levels were immediately reported via phone to an on-site Registered Nurse from the Endocrinology Department at The Hospital for Sick Children. If blood glucose levels were out of range or nearing 4 or 10 mmol/L, the Registered Nurse provided consultation for
maintaining blood glucose levels between 4 and 10 mmol/L. If blood glucose levels were relatively low or high, or if the rate of change was large, then the testing session was stopped to ensure participant safety. Methods to control blood glucose levels included fruit juice, cookies, exercise when appropriate and insulin bolus. Testing resumed after achieving blood glucose levels within range.

4.7.2 Clinical Assessments
In order to enroll the individual in the study, clinical assessments were conducted to determine if inclusion and exclusion criteria were met. One eye from each participant was selected for assessment based on a random binary sequence where “0” referred to the left eye and “1” referred to the right eye. If the participant had participated previously in the ongoing longitudinal assessment of neuro-retinal markers in the Westall Lab, then the same eye was selected for consistency. In addition to personal and family medical and drug history, other clinical assessments included, in the order they were completed, refraction, visual acuity, contrast sensitivity, colour vision and axial length (see Appendix K. Case Report Form). All clinical assessments were conducted in a single visit and required approximately 30 minutes.

Medical History
Medical history, as well as family medical history, were discussed orally and recorded in writing (see Appendix H. Information Intake Form). Systemic diseases and medications and any other factors known to affect vision were addressed. Only controls were given an additional assessment to screen for undiagnosed diabetes (see Appendix I. Diabetes Screening Questionnaire).

HbA1c Levels
Written authorization for access to HbA1c values was obtained from participants with T1D who were seen by a physician external to The Hospital for Sick Children (see Appendix G. Authorization Letter for HbA1c). HbA1c values and date of measurement were accessed and recorded.
**Refraction**

Refraction was measured if the participant presented with spectacles or no spectacles and visual acuity worse than 0.3 logMAR. The spherical and cylindrical powers of the spectacles were determined by a Huvitz auto-lensmeter CLM-4000 (Huvitz Co., Ltd., Gunpo, South Korea); otherwise, an Ophthalmologist or Orthoptist performed the refraction. Visual acuity, contrast sensitivity and colour vision were measured with best-corrected visual acuity.

**Visual Function**

Measures of visual function included visual acuity and contrast sensitivity. Visual acuity refers to the ability of the visual system to resolve fine detail spatially (Kalloniatis & Luu, 2007). Tests of visual acuity may require the recognition of letters, where the letters have equal legibility, same number of letters on each row, uniform spacing between letters and between rows and letter sizes that progress logarithmically (Bailey & Lovie, 1976). Contrast sensitivity refers to the ability of the visual system to distinguish luminance differences between object and background (borders) (Wei et al., 2012). Tests of contrast sensitivity may involve the recognition of letters of constant size against changing luminance.

The M&S Smart System II (MSSSII) (M&S Technologies, Inc., Niles, IL, USA) was used to test visual acuity and contrast sensitivity in participants. It is a computerized vision testing system that accommodates for non-standard testing distances and has intrinsic luminance (85 candelas/m²), no expiration and randomized optotypes (Chandrakumar, Colpa, Reginald, Goltz, & Wong, 2013). The MSSSII system is comprised of a computer processor, 17 inch wall-mounted flat LCD screen monitor and interactive keypad controller. Visual tests were displayed on the screen monitor.

Gold standards for testing visual acuity and contrast sensitivity include the printed revised ETDRS charts and Pelli-Robson chart, respectively (McClenaghan, Kimura, & Stark, 2007; Pelli, Robson, & Wilkins, 1988). Compared to the revised ETDRS charts, the MSSSII test of visual acuity has equivalent accuracy and clinical repeatability in well-corrected and visually normal young adults (McClenaghan et al., 2007). Compared to the Pelli-Robson chart, the MSSSII test of contrast sensitivity is a clinically valid tool and alternative to the Pelli-Robson chart, with comparable contrast sensitivity results (Chandrakumar et al., 2013).
For both visual acuity and contrast sensitivity testing, participants were seated in a dark room four meters from the screen monitor with monocular viewing aligned to the screen. An opaque paddle occluded the non-tested eye. Letters displayed were standard Snellen and Sloan letters. To test visual acuity, participants were asked to identify the letters on a chart displayed on the monitor. The chart measured eight rows of five letters. The top row corresponded to a 0.5 logMAR. Entire rows correctly identified corresponded to a 0.1 logMAR deduction and each letter correctly identified in that row corresponded to a 0.02 logMAR deduction. To test contrast sensitivity, a single letter of maximum size (180) was displayed on the screen monitor. Participants were asked to identify the letter at different contrast manipulations. The contrast level to which the participant could identify before incorrectly identifying the letter twice was recorded as their contrast sensitivity.

**Colour Vision**

Colour vision was tested using the Mollon-Reffin Minimal Test for colour deficiencies (Mollon 1991). This test is comprised of a series of nine achromatic caps (saturations N4.0 to N6.0; 0.25 increments), and three series of six chromatic caps (1-least saturated to 6-most saturated). Each series of chromatic caps represent deutan (red), tritan (blue-yellow), and protan (green) colour axes.

To test colour vision, a chromatic cap was placed amongst the nine achromatic caps and shuffled. Under normal luminance levels, the participant was encouraged to identify the chromatic cap with the test eye. The non-tested eye was occluded with an opaque paddle. The chromatic cap was alternated between each chromatic series and saturation, from most to least saturated. The least saturated cap in each chromatic series that the participant could correctly identify was recorded as their colour vision. Correctly identifying the least saturated cap in each chromatic series was defined as no colour deficiencies detected.

**Axial Length**

Axial length refers to the distance between the anterior surface of the cornea and the fovea. The I3 System-ABD ultrasound system (Innovative Imaging Inc.; Brooklyn, NY, USA) was used to measure axial length. Before measuring axial length, a drop of 0.5% proparacaine hydrochloride
(Alcaine®; Alcon Canada Inc., Mississauga, Canada) was topically instilled on the eye to control comfort. Participants were encouraged to fixate on a red light target at the blunt end of an ultrasound pen. Then the blunt end of the ultrasound pen was pressed gently on the surface of the cornea to produce an axial length measurement. The ultrasound pen was aligned parallel to the participant’s axis of vision. Reported axial lengths were the average of three measurements.

**Anesthesia, Mydriasis & Cycloplegia**

A combination of three topical ophthalmic medicated drops were used to induce comfortable pupil dilation for optimal ophthalmic imaging. A single drop of 0.5% proparacaine hydrochloride surface anesthetic (Alcaine®; Alcon Canada Inc., Mississauga, Canada), 1.0% tropicamide cycloplegic-mydratic (Mydriacyl®; Alcon Canada Inc., Mississauga, Canada) and 2.5% phenylephrine hydrochloride vasoconstrictor-mydratic (Mydfrin®; Alcon Canada Inc., Mississauga, Canada) were topically instilled to the conjunctival fornix of the test eye to temporarily relieve pain, paralyze the iris sphincter muscle and ciliary body muscle and produce local vasoconstriction, respectively. Generally, pupils were dilated to 8 mm in diameter, 15-20 minutes after instillation.

**Fundus Photography**

Seven-standard field, stereoscopic-colour fundus photography, the current gold standard, was used to screen for indications of diabetic retinopathy (Boyd, Advani, Altomare, & Stockl, 2013). All participants received fundus photography imaging after anesthesia, mydriasis and cycloplegia, using Zeiss Digital FF450 or Zeiss Visucam 200 fundus cameras (Carl Zeiss Meditec) (see Appendix M. Ophthalmic Imaging Form). In addition to seven-standard field photographs, fundus red reflex photographs (45° field) were taken to assess lenticular opacity. All fundus photographs were taken by a Medical Imaging Specialist and interpreted by a Retina Specialist.

**4.7.3 Optical Coherence Tomography**

All participants underwent SD-OCT imaging on Cirrus HD-OCT 5000 (Carl Zeiss Meditec) (see Appendix M. Ophthalmic Imaging Form). A standardized 512 x 128 Macular Cube protocol was used to scan a 3D volumetric region of the retina, measuring 6 x 6 x 2 mm and centered on the fovea. The scan had 128 equally spaced raster B-scans, each comprised of 512 A-scans, giving a
total of 65536 A-scans per 3D volume. This protocol was chosen, as opposed to an alternate 200 x 200 protocol, because it scans with almost 3-fold higher resolution laterally and is superior for detailed assessment of retinal thickness (Nittala, Konduru, Ruiz-Garcia, & Sadda, 2011).

The participant was asked to place his/her chin on the appropriate chin cup for left or right eye testing and forehead in contact with the headrest. Head position was adjusted to find the iris and pupil and adjusted again to obtain a clear focus on the iris. Auto focus and optimize functions were used to optimize fundus and B-scan images, respectively. Manual adjustments were made when necessary. Finally, the participant was asked to blink, then open his/her eyes wide and fixate on an internal target. Acquisition of the fundus and B-scans required a duration of 2.4 seconds. The acquired scan was reviewed before saving or trying again. Scans were repeated if saccades, blinks or any eye or head movements occurred or if scan quality was low. Breaks were offered to the participant between scans.

4.7.4 Retinal Layer Segmentation & Thickness

Raw training and validation set OCT scans were extracted from Cirrus HD-OCT and sent to the Doheny Eye Institute (University of California, Los Angeles) and Vision & Imaging Laboratory (Duke University), respectively, for file decryption (see Appendix N. Letter of Agreement from Doheny Eye Institute (UCLA) and Appendix O. Letter of Agreement from Vision & Imaging Laboratory (Duke University)). The Iowa Reference Algorithm (Retinal Image Analysis Lab, Iowa Institute for Biomedical Imaging, Iowa City, IA) is an automated 3D retinal layer segmentation software that was used to objectively discriminate retinal layers from the 3D volumetric OCT scans (Abramoff, Garvin, & Sonka, 2010; Garvin et al., 2009; Kang, Wu, Chen, & Sonka, 2006). Ten retinal layers were identified, which included the RNFL, GCL, IPL, INL, OPL, ONL, ELM, ISL, OSL and RPE. This algorithm determined the boundaries between adjacent retinal layers based on the analysis of surrounding pixel intensities in three dimensions and subsequently calculated retinal thickness by a difference of adjacent boundaries. A total of 65536 retinal thicknesses were obtained for each retinal layer in each participant. The retinal thicknesses corresponded to the 512 A-scans in each of the 128 B-scans.
4.7.5 MATLAB® Computation

MATLAB® (R2014b; The MathWorks Inc.), a high-level language and data processing software, was used to 1) organize retinal thickness data from retinal layer segmentation, 2) calculate mean retinal layer thicknesses within-group for training and validation datasets, 3) calculate mean retinal layer differences between-groups by subtraction and \( t \)-statistic, 4) delineate ETDRS regions and 5) assign retinal thickness values to corresponding ETDRS regions for model analyses. Organizing retinal thickness data refers to generating lists of cells containing participant retinal thicknesses, organized by retinal location, retinal layer and training/validation set. Additionally, all right eye data were reflected along a vertical axis to achieve left eye consistency across analyses. The subtraction between-groups was calculated by subtracting the mean retinal thickness of the control group from the T1D group at every retinal location. The \( t \)-statistic was calculated with an unpaired Student’s \( t \)-test for retinal thicknesses at each retinal location. All custom MATLAB® scripts written for this thesis are provided in the appendix (see Appendix P. Custom MATLAB® Scripts).

4.8 Statistical Analysis

Linear mixed-effects (LME) models were generated using ‘R’ (Version 3.2.2; R Foundation for Statistical Computing, Vienna, Austria), a statistical computing software, to 1) determine estimates of mean retinal thickness in each ETDRS region, 2) calculate training set \( p \)-values to determine regions of significant retinal thickness irregularities \( (p<0.05) \) and 3) calculate validation set \( p \)-values in the same regions. All custom ‘R’ scripts written for this thesis are provided in the appendix (see Appendix Q. Custom ‘R’ Scripts).

The LME model is retinal thickness by diabetes status, repeated for each of the nine ETDRS regions in each of 10 retinal layers. The LME model is similar to an ANOVA with repeated measures, but the LME model treats participants as a random effect rather than a fixed effect. In the LME model, \( p \)-values less than 0.05 may not be strictly significant in the presence of multiple comparisons. However, \( p \)-values less than 0.05 were intended for identifying regions of interest for verification by the validation set.
For every $p$-value generated by the LME model, a corresponding effect size was calculated by Cohen’s $d$, given by the formula $d = (\text{mean}_1 - \text{mean}_2) / \text{SD}_{\text{pooled}}$, where $\text{mean}_1$ and $\text{mean}_2$ are the means of mean retinal thickness in a region and $\text{SD}_{\text{pooled}}$ is the pooled standard deviation, given by $\text{SD}_{\text{pooled}} = \sqrt{\frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2}}$, where $n_1$ and $n_2$ are the sample sizes and $s_1$ and $s_2$ are the variances for T1D and control groups (Sullivan & Feinn, 2012).

It is important to note that the $p$-values reported by the LME model do not identify the direction of retinal thickness irregularities (thinning or thickening). Estimates of mean retinal thickness generated from the LME model for T1D and control groups in the training and validation sets were compared to determine the direction of retinal thickness irregularities (see Appendix R. Estimates of Mean Retinal Thickness for Training Set and Appendix S. Estimates of Mean Retinal Thickness for Validation Set).

5 Results

5.1 Participants Recruited

A total of 90 participants were recruited and scheduled for testing sessions between March 21, 2014 and March 20, 2015. Of the 45 participants with T1D recruited, 15 cancelled their testing session, five did not show and eight were excluded because of Addison disease (one), amphetamine medication (one), blood sugar levels that could not be safely brought within range given the allotted time (three) and incomplete testing (three) (Figure 4). Of the 45 controls recruited, nine cancelled their testing session, three did not show and two were excluded because of hemoglobinopathy (beta-thalassemia) (one) and incomplete testing (one) (Figure 4).
Figure 4. Summary of participants recruited. **Left.** Recruited participants with T1D. **Right.** Recruited control participants. Pie-chart lists the number and proportion of enrolled and non-enrolled participants.

**Enrolled**

A total of 17 participants with T1D with no or minimal DR and 31 controls were enrolled in the study. The training set was composed of five participants with T1D and eight controls and the validation set was composed of 12 participants with T1D and 23 controls, two of which were siblings (Figure 5). All enrolled participants completed each component of the testing session and met all inclusion and exclusion criteria. No participants appeared more than once in the training and validation sets.

Figure 5. Summary of participants enrolled. **Left.** Training set. **Right.** Validation set. Pie-chart lists the number and proportion of enrolled participants by diabetes status and data set.
5.2 Demographics and Clinical Assessment

Demographics and clinical assessments were obtained from all enrolled participants where applicable (Table 3). All participants enrolled were adolescents and young adults (12 to 35 years old). T1D and control groups were age-similar. Both T1D and control groups in training and validation sets had a sex bias towards females.

<table>
<thead>
<tr>
<th></th>
<th>Training Set, $n = 13$</th>
<th>Validation Set, $n = 35$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1D, $n = 5$</td>
<td>Control, $n = 8$</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>1:4</td>
<td>1:7</td>
</tr>
<tr>
<td>Age at Testing, years</td>
<td>23.20 ± 1.79</td>
<td>23.50 ± 4.75</td>
</tr>
<tr>
<td>Duration of T1D, years</td>
<td>14.00 ± 2.92</td>
<td>-</td>
</tr>
<tr>
<td>No DR</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Minimal DR</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>HbA$_1$c, %</td>
<td>7.22 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>Refractive Error, diopters</td>
<td>2.50 ± 4.24†</td>
<td>-3.75 ± 1.98††</td>
</tr>
<tr>
<td>Visual Acuity, logMAR</td>
<td>0.01 ± 0.04</td>
<td>0.05 ± 0.12</td>
</tr>
<tr>
<td>Contrast Sensitivity</td>
<td>4.6 ± 2.6</td>
<td>5.1 ± 2.2</td>
</tr>
<tr>
<td>Axial Length, mm</td>
<td>22.31 ± 1.30††</td>
<td>25.63 ± 0.12†</td>
</tr>
</tbody>
</table>

Table 3. Demographic and clinical assessment data for participants enrolled in training and validation sets. Data are presented as mean ± SD. Dash (-) refers to non-applicable fields. Data was calculated for only †2, ††3, †††8 or ††††9 participants within each specific group, depending on available data; otherwise, data was calculated from all participants within each specific group. Blood glucose level monitoring, medical history and colour vision results for all participants were within the parameters of this study (results not shown).

Training and validation set participants with T1D had been living with T1D on average for 14.0 ± 2.9 and 12.3 ± 6.6 years, respectively. Despite the long duration of diabetes, fundus photography detected no clinically visible signs of DR in any participant, except for two participants with T1D in the validation set. One participant had scattered intraretinal hemorrhages/microaneurysms and one participant had a single intraretinal hemorrhage/microaneurysm. Both of these participants had minimal DR and were included in the validation set T1D group. All other participants had no signs of microvascular lesions. Mean
HbA1c levels were calculated from available data; missing values were a result of requested data not sent from private clinics. In both data sets, mean HbA1c levels were above the recommended target of <7% (American Diabetes Association, 2014b).

The refractive error, visual acuity, contrast sensitivity and axial length measurements for each participant were all within the parameters of this study and mean values are detailed in Table 3. Missing values resulted from an improvement of protocol, where certain criteria were not initially measured.

The blood glucose levels of all participants were monitored and maintained between 4 and 10 mmol/L for the duration of the testing session. Enrolled participants did not have any systemic disease or medications that affected eye function and structure. Furthermore, none had any colour deficiencies. All participants met the conditions of the inclusion/exclusion criteria.

5.3 Retinal Layer Thicknesses
All 3D volumetric OCT scans used to measure retinal thicknesses were focused, optimized and complete with no scanning frame interruptions. All OCT frames were successfully decrypted and segmented (Figure 6); retinal thicknesses were generated for all 65,536 retinal locations in each retinal layer for all participants.
Figure 6. Segmented B-scan delineating 10 retinal layers. Retinal layers identified, from top to bottom, were the RNFL, GCL, IPL, INL, OPL, ONL, ELM, ISL, OSL and RPE (Participant 560, frame 64).

Training and validation set mean retinal layer thicknesses for each group and retinal layer are shown in Figure 7 to Figure 16. Mean retinal layer thicknesses are presented as 20 x 20 degree thickness maps centered on the fovea, superimposed with the ETDRS grid. The X and Y-axes are degree coordinates from the fovea. Retinal thicknesses are colour-weighted using a parula colourmap as opposed to the more conventional rainbow colourmap. Rainbow colourmaps may misrepresent continuous data because of random colour ordering, which lead to the appearance of pseudo-features, obscurance of detail and de-emphasis of high and low values (Eddins, 2014). Parula colourmaps avoid these issues and facilitate a natural perceptual viewing of areas of thin, medium and thick retina. Given the number of figures presented, the parula colourmap expedites a timelier interpretation of the spatial distribution of retinal thicknesses within a layer.

Each retinal layer had a unique range and distribution of retinal thicknesses, which were similar between T1D and control groups and between training and validation sets. The RNFL and ONL were generally thick retinal layers (thickness range up to 70 pixels); the GCL, IPL, INL and OPL were generally medium retinal layers (thickness range up to 33 pixels); and the ELM, ISL, OSL and RPE were generally thin retinal layers (thickness range up to 14 pixels).
distribution, there were areas of thinner and thicker retina within each retinal layer. The RNFL was thinnest at the fovea and progressively thicker from the temporal to nasal edge of the scan (Figure 7). In the validation set, the thicker nasal corners of the scan were determined to be a result of traversing retinal blood vessels. The GCL had relatively thinner foveal region and thicker pericentral regions (Figure 8). The transitions from foveal to pericentral and pericentral to peripheral regions were gradual across the ETDRS divisions. The IPL had relatively thicker pericentral and peripheral regions (Figure 9). The INL had relatively thicker pericentral regions that diffused into the peripheral regions (Figure 10). The OPL in the training set had an area of thicker retina shared by the Si and Ti regions (Figure 11). In the validation set, that area of thicker retina was more concentrated in the Si region. Similar to the OPL, the ONL had an area of thicker retina. This thicker area was encompassed primarily by the foveal region and partly by the Ti, Ii and Ni regions. The ELM was relatively thin within the ETDRS regions and thicker towards the edges of the scan (Figure 13). Both the ISL and OSL had scattered areas that were either relatively thinner or thicker (Figure 14 and Figure 15). The ISL and OSL were reciprocal in that areas that were thicker in the ISL were thinner in the OSL and vice versa. In the ISL only, the foveal region was relatively thicker (Figure 14). The RPE had small areas of relatively thicker retina throughout the scanned area (Figure 16).
Figure 7. Mean RNFL thickness for T1D and control groups in training and validation sets. The RNFL was thinnest at the fovea and progressively thicker from the temporal to nasal edges of the scan. In the validation set, thicker retina at the nasal corners of the scan was seen to result from traversing retinal blood vessels. The range of thicknesses was 0 – 70 pixels.
Figure 8. Mean GCL thickness for T1D and control groups in training and validation sets. The GCL had a relatively thin foveal region and thicker pericentral region. The transitions between foveal, pericentral and peripheral regions were gradual across the ETDRS divisions. The range of thicknesses was 0 – 33 pixels.
Figure 9. Mean IPL thickness for T1D and control groups in training and validation sets. The IPL had relatively thicker pericentral and peripheral regions. The range of thicknesses was 2 – 24 pixels.
Figure 10. Mean INL thickness for T1D and control groups in training and validation sets. The INL had relatively thicker pericentral regions that diffused into the peripheral regions. The range of thicknesses was 1 – 25 pixels.
Figure 11. Mean OPL thickness for T1D and control groups in training and validation sets. The training set OPL had an area of thicker retina within the $S_i$ and $T_i$ regions. In the validation set, the area of thicker retina was more concentrated in the $S_i$ region. The range of thicknesses was $6 – 24$ pixels.
Figure 12. Mean ONL thickness for T1D and control groups in training and validation sets. The ONL had an area of thicker retina primarily within the foveal region and partly within the T$_i$, I$_i$ and N$_i$ regions. The range of thicknesses was 28 – 64 pixels.
Figure 13. Mean ELM thickness for T1D and control groups in training and validation sets. The ELM was relatively thin within the ETDRS regions but relatively thicker near the edges of the scan. The range of thicknesses was 4 – 11 pixels.
Figure 14. Mean ISL thickness for T1D and control groups in training and validation sets. The ISL had a relatively thicker foveal region and scattered areas of thinner or thicker retina. The range of thicknesses was 3 – 14 pixels.
Figure 15. Mean OSL thickness for T1D and control groups in training and validation sets. The OSL had scattered areas of thinner or thicker retina that were opposite to the ISL. The range of thicknesses was 4 – 14 pixels.
Figure 16. Mean RPE thickness for T1D and control groups in training and validation sets. The RPE had small areas of relatively thicker retina throughout the scanned area. The range of thicknesses was 6–9 pixels.
5.4 Between-Group Comparisons

The between-group comparisons were analyzed by subtraction and \( t \)-statistic. Retinal thickness differences by subtraction were generated from location specific subtraction of mean control retinal thickness from mean T1D retinal thickness to determine the magnitude of retinal thickness differences exclusive to T1D. The parula colourmap was used and thinner regions in T1D are blue and thicker regions in T1D are yellow. Retinal thickness differences by \( t \)-statistic were generated from location specific unpaired Student’s \( t \)-test, which identified the magnitude of retinal thickness differences in T1D after factoring retinal variation. For instance, large thickness differences between-groups at locations where retinal thicknesses are variable were de-emphasized. Similarly, large differences at locations with little variation were not de-emphasized. Additionally, the \( t \)-statistic is bidirectional—negative values (blue) refer to retinal thinning and positive values (red) refer to retinal thickening in T1D relative to control. Therefore, larger \( t \)-statistic values in either direction were thickness differences that were relatively more significant. The blue-white-red colourmap used to colour-weight \( t \)-statistic values had equal gradient steps similar to the parula colourmap.

The retinal thickness differences by subtraction and \( t \)-statistic in the training set were uniquely distributed in each retinal layer and are shown in Figure 17 to Figure 26. In the RNFL, differences were present in the nasal and temporal hemispheres. Differences by subtraction were thinner nasal and thicker temporal areas towards the edges of the scanned area in T1D (Figure 17). Differences by \( t \)-statistic were thinner nasal and thicker temporal hemispheres without the edge patterns as seen by subtraction. In the GCL, differences were scattered areas of thicker retina in T1D (Figure 18). These thicker areas were most concentrated in the \( T_o \) region in both subtraction and \( t \)-statistic comparisons. In the IPL, differences by subtraction and \( t \)-statistic were a thinner \( T_o \) region in T1D (Figure 19). Other differences by subtraction were not apparent. However, differences by \( t \)-statistic revealed an area of thinner retina in the \( S_o \) region. In the INL, no large differences by subtraction or \( t \)-statistic existed in any region (Figure 20). In the OPL, differences by subtraction were scattered areas of thicker retina, which were greatest in the \( S_i \) region (Figure 21). Differences by \( t \)-statistic were areas of thicker retina in the superior hemisphere. In the ONL, differences by subtraction were an area of thicker retina within the foveal, \( T_i \) and \( I_i \) regions and an area of thinner retina within the \( S_i \) region in T1D (Figure 22). There were also areas of thicker retina towards the nasal corners of the scan. Differences by \( t \)-
statistic were more diffuse—the inferior hemisphere was thicker in T1D. In addition, the areas of thicker and thinner retina seen by subtraction were de-emphasized by *t*-statistic. Comparatively, the OPL and ONL seemed to have reciprocal differences. In the ELM and RPE, differences by subtraction and *t*-statistic did not exist in T1D (Figure 23 and Figure 26). In the ISL, differences by subtraction and *t*-statistic both revealed scattered areas of thinner and thicker retina. Some of these areas were at the divisions between foveal and pericentral and pericentral and peripheral regions (Figure 24). Similarly, the OSL had scattered areas of thinner and thicker retina at the divisions between foveal and pericentral and pericentral and peripheral regions by subtraction and *t*-statistic (Figure 25). However, where the ISL was thinner, the OSL was thicker and where the ISL was thicker, the OSL was thinner. The ISL and OSL have reciprocating retinal thickness differences.
Figure 17. Mean RNFL thickness differences between groups in training set. Differences by subtraction were thinner nasal and thicker temporal areas towards the edges of the scan in T1D. Differences by \( t \)-statistic were thinner nasal and thicker temporal hemispheres.

Figure 18. Mean GCL thickness differences between groups in training set. Differences by subtraction and \( t \)-statistic were scattered areas of thicker retina in T1D. The \( T_o \) region had a higher concentration of these thicker areas.
Figure 19. Mean IPL thickness differences between groups in training set. No large retinal thickness differences were present, except for a thinner T₀ region (by subtraction and t-statistic) and an area of thinner retina in the S₀ region (more apparent by t-statistic).

Figure 20. Mean INL thickness differences between groups in training set. No large retinal thickness differences were present.
Figure 21. Mean OPL thickness differences between groups in training set. Differences by subtraction were an area of thicker retina in the Si in T1D. Differences by t-statistic were a thicker superior hemisphere in T1D.

Figure 22. Mean ONL thickness differences between groups in training set. Differences by subtraction were an area of thicker retina in the foveal, Ti and Ii regions and an area of thinner retina in the Si region in T1D. However, differences by t-statistic were a thicker inferior hemisphere and de-emphasized the thicker and thinner areas shown by subtraction.
Figure 23. Mean ELM thickness differences between groups in training set. Differences by subtraction and \( t \)-statistic do not have large retinal thickness differences in T1D.

Figure 24. Mean ISL thickness differences between groups in training set. Differences by subtraction and \( t \)-statistic were scattered areas of thinner and thicker retina, some of which are at the division between foveal and pericentral and pericentral and peripheral regions.
Figure 25. Mean OSL thickness differences between groups in training set. Differences by subtraction and \( t \)-statistic were scattered areas of thinner and thicker retina, some of which are at the division between foveal and pericentral and pericentral and peripheral regions. The differences were opposite to the ISL.

Figure 26. Mean RPE thickness differences between groups in training set. No large retinal thickness differences were present.
5.5 Regions of Interest

Regions of interest were determined from the training set data using an LME model. The model compared retinal thicknesses within a region by diabetes status (T1D or control) and was repeated for each of the ETDRS regions for all retinal layers. Each region was assigned a significance value, where \( p < 0.05 \) were regions in T1D that had significant retinal thickness irregularities. The significance values are represented visually on a gray colourmap in Figure 27. The significance of the \( p \)-value is correlated with the level of darkness. The significance values are provided numerically in Table 4. From the LME model, estimates of mean retinal thickness were generated and compared to determine the direction of the retinal thickness irregularity (thinning or thickening) (see Appendix R. Estimates of Mean Retinal Thickness for Training Set). The direction of the retinal thickness irregularity is denoted above each significance value in Table 4—minus (-) symbol for thinning or plus (+) symbol for thickening in T1D relative to control.

Only four regions (4.4\%) reached significance \( (p < 0.05) \). These regions were the \( T_o \) region in the GCL \( (p < 0.05, \text{ effect size} = 1.7) \), foveal region in the INL \( (p < 0.05, \text{ effect size} = 1.3) \) and \( N_i \) and \( S_o \) regions in the OPL \( (p < 0.05, \text{ effect size} = 1.2 \) and \( p < 0.05, \text{ effect size} = 1.4, \) respectively). Comparing the estimates of mean retinal thickness in each significant region between-groups revealed that all four regions experienced irregular thickening in T1D.
Figure 27. Training set retinal thickness irregularities by region and retinal layer. Linear mixed-effects modelling of retinal thickness by diabetes status determined the significance of retinal thickness irregularities in T1D. X- and Y-axes specify the ETDRS region and retinal layer, respectively, and colour-scale depicts magnitude of $p$-value, where darker is more significant.
Table 4. Specific p-values of training set retinal thickness irregularities by region and retinal layer. Linear mixed-effects model analyses of retinal thickness by diabetes status determined the significance of retinal thickness irregularities in T1D. X- and Y-axes specify the ETDRS region and retinal layer, respectively. The minus (-) or plus (+) symbol above each p-value indicates thinning or thickening, respectively, determined from comparisons of estimates of mean retinal thickness. Retinal regions where p<0.05 are shaded.
5.6 Verification

The LME model was used to analyze the validation set to verify the four regions of retinal thickness identified by the training set. The \( p \)-values and effect size for both the training and validation sets in the four regions are provided in Table 5. In the validation set, the To region in the GCL (\( p \geq 0.05 \), effect size = -0.2), foveal region in the INL (\( p \geq 0.05 \), effect size = 0.5) and Ni and So regions in the OPL (\( p \geq 0.05 \), effect size = -0.03 and \( p \geq 0.05 \), effect size = 0.4, respectively) did not reach significance. Comparing the estimates of mean retinal thickness between-groups in the validation set revealed that the foveal region in the INL and So region in the OPL experienced irregular thickening in T1D, which was consistent with the training set (see Appendix S. Estimates of Mean Retinal Thickness for Validation Set). On the other hand, the To region in the GCL and Ni region in the OPL experienced irregular thinning in T1D, which was not consistent with the training set.

<table>
<thead>
<tr>
<th>Region of Interest</th>
<th>Training Set</th>
<th>Validation Set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( p )-value</td>
<td>effect size</td>
</tr>
<tr>
<td>To of GCL</td>
<td>0.01*</td>
<td>1.7</td>
</tr>
<tr>
<td>Fovea of INL</td>
<td>0.04*</td>
<td>1.3</td>
</tr>
<tr>
<td>Ni of OPL</td>
<td>0.0499*</td>
<td>1.2</td>
</tr>
<tr>
<td>So of OPL</td>
<td>0.03*</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 5. Comparing training and validation set \( p \)-values in regions of significant retinal thickness irregularities determined by training set data. *\( p < 0.05 \).

5.7 Exploratory Analysis

Since the regions of significant retinal thickness irregularities identified from the training set were not verified by the validation set, the validation set was analyzed by region and layer to investigate possible differences.

5.7.1 Between-Group Comparisons

The between-group comparisons in the validation set were analyzed by subtraction and \( t \)-statistic. The differences by subtraction (left) and \( t \)-statistic (right) are shown for each retinal
layer in Figure 28 to Figure 37. Compared to the training set, there were between-group similarities and differences.

In the RNFL, differences by subtraction were small areas of thinner and thicker retina in the nasal corners of the scan in T1D (Figure 28). Otherwise, there were no large thickness differences by subtraction or t-statistic. The thinner nasal and thicker temporal differences in the training set RNFL (Figure 17) were not present in the validation set. In the GCL, no large differences by subtraction and t-statistic were present (Figure 29). Differences by t-statistic were trending towards thinner GCL in all regions in T1D. The scattered areas of thicker GCL in the training set were not present in the validation set (Figure 18). In the IPL and INL, there were scattered areas of thinner and thicker retina; however, these thickness differences were small (Figure 30 and Figure 31). The thinner T0 region and thinner area in the S0 region in the training set IPL were not present in the validation set (Figure 19). The training and validation set INL were similar (Figure 20). In the OPL, differences by subtraction were a thicker S1 region in T1D (Figure 21). Differences by t-statistic were a thicker superior and thinner inferior hemisphere. In the ONL, differences by subtraction were a thinner S1 region in T1D (Figure 22). Differences by t-statistic were a thinner superior and thicker inferior hemisphere. The thickness differences of the OPL and ONL were reciprocal. The thickness differences in the OPL and ONL and the reciprocity of these differences in the validation set were similar to the training set (Figure 21 and Figure 22). In the ELM, there were no large differences by subtraction (Figure 34). Differences by t-statistic were thicker retina along the division between the pericentral and peripheral regions, which was not present in the training set (Figure 23). In the ISL and OSL, differences by subtraction and t-statistic were areas of thinner and thicker retina (Figure 35 and Figure 36). These thickness differences were not large and are more visible by t-statistic. The thinner and thicker areas in the ISL were concentrated in the nasal and temporal hemispheres, respectively. The thinner and thicker areas in the OSL were concentrated in the temporal and nasal hemispheres, respectively. In the training set, the distribution of thinner and thicker areas in the ISL and OSL were not concentrated; rather, they were scattered (Figure 24 and Figure 25). Similar to the training set, the validation set ISL and OSL had reciprocal differences. In the RPE, no large differences were present by subtraction or t-statistic (Figure 37). The training and validation set RPE were similar (Figure 26).
Figure 28. Mean RNFL thickness differences between groups in validation set. Differences by subtraction revealed small areas of thinner and thicker retina in the nasal corners of the scan in T1D. Otherwise, no large thickness differences were present.

Figure 29. Mean GCL thickness differences between groups in validation set. No large differences were present. Differences by $t$-statistic were trending towards a thinner GCL in all regions in T1D.
Figure 30. Mean IPL thickness differences between-groups in validation set. Scattered areas of weak IPL thinning and thickening were present in T1D.

Figure 31. Mean INL thickness differences between-groups in validation set. Scattered areas of weak INL thinning and thickening were present in T1D.
Figure 32. Mean OPL thickness differences between-groups in validation set. Differences by subtraction were a thicker Si region and differences by $t$-statistic revealed a thicker superior and thinner inferior hemisphere in T1D.

Figure 33. Mean ONL thickness differences between-groups in validation set. Differences by subtraction were a thinner Si region and differences by $t$-statistic revealed a thinner superior and thicker inferior hemisphere in T1D.
Figure 34. Mean ELM thickness differences between-groups in validation set. Differences by subtraction were no large differences between-groups, but differences by $t$-statistic revealed thicker retina along the division between pericentral and peripheral regions.

Figure 35. Mean ISL thickness differences between-groups in validation set. Areas of thinner and thicker retina were present in the nasal and temporal hemispheres, respectively. These thickness differences were not large and were more visible by $t$-statistic.
Figure 36. Mean OSL thickness differences between groups in validation set. Areas of thinner and thicker retina were present in the temporal and nasal hemispheres, respectively. These thickness differences were not large and were more visible by \( t \)-statistic.

Figure 37. Mean RPE thickness differences between groups in validation set. No large thickness differences were present.
5.7.2 Regions of Interest

The LME model was used to analyze the validation set data to determine the significance of retinal thickness irregularities in T1D for each region in each layer. The significance values are represented in Figure 38 and numerically in Table 6. The only region that reached significance was the N_i region in the GCL (p<0.05, effect size=-1.0). Comparing estimates of mean retinal thickness revealed that this region had irregular thinning in T1D (see Appendix S. Estimates of Mean Retinal Thickness for Validation Set).
Figure 38. Validation set retinal thickness irregularities by region and retinal layer. Linear mixed-effects modelling of retinal thickness by diabetes status determined the significance of retinal thickness irregularities in T1D. X- and Y-axes specify the ETDRS region and retinal layer, respectively, and colour-scale depicts magnitude of $p$-value, where darker is more significant.
Table 6. Specific $p$-values of validation set retinal thickness irregularities by region and retinal layer. Linear mixed-effects model analyses of retinal thickness by diabetes status determined the significance of retinal thickness irregularities in T1D. X- and Y-axes specify the ETDRS region and retinal layer, respectively. The minus (-) or plus (+) symbol above each $p$-value indicates thinning or thickening, respectively, determined from comparisons of estimates of mean retinal thickness. Retinal regions where $p<0.05$ are shaded.
6 Discussion

The primary finding of this investigation was that retinal thickness irregularities in preclinical DR were present in specific retinal regions and layers. By comparing T1D and control retinal thicknesses in a training set, significant regions were identified for verification in a validation set. In the training set, four retinal regions presented with significant retinal thickness irregularities. These regions were the T₀ region in the GCL, foveal region in the INL and N₁ and S₀ regions in the OPL. In all four regions, there was irregular retinal thickening in T1D relative to control. The validation set had a larger sample size and similar composition to the training set, but the four regions were not significant in the validation set.

6.1 Participants with T1D

Age

All enrolled participants were adolescents or young adults. Compared to other investigations, this study had younger diabetes and control groups (Table 1). Younger participants avoided retinal thickness changes associated with increasing age. Regions in the RNFL, GCL, IPL and OSL have been shown to thin and regions in the RPE have been shown to thicken with increasing age (Demirkaya et al., 2013). The retinal thickness irregularities identified in this study were attributed to the presence and duration of diabetes and not the effect of increasing age. Furthermore, younger participants were less likely to have high blood pressure, cataracts and other systemic diseases and medications that may affect retinal thickness or its measurement (Matthews et al., 2004). Regardless, clinical assessments were completed for assurance.

Duration of Diabetes

All participants with diabetes had T1D. Since the duration of diabetes is correlated with the onset and progression of DR-related complications, it is important to consider the duration of diabetes of participants with diabetes (Goldenberg & Punthakee, 2013). The duration of diabetes is the amount of time since the date of diagnosis to the date of testing. Since the date of testing is absolute, it is the date of diagnosis that determines the accuracy of the duration of diabetes. T1D is readily diagnosable by markers of immune destruction of β-cells whereas T2D may be asymptomatic and undiagnosed for several years (American Diabetes Association, 2014a, 2014b).
In most cases of T2D, the date of diagnosis is not the date of diabetes onset. The presence of undiagnosed diabetes leads to an underestimation of the duration of diabetes and its contribution to retinal thickness irregularities. Therefore, it is advantageous to study a participant group that is homogeneous for T1D, rather than one that is homogeneous for T2D or a mix of T1D and T2D.

**Severity of DR**

All participants with diabetes had preclinical DR, which was defined as diabetes with no or minimal DR. In the training set, all participants with T1D had no clinical signs of DR, whereas in the validation set, the majority had no clinical signs of DR (88%) and two participants had minimal DR (12%). This composition of participants with T1D met inclusion/exclusion criteria. However, for the purposes of detecting retinal thickness irregularities, it might have been more appropriate to study a participant group consisting mostly of participants with T1D with minimal DR. Other investigations of retinal thickness irregularities found significant retinal thickness irregularities in participants with diabetes with minimal DR rather than no DR (Biallusterski et al., 2007; van Dijk et al., 2009, 2010). The T1D group in this investigation may have been too early in the neurodegenerative process to consistently identify regions of retinal thickness irregularities, which may explain the discrepancy between training and validation sets. Without compromising a younger T1D group, participants with a longer duration of diabetes or poorer glycemic control (higher HbA1c levels) would remedy this issue.

**Sample Size**

A limiting factor of this investigation was the small sample size in the training and validation sets. The regions of interest identified by the training set may be irreproducible because of the small sample size and may explain the discrepancy between the training and validation sets. The validation set, which has a larger sample size than the training set, had results similar to other investigations. In the validation set, the \( N_i \) region in the GCL was significant for retinal thickness irregularities in T1D and exhibited irregular thinning. Other studies also found irregular thinning in the GCL (van Dijk et al., 2009, 2010). Although the training set also identified the \( T_o \) region in the GCL as significant, it exhibited irregular thickening. It is worth considering the validation set as the training set and collecting more data for a new validation set.
6.2 Retinal Layer Thicknesses

High-Resolution Approach

The 512 x 128 Macular Cube protocol achieved retinal scans with lateral resolutions of 12 and 47 µm in the X- and Y-axes, respectively. Each retinal layer was composed of 65,536 retinal thickness measurements at unique locations on the retina. Retinal layer segmentation, retinal thickness generation, comparisons of retinal layer thicknesses and LME model analyses in each region all operated on individual retinal thicknesses. For example, between-group comparisons by subtraction and t-statistic calculated subtraction and unpaired Student’s t-test for each retinal location. Each retinal location was represented by a retinal thickness, rather than one mean retinal thickness for an entire region. Furthermore, no interpolation was used. This high-resolution approach preserved retinal information.

Distribution of Thicknesses within a Retinal Layer

In this study, each retinal layer had a unique range and distribution of retinal thicknesses. In general, the RNFL and ONL were thicker retinal layers, the ELM, ISL, OSL and RPE were thinner retinal layers and the GCL, IPL, INL and OPL were in-between. In terms of thickness distributions, all retinal layers had point symmetry (symmetry about the foveal center) except for the OPL and ONL. The RNFL was progressively thicker away from the fovea, the GCL was thickest at the pericentral regions, the IPL and INL were thicker in the pericentral and peripheral regions, the OPL and ONL had specific regions of thicker retina that mirrored each other, the ELM was relatively thin except for thicker areas near the edges, the ISL and OSL had scattered, but reciprocal areas of thinner and thicker retina and the RPE had small scattered areas of thicker retina. It appeared that the RNFL, GCL, IPL, INL, ELM and RPE had independent thickness distributions whereas the OPL and ONL and ISL and OSL had associated distributions. The OPL and ONL had mirroring thickness distributions about a horizontal plane and the ISL and OSL had reciprocal thicknesses, where a thicker region in one layer was thinner in the other and vice versa.

Between-Group Differences by Subtraction and t-statistic

Between-group comparisons of retinal layer thicknesses by subtraction and t-statistic identified thickness irregularities unique to T1D. By subtracting the mean control thicknesses from the mean T1D thicknesses, only the differences or irregular thicknesses remained. On the other hand,
*t*-statistic compared the differences between control and T1D thicknesses while considering the thickness variability at that location. For most retinal layers, the areas of thickness irregularities by subtraction and *t*-statistic were similar, but the magnitude of thickness irregularities within the areas was not. For instance in the RNFL, differences by subtraction identified large thinner nasal and thicker temporal areas toward the edges of the scan in T1D, but differences by *t*-statistic showed thinner nasal and thicker temporal hemispheres without the intense edges (Figure 17). The areas toward the edges in the RNFL were relatively variable and consequently, the magnitude of its thickness differences was de-emphasized by *t*-statistic. Some regions that did not have large thickness differences by subtraction had stronger irregularities by *t*-statistic. For example in the OPL, differences by subtraction revealed scattered areas of thicker retina, which were greatest in the $S_1$ region, but differences by *t*-statistic identified a thicker superior hemisphere (Figure 21). By inference, these regions had little variation. Regions with medium or small thickness differences were revealed by *t*-statistic, so long as the thicknesses between-groups were stable. The *t*-statistic was a more reliable estimation of thickness irregularities. Subtraction revealed areas of retinal thickness irregularities between-groups and approximated the magnitude of thickness differences, but it did not consider the thickness variations between participants. The *t*-statistic also revealed areas of retinal thickness irregularities between-groups, but it factored the amount of variation at that location. The thickness irregularities by *t*-statistic were appropriately scaled by how large and reliable the differences were. Though *t*-statistic comparisons illustrated the size, shape and location of retinal thickness irregularities in each retinal layer, they did not determine the significance of retinal thickness irregularities within the ETDRS regions.

Visualizing the thickness differences between-groups emphasized the importance of studying individual ETDRS regions. In other studies, superior, temporal, inferior and nasal regions were merged to form whole pericentral and peripheral regions (Biallosterski et al., 2007; van Dijk et al., 2009, 2010). With this approach, retinal thickness irregularities may have been masked. For example, the thinner nasal and thicker temporal areas in the RNFL (Figure 17), the thicker superior and thinner inferior areas in OPL (Figure 21) and the thinner superior and thicker inferior areas in the ONL (Figure 22) would not have been detected by whole pericentral and peripheral regions. The summative effect of hemispheric thickness differences would have offset each other and appeared as no retinal thickness irregularities in that region. For other retinal
thickness irregularities that were confined to specific regions, their presence would have been diluted in whole pericentral and peripheral regions.

Similarly, the results of thickness differences between-groups highlighted the necessity to study individual retinal layers. In other studies, some individual retinal layers were combined and treated as a single layer (Biallosterski et al., 2007; van Dijk et al., 2009, 2010; Vujosevic & Midena, 2013). Combining retinal layers and treating them as one has a similar issue as merging ETDRS regions, where retinal thickness irregularities are masked. This is most obvious for the ISL and OSL. Between-group comparisons of thickness irregularities revealed scattered areas of thinner and thicker retina in both the ISL and OSL (Figure 24 and Figure 25). Comparing the ISL and OSL, areas of ISL thinning were areas of OSL thickening and vice versa. If the ISL and OSL were combined and treated as one retinal layer, then the thinner areas in one layer would be compensated by the thicker areas in the other layer and vice versa. The thickness irregularities would have been normalized and detected as no thickness irregularities. A similar scenario would have occurred with the OPL and ONL (Figure 21 and Figure 22). Each pair of retinal layers were adjacent, with reciprocal thickness differences. Masking retinal thickness irregularities by combining retinal layers is most serious when total retinal thickness is measured. Any subtle retinal thickness irregularities could be compensated by a retinal thickness irregularity in the opposite direction in any of the other retinal layers.

It is important to consider the density by which the retina is partitioned when studying retinal thickness irregularities in preclinical DR. The combination of regions or retinal layers may mask and misrepresent retinal thickness irregularities. Despite the need for a larger sample size, this study investigated individual ETDRS regions and individual retinal layers.

### 6.3 Regions of Significant Retinal Thickness Irregularities

The LME model was used to determine the significance of retinal thickness irregularities by region, without compromising high-resolution analyses or reliability. The model compared individual retinal thicknesses and variation within a region by diabetes status (T1D or control) and assigned a significance value for that region. The LME model was similar to an ANOVA with repeated measures, but it treated participant as a random effect rather than a fixed effect, which was useful since natural retinal variation across participants cannot be controlled. The
LME model was repeated for each region in each retinal layer. In this study, there were a total of 90 regions—nine ETDRS regions in each of 10 retinal layers. To our knowledge, this is the most region-specific investigation of retinal thickness irregularities in preclinical DR. In 2007, Biallosterski et al. studied four regions (total retinal thickness in central, foveal, pericentral and peripheral regions); in 2009, van Dijk et al. studied 21 regions (foveal, pericentral and peripheral regions in seven individual and composite retinal layers, including total retinal thickness); in 2010, van Dijk et al. studied 18 regions (pericentral and peripheral regions in nine individual and composite retinal layers, including total retinal thickness); and in 2013, Vujosevic et al. studied 45 regions (nine ETDRS regions in each of five composite retinal layers).

In the training set, four regions were significant for retinal thickness irregularities in T1D. They were the T0 region in the GCL, foveal region in the INL and N1 and S0 regions in the OPL. Comparisons of estimates of mean retinal thickness in each region between-groups revealed that all four regions were irregular thickening in T1D. In the validation set, none of these four regions were significant. Rather, the N1 region in the GCL was significant for retinal thickness irregularities in T1D. This region had irregular thinning in T1D.

6.4 Neurodegeneration in Specific Retinal Layers
The results of this study can be partially explained by the neurodegenerative pathways in preclinical DR. Regardless of the discrepancy between training and validation sets, the GCL, INL and OPL were retinal layers that were susceptible to the development of regional thickness irregularities. Neurodegenerative pathways, including oxidative stress, glutamate excitotoxicity and loss of neuroprotective factors, are associated with these retinal layers.

First, oxidative stress may be indirectly interfering with normal photoreceptor physiology. In preclinical DR, oxidative stress produces toxic ROS, which is especially prevalent as mtROS from mitochondria in the highly metabolic RPE (Jarrett et al., 2008). The RPE has major roles in photoreceptor homeostasis and therefore, loss of RPE function from oxidative stress may result in photoreceptor degeneration. It is a common feature across neuronal cells that neuronal insults often begin with the neurodegeneration of their cell projections (Wang, Medress, & Barres, 2012). Cell projections may experience morphological changes such as inflammation, disassembly and fragmentation prior to cell death. In terms of the retina, photoreceptor
degeneration may initially present as the degeneration of its projections. Since the OPL is composed of photoreceptor projections, it is susceptible to oxidative stress and early degeneration in preclinical DR. In this study, irregular thickening of the OPL in T1D was observed. The thickening of the OPL may be a result of subclinical inflammation secondary to early photoreceptor degeneration.

Second, glutamate excitotoxicity in preclinical DR may be disrupting the photoreceptor projections and GCs. In preclinical DR, excess glutamate in the extracellular and synaptic spaces can become toxic to retinal cells(Ambati et al., 1997; Kowluru et al., 2001; E Lieth et al., 1998; Erich Lieth et al., 2000; Pulido et al., 2007). Chronic glutamate excitotoxicity can lead to uncontrolled retinal cell death. In the retina, glutamate signaling is the primary means of communicating visual information between photoreceptors and GCs. The photoreceptor projections (OPL) release glutamate into the synapse with GCs (GCL). This synapse is susceptible to glutamate excitotoxicity and may cause GCL and OPL cell death in preclinical DR. In this study, retinal thickness irregularities were observed in the GCL and OPL and may be explained by glutamate excitotoxicity. In the training set, the GCL and OPL experienced regions of irregular thickening, whereas in the validation set, the GCL experienced a region of irregular thinning. The different directions of retinal thickness irregularities may be from the same neurodegenerative pathway but at different stages of cell death.

Third, the loss of neuroprotective factors in preclinical DR affects Müller cell function. Under normal physiological conditions, specific neuroprotective factors regulate the expression of glutamine synthetase, a Müller cell-specific enzyme responsible for recycling glutamate to glutamine(Barnstable, 2004; E Lieth et al., 1998; Erich Lieth et al., 2000). Therefore, the loss of neuroprotective factors in preclinical DR reduces glutamate recycling and augments glutamate excitotoxicity. In this way, the loss of neuroprotective factors contributes to glutamate-associated degeneration in the GCL and OPL.

In addition to augmenting glutamate excitotoxicity, Müller cells may have a second contribution to retinal thickness irregularities in preclinical DR. Under diabetic and hyperglycemic conditions, retinal Müller cells exhibit altered reactivity(Mizutani, Gerhardinger, & Lorenzi, 1998; Rungger-Brandle, Dosso, & Leuenberger, 2000). Although Müller cells have projections
that span the retinal layers, their cell bodies are situated in the INL. The exact mechanism and consequence of altered Müller cell activity on retinal morphology and function is unknown, but it may be associated with retinal thickness irregularities in the INL. In this study, the INL had regions of irregular thickening.

6.5 Neurodegeneration in Specific Retinal Regions

The neurodegeneration underlying the lateral distribution of thickness irregularities within a retinal layer is unclear. Since the type of retinal cells within a retinal layer is consistent, there must be factors other than retinal cell type that contribute to the differences in regional susceptibility to retinal thickness irregularities. Such factors could be related to the morphological, functional and vascular features of the retina.

Morphological Susceptibility

The morphology of each retinal layer may indicate its susceptibility to regional thickness irregularities. Unique distributions of retinal thicknesses in each retinal layer were observed in this study (Figure 7 to Figure 16). In retinal layers that are susceptible to neurodegeneration, it can be speculated that naturally thinner or thicker regions within the layer are related to the magnitude of retinal thickness irregularities. For instance, the GCL is naturally thinnest at the fovea because of the lateral displacement of its cells, thickest in the parafoveal areas (4-6 layers of GCs and 7-11 layers of BCs) and medium thickness in the perifoveal areas (several layers of GCs and 6 layers of BCs)(Schubert, 2009). If the GCs within the GCL were equally exposed to diabetes and susceptible to neurodegeneration, then thicker areas would exhibit more apparent thickness changes. Since thicker areas have a higher density or more layers of GCs, an equal change in each GC would exhibit overall greater thickness changes than areas with a lower density or fewer layers of GCs.

Simply put, retinal regions that are naturally thicker have a greater density of a particular retinal cell. If that particular cell type is susceptible to neurodegeneration, then there are more cells experiencing neurodegeneration relative to a naturally thinner region with less cells. The overall contribution of neurodegeneration from thicker regions may be more detectable than neurodegeneration from thinner regions within a retinal layer. If that is the case, then retinal layers with consistently thin retinal thickness across its layer may be less telling of retinal
thickness irregularities and therefore less useful for the study of retinal thickness irregularities in preclinical DR. In this study, retinal thickness irregularities were observed in the $T_o$ (training set) and $N_i$ (validation set) regions in the GCL. The $T_o$ and $N_i$ regions are within the thicker parafoveal and perifoveal areas of the GCL. The region-specific manifestations of retinal thickness irregularities may be a result of detectability associated with the intrinsic distribution of retinal thicknesses within a layer.

**Functional Susceptibility**

Functionally, the retina can be divided into regions of varying levels of activity. During photopic conditions or daytime vision, light rays are preferentially focused on the fovea. The fovea contains the highest density of cone photoreceptors, which are responsible for high visual acuity. Therefore, during photopic conditions, the fovea has a relatively high level of activity compared to other regions under the same conditions. Consequently, functionally-associated GCs, BCs and their processes have a high level of activity too. Depending on how laterally displaced these second- and third-order neurons are, areas of higher activity may be in the parafovea or perifovea. In preclinical DR, a retinal layer that is susceptible to neurodegeneration may initially develop retinal thickness irregularities in regions that have high levels of activity.

To better illustrate this concept, consider the analogy of a major city highway receiving heavy traffic and a rural street receiving less traffic. If a pothole develops on both roads, the number of incidents and complaints related to the pothole would be greater for the highway since it receives more traffic than the street. Eventually, the pothole on the busy highway will worsen as a result of the number of vehicles driving over it and pavers will be required to repair the pothole. However, in order to repair the pothole, pavers must slow the traffic. In terms of the retina, regions that normally have high levels of activity would be more frequently dysfunctional under neurodegenerative conditions compared to areas with lower levels of activity. Regions with higher levels of activity would constantly flag retinal dysfunction and worsen over time or attract repair mechanisms. Like the pavers slowing traffic to repair the pothole, repair mechanisms in the retina may attempt to repair the neurodegeneration by attracting different cell types and causing local inflammation. In the retina, regions with higher levels of activity may have more detectable thickness irregularities as a result of more frequent dysfunction.
Vascular Susceptibility

The arrangement of the blood vessels in the retina may allude to why certain retinal areas are more susceptible to retinal thickness irregularities. The retinal blood supply to the inner retinal layers emerges from the optic nerve as superior and inferior arteries, which travel toward the nasal hemisphere without crossing the horizontal raphé (Hayreh, 1962). The retinal blood vessels form a laminar meshwork of retinal capillaries which supply the retina with nutrients, such as oxygen (Shimizu & Ujiie, 1978). However, a FAZ measuring 400-500 µm in diameter exists where there is no laminar meshwork. Consequently, the FAZ relies on the diffusion of nutrients from neighbouring capillaries. On the other hand, the choroidal blood supply to the outer retinal layers is more consistent. It has an abundant arterial blood supply, blood vessels that anastomose and a segmental structure, such that there is adequate perfusion of outer retinal layers across the retina (Ring & Fujino, 1967; Weiter & Ernest, 1974). Nonetheless retinal and choroidal blood supplies are altered in preclinical DR (Dimitrova et al., 2001; Nagaoka et al., 2010). However, regarding regional susceptibility to neurodegeneration, the retinal blood supply is more likely to contribute to regional retinal thickness irregularities since it is inconsistent across the retina.

With respect to the arrangement of the retinal blood vessels, temporal regions may be more susceptible to neurodegeneration. In preclinical DR, early impairment of retinal blood flow autoregulation or loss of neurovascular coupling could limit the delivery of nutrients that support and maintain the retina (Clermont et al., 1997; Grunwald et al., 1986; Lecleire-Collet et al., 2011). Physically, retinal blood must travel a greater distance to reach the temporal retina than the nasal retina. Both the loss of retinal blood flow autoregulation and neurovascular coupling may result in insufficient blood pressure, which may still be able to supply the more proximal nasal areas, but not the more distal temporal areas. The consequences of hemodynamic changes or insufficient blood supply are more likely to be experienced by the temporal retina.

Specific regions within a retinal layer may be more susceptible to retinal thickness irregularities depending on morphological, functional and vascular features in the retina. At the moment, much is based on speculation. This is an area of research that has not been elucidated and there may be other factors that contribute to region-specific susceptibility. It is also unknown how much each factor contributes to region-specificity and their interaction. Research concerning the underlying
susceptibility of regions within a retinal layer will be required to understand why retinal thickness irregularities are present in some regions but not others.

6.6 Assumptions Associated with ETDRS Regions

In using the ETDRS grid design to regionalize the retina, a major assumption was that the regions are universally appropriate for all retinal layers. The ETDRS grid was originally designed for grading fluorescein angiograms and subsequently adopted for fundus photography grading of field 2 by the ETDRS (Early Treatment Diabetic Retinopathy Study Research Group, 1991b). In fundus grading, the ETDRS regions divide the macula into subfields to describe the location of edematous retinal thickening and hard exudates. In this investigation, the foveal region approximates the anatomical fovea in the RNFL and GCL accurately. The pericentral and peripheral regions also approximate the anatomical parafovea and perifovea in the GCL accurately. But for all other retinal areas and layers, the ETDRS regions seem arbitrary. Even though the ETDRS grid design is not a “one size fits all” for the retinal layers, it is currently the best design available. Designing a more appropriate grid for studying retinal thickness is not within the scope of this thesis, but would be immensely useful for future investigations of retinal thickness irregularities.

7 Conclusions

In preclinical DR, retinal thickness irregularities were present in specific retinal regions and layers. In this investigation, four regions of retinal thickness irregularities were identified in the training set. Although these four regions were not verified by the validation set, retinal thickness irregularities were without a doubt non-uniform across the retina and within specific retinal layers. This investigation highlighted the use of high-resolution analyses for individual retinal regions and individual retinal layers, while considering the natural variability of the retina. An important consideration for future studies is the specificity by which the retina is partitioned so that retinal thickness irregularities are not masked. Earlier investigations placed less emphasis on these factors and may therefore weaken the accuracy of their results.

With the emerging importance of earlier detection methods for DR, the significance of identifying and localizing preclinical retinal thickness irregularities is rising. Clinically, if retinal
thickness irregularities were detected before the onset of sight-threatening microvascular lesions, then the screening frequency or monitoring level could be increased to catch DR at its onset, when the risk of vision loss is lowest. This investigation does not immediately impact clinical practice, but it serves as an important stepping stone for future investigations in this field. Although considerable emphasis cannot be given to the specific regions of retinal thickness irregularities, this investigation has been useful in describing a valid analytical approach for identifying regions and layers of interest in high-resolution OCT scans and highlighting the non-uniformity of retinal thickness irregularities in preclinical DR.

8 Future Directions
There are three projects that I would like to investigate if given the opportunity to pursue them. The first project is concerned with correlating regions of retinal thickness irregularities with regions of functional deficits in preclinical DR. The second project is identifying regions that exhibit changes in vascular perfusion in preclinical DR. The third project is optimizing the grid design used to regionalize retinal thicknesses.

Correlating Retinal Thickness and Functional Irregularities
The first project investigates the correlation between regions of retinal thickness irregularities and regions of functional deficits in preclinical DR. In most cases, structure is associated with function. For example, a fishing net can be used to catch fish if the net is not torn. Similarly, a retinal cell can receive input and send downstream signals if the cell is structurally intact. There are multiple investigations that have identified functional deficits in retinal regions (Bearse et al., 2006; Harrison et al., 2011; Tan et al., 2014). It would be interesting to see if retinal thickness irregularities occur in the same retinal regions as functional deficits. If regions of retinal thickness irregularities and functional deficits are spatially superimposable, then it would also be interesting to identify the retinal layer that is most correlated with regions of functional deficit. In this investigation, functional mfERG data was collected from all participants as part of another investigation.

Vascular Perfusion across the Retina
The second project investigates the vascular perfusion across the retinal tissue. The retinal and choroidal blood supplies are essential for maintaining retinal physiology by supplying nutrients
and removing wastes. Specifically, the retinal capillaries supply the inner retinal layers and the choroidal choriocapillaris supply the outer retinal layers. In preclinical DR, both blood supplies are affected (Dimitrova et al., 2001; Nagaoka et al., 2010). However, it is unknown how the blood supplies are affected laterally in the retina. Since oxygen is essential for retinal physiology and is delivered by blood, it may be an appropriate marker for the quality of vascular perfusion across the retina. It is important to make the distinction that oxygen saturation is not the same as oxygen consumption. Oxygen saturation is simply the amount of oxygen delivered to a retinal area and oxygen consumption is the demand for oxygen by retinal cells. A shift in either could be present in preclinical DR, but a project investigating vascular perfusion across the retina would be focused on oxygen delivery and saturation. Ultimately this could explain the vascular contribution to regional retinal susceptibility to retinal thickness irregularities.

**Optimizing the Grid Design**

The major assumption associated with using the ETDRS grid in studies of retinal thickness was that the grid is appropriate for all retinal layers. The results of this investigation have illustrated that there is much variability in each retinal layer, such that the ETDRS grid would not generate accurate results if applied universally. There are two possible ways to improve the ETDRS grid design based on the distribution of retinal thicknesses observed in this investigation.

The first is to define regions by hemispheric differences. This can be as simple as rotating the ETDRS grid design by 45°, such that the pericentral and peripheral ring areas are divided by meridians, rather than diagonals. Instead of superior, temporal, inferior and nasal inner and outer regions, there would be superior-temporal, inferior-temporal, inferior-nasal and superior-nasal inner and outer regions. The rationale behind this is that the rotated ETDRS grid follows the natural hemispheric differences in the retina, such as retinal structure, function and vasculature. Given any combination of superior and inferior and nasal and temporal features that may be susceptible to retinal thickness irregularities in preclinical DR, the only possible regional combinations are those of the rotated ETDRS grid.

For example, the superior papillary arteries are retinal vessels that supply the superior hemisphere and branch from the nasal hemisphere towards the temporal hemisphere (Hayreh, 1962). Hypothetically, if diabetes and hyperglycemia in preclinical DR weakened the superior
papillary arteries’ ability to supply nutrients to the retina, then the more distant temporal branches would be affected first. In this scenario, the superior-temporal region would be malnourished. If the retinal malnourishment resulted in retinal neurodegeneration and retinal thickness irregularities in the superior-temporal region, then the original ETDRS grid would only encompass 50% of the retinal thickness irregularities in the superior regions and the other 50% in the temporal regions. The other 50% of the superior and temporal regions would diminish the retinal thickness irregularities. On the other hand, the rotated ETDRS grid would encompass 100% of the retinal thickness irregularities in the superior-temporal regions.

In this investigation, some regions may become significant for retinal thickness irregularities with a rotated, hemispheric ETDRS grid. In between-group comparisons of retinal thickness by subtraction and $t$-statistic, the RNFL had nasal and temporal hemispheric differences in the training set (Figure 17) and OPL and ONL had superior and inferior hemispheric differences in the training set (Figure 21 and Figure 22) and validation set (Figure 32 and Figure 33).

The second is to better approximate the anatomical regions of the retina. At the moment, the ETDRS foveal, pericentral and peripheral areas approximate the foveal, parafoveal and perifoveal anatomical regions, respectively. The division between the foveal and pericentral areas and pericentral and peripheral ring areas are situated at the variable regions of the retina, where retinal thickness transitions occur. This is evident in the mean retinal thickness maps for the GCL, IPL, INL, ONL and OSL (Figure 8 to Figure 10, Figure 12 and Figure 15). This is also evident in the comparisons between-groups by subtraction and $t$-statistic for the ISL and OSL in the training set (Figure 24 and Figure 25). The divisions fall on areas of diffuse retinal thickness, where the primary retinal thickness in one region “bleeds” into another region. Depending on the retinal structure of an individual, an ETDRS region may encompass more or less trivial retinal thicknesses, thereby shifting the “true” retinal thickness in that region.

The aim would be to have regions that contain less of the trivial retinal thicknesses and more of the retinal thicknesses that represent that region. To remove the more variable regions from the analysis of retinal thickness irregularities within regions, the foveal, pericentral and peripheral areas could be made narrower. For example, rather than a 1 mm diameter for the foveal region, 1 and 3 mm diameter for the inner and outer boundaries of the pericentral region and 3 and 6 mm
diameter for the inner and outer boundaries of the peripheral region, the foveal region could be 0.75 mm in diameter, inner and outer boundaries of the pericentral region 1.25 and 2.75 mm in diameter and inner and outer boundaries of the peripheral region 3.25 and 6 mm in diameter. In this example, there is a 0.5 mm wide belt between adjacent retinal regions, where the retinal thicknesses are trivial and not incorporated in the analysis of retinal thickness irregularities. The width of the belt could be increased or decreased to see how that affects the results.

In either grid design, the aim is to better define regions for identifying retinal thickness irregularities. The rationale is not to find a region design that returns the most significant results, but rather to have regions more closely represent the retinal thickness of their anatomical counterpart. For me, it would be exciting to find a region design that is both better at identifying retinal thickness irregularities and associated with retinal anatomy. If retinal thickness irregularities are found in specific regions, then the value in regions associated with retinal anatomy is that it may lend insight into why specific retinal regions are more susceptible to retinal thickness irregularities within a retinal layer.
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doi:10.1016/S0092-8674(00)81106-X


Appendices


RESEARCH ETHICS BOARD

September 13, 2013

Dr. Carol Westall
Ophthalmology
The Hospital for Sick Children

Dear Dr. Westall,

Your study "Structural and Functional Markers of Neuro-Retinal Complications in Adolescents with Early Type 1 Diabetes"

REB File No.: 1000017647

On behalf of the REB, I am writing to confirm that the above noted study was re-approved by the REB for one year ending in September 2014. The REB approved continuing review at level 2D. As necessary, the Clinical Research Office will be contacting you to arrange follow-up.

Please note that, in accordance with the Personal Health Information Protection Act of Ontario, you are responsible for adhering to all conditions and restrictions imposed by the REB governing the use, security, disclosure, return and disposal of the research subjects' personal health information. You are also responsible for reporting immediately any privacy breaches to the REB Chair and to Janice Campbell, the Sick Kids privacy officer.

Yours truly,

Richard Sugarman
Chair, Research Ethics Board

Co-Investigator(s): Thomas Wright

THE HOSPITAL FOR SICK CHILDREN

155 University Ave
Toronto, Ontario
Canada M5G 1X8

www.sickkids.ca

RESEARCH ETHICS BOARD

September 12, 2014

Dr. Carol Westall
Ophthalmology
The Hospital for Sick Children

Dear Dr. Westall:

Your study “Structural and Functional Markers of Neuro-Retinal Complications in Adolescents with Early Type 1 Diabetes”

REB File No.: 1000017647

On behalf of the REB, I am writing to confirm that the above noted study was re-approved by the REB for one year ending in September 2015. The REB approved continuing review at level 2D. As necessary, the Clinical Research Office will be contacting you to arrange follow-up.

Please note that, in accordance with the Personal Health Information Protection Act of Ontario, you are responsible for adhering to all conditions and restrictions imposed by the REB governing the use, security, disclosure, return and disposal of the research subjects' personal health information. You are also responsible for reporting immediately any privacy breaches to the REB Chair and to Janice Campbell, the Sick Kids privacy officer.

Yours truly,

Ronald Grant, M.D.
Chair, Research Ethics Board

Co-Investigator(s): Thomas Wright

555 University Ave
Toronto, Ontario
Canada M5G 1X8

www.sickkids.ca
Appendix C. Note to File Form.

Note to File

Date:

Participant Study ID # (if applicable):

Re:

Completed By:

Name [Position] ____________________________________________________________________________________________

Signature

Reviewed By:

Name [Position] ____________________________________________________________________________________________

Signature

SickKids

Note to File Template
Version Date: April 19, 2013
Appendix D. Quality Improvement Sign-Off by Principal Investigator Form.

Participant Study ID# ________________________

PRINCIPAL/QUALIFIED INVESTIGATOR’S STATEMENT

By signing this form I acknowledge that I have reviewed pages ______ through ________ of this participant’s case report forms and to the best of my knowledge they are accurate and complete.

Principal Investigator signature: ______________________

Date: ________________ (dd/mmm/yyyy)
Appendix E. Research Study Information Pamphlet.

1. **Diabetic Retinopathy**
   - Diabetes affects the whole body, including the eyes. The most common serious eye complication of diabetes is diabetic retinopathy, which is the medical term for damage to the blood vessels of the retina. The retina is the layer at the back of the eye which acts like camera film – it captures what we see and then sends these images to the brain.

2. **Electroretinogram (ERG)**
   - This test allows us to see how cells in the retina respond to light and colour. We will place a small contact lens sensor at the front of the participant’s eye which will pick up electrical signals from the retina.

3. **Multifocal Electroretinogram**
   - In this test, we will ask the participant to watch a pattern of flashing hexagons on the computer screen. This test allows us to record the electrical responses originating from the eye while the participant watches the pattern.

4. **Adaptive Optics**
   - This is a novel non-invasive technology that allows experts to see and record microstructures inside a participant’s retina in real time.

5. **Vision Research at SickKids**
   - The purpose of this research study is to find ways to detect diabetic retinopathy before the retina becomes damaged. Our scientists will perform special tests on the eye to detect diabetic retinopathy. These tests are safe and do not hurt.

**Type 1 Diabetes Study**

**Markers of Retinal Function in Early Diabetes**

**Westall Lab**

**Diabetes and Vision**

**Research Study**

In addition to these tests, we will perform the following standard clinical tests:

- **Ophthalmoscopy**
  - All eye doctors will shine a light into the participants’ eyes to examine the retina.

- **Visual Acuity**
  - We will check how well participants can see at a distance and up close.

- **Fundus Photography**
  - We will take a color photograph of the retina.

**Questions?**

If you have any questions about our research or would like to participate in this study, please contact Aparna Bhan at 416.813.9124 ext. 294170 or via email at: aparna.bhan@sickkids.ca

For more information please visit: http://www.sickkids.ca/visionresearch/
Appendix F. Research Study Advertisement.

VOLUNTEERS NEEDED
FOR STUDIES ON VISION

We are looking for volunteers between the ages of 12 and 45 with or without Type 1 diabetes to participate in our research on visual structure and function. We are investigating a new imaging tool that may be able to diagnose diabetic retinopathy in its early stages. If you agree to participate, we will examine your eyes by carrying out a number of non-invasive vision tests at the Hospital for Sick Children. This normally takes about 3 hours. You will be compensated for your participation in this study.

For more information about this study, please contact:

Aparna Bhan: 416-813-1500 ext. 20470 or aparna.bhan@sickkids.ca

Coor Westall, Ph.D., Principal Investigator Department of Ophthalmology and Vision Sciences, Hospital for Sick Children Toronto, ON, 555 University Avenue, Phone 416-813-6016

Authorization Letter for Disclosure of Personal Health Information for Research Purposes

Dear Dr. ____________________________,

(Doctor’s Name)

I would like to notify you of my participation in the Diabetes and Vision research study at the Visual Electrophysiology Unit (VEU) and Retinal Imaging Lab at the Hospital for Sick Children, under the direction of Dr. Carol Westall.

This letter is to request the release of hemoglobin A1c (HbA1c) values from your files for the purposes of this study, from the time of type 1 diabetes diagnosis until the end of my participation in this study. I recognize that this information reflects long-term blood glucose control. I, ____________________________________________

(Full Legal Name)

indicate that I voluntarily consent to this release of information with my signature below.

Sincerely,

__________________________________________   ____________________________

(Signature)   (Date of Signature: mm/dd/yy)

Last Revised: March 13, 2013
Appendix H. Information Intake Form.

![Information Intake Form](image-url)
Appendix I. Research Consent Form (T1D).

Research Consent Form (Patients with ability to give consent)

Structural and functional markers of neuro-retinal complications in adolescents with early Type 1 diabetes

Investigators:

Principal and Qualified Investigator
Dr. Carol Westall Principal Investigator

Co-/Sub-Investigator
Dr. Melanie Campbell Professor, University of Waterloo

Other Study Team Members
Thomas Wright Research Manager
Yaiza Garcia-Sanchez Research Technologist II
Aparna Bhan Research Coordinator
Alan Poon Graduate Student

Purpose of the research:

The purpose of this study is to help us understand the earliest changes that occur in the eyes of young people with Type 1 diabetes. Current tests for detecting diabetic eye disease look for damage to the blood vessels in the retina (the back of the eye). Based on this treatment is often given late in the disease when there is a risk of vision loss.

Recent evidence indicates that there may be changes to the retina that occur before the blood vessels become damaged. The adaptive optics corrected imaging system will allow us to see the retina in much more detail than is normally possible. This will allow us to see any changes that are happening to the retina before diabetic retinopathy has begun.

We will also evaluate three nerve function tests together with the retinal images to see how well they detect diabetic damage, and how well they can follow changes over time. These tests are the multifocal electroretinogram, the slow-flash multifocal electroretinogram and the full field electroretinogram. This has not been done before.

There are suggestions that diet is important in the management of blood sugar in young people with Type 1 diabetes. We will use a questionnaire to evaluate the food frequency and consumption of fat, fibre, fruits and vegetables. The results of the questionnaire will be compared to information from the nerve function test to determine whether there is any potential effect of dietary habits on the retina. This has not been done before.
We will take detailed images of several areas of your eye, these will be compared with information from the nerve function tests. We will use this information to identify what are the first changes that occur in the retina and to see if any changes get worse over time. This has not been done before.

This study does not take the place of a clinical exam. The clinical guideline is that persons with diabetes have an eye exam at least once per year or more often if recommended by their eye care practitioner.

**Description of the research:**

To take part in this study we will need you to visit the hospital once every year for approximately 3 hours. The study may continue for up to 5 years.

At the first visit the study will be described to you by one of the research team. We will ask you some questions to ensure that you can take part in the study. Any questions you have will be answered and you will be asked to sign this consent form. We will choose one of your eyes that we are going to examine.

At every visit we will do the following tests:

1) We will use a finger prick test to test your blood glucose.

2) We will measure your blood pressure.

3) Vision: We will examine your vision by asking you to read some letters on a regular vision chart to measure what you can see. We will also test how well you can see colour and read fading letters.

4) We will dilate your eye. This involves putting drops into your eye which will make your pupil bigger. This will take about 20 minutes to take effect and may last for 4-8 hours afterwards. While your eye is dilated your vision may be blurred.

5) We will ask you to complete a questionnaire about your dietary habits.

6) Electroretinography. Three different tests will look at the nerve function in your eye. For all these tests we will place a small contact lens sensor (electrode) on the surface of your eye. We will use an anesthetic drop to numb the front of your eye.

   a. Multifocal Electroretinogram (mFERG) and Slow-Flash Electroretinogram (sFERG). For both of these tests we will ask you to watch a pattern of flashing hexagons while we record the electrical responses that your eye makes. Each test will take about 10 minutes.

   b. Photopic Negative Response (PhNR). This test will use a different machine to the mFERG and sFERG, however the contact lens sensor will be the same. You will be asked to look at a blue background light while we flash several dim red lights. Again this test will take about 10 minutes.
7) Fundus photography. We will take some pictures of the inside of your eye. These will be examined by a doctor to identify any problems that might already exist.

8) Refraction. We will test if you are near sighted (able to see things better up close than far away), far sighted (able to see things better far away than close), or if you have clear vision for both close and far. We will then shine a light into your eyes and hold lenses in front of your eyes.

9) Adaptive optics enhanced imaging. The imaging process is similar to having photographs taken of the back of your eye. You will be asked to sit and look at a small light in the machine. For some of the images the light may move and you will be asked to follow it with your eye. The testing will be painless and is not dangerous. It will not be possible to recognize you from the images we take. We expect the imaging to take about one hour, however there will be plenty of breaks during this time.

We will need to gather some more information about your blood glucose control from your hospital records. If you have taken part in any other studies with our group we will also compare your results from this study with that data.

For this study we will be testing 180 people with diabetes as well as 180 people without diabetes.

**Potential Harms:**

We know of no harms that this study could cause you. There may be harms we do not know about.

**Potential Discomforts or Inconvenience:**

Testing will take about 3 hours in total and you will need to come to the hospital.

Your eye may sting when we first put in the drops to dilate you. This is similar to getting grit in your eye but will only last for a few seconds.

While your eye is dilated your vision may be affected. This may stop you doing things like reading a book. You can also be sensitive to bright lights. These effects may last until the next day but usually your eye will return to normal after 4-8 hours.

**Potential Benefits:**

To you:

You will not benefit directly from participating in this study. If we identify any special problems with your eyes we will give you special help if needed.

To Society:

Consent Form 9th May 2012

Patients with the ability to give consent

Page 3 of 6
This research will help us to understand the earliest changes that happen to the eyes of people with diabetes. In the future this may help the development of new treatments for diabetic retinopathy.

Confidentiality:

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless required by law. For example, the law could make us give information about you if a child has been abused, if you have an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

Sick Kids Clinical Research Monitors, employees of the funder or sponsor [Canadian Institute of Health Research], or the regulator of the study may see your health record to check on the study. By signing this consent form, you agree to let these people look at your records. For example, people from Health Canada Health Products and Food Branch, if necessary, may look at your records. We will put a copy of this research consent form in your patient health record and give you a copy as well.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study the data will be kept as long as required then destroyed as required by Sick Kids policy. Published study results will not reveal your identity.

Reimbursement:

We will reimburse you for all your reasonable out of pocket expenses for being in this study eg., meals, babysitters, parking and getting you to and from Sick Kids. If you stop taking part in the study, we will pay you for your expenses for taking part in the study up until that point.

We will also provide you with some compensation, $10 / hour, in recognition of your time and effort.

Participation:

It is your choice to take part in this study. You can stop at any time. The care you get at Sick Kids will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

Consent Form 9th May 2012
Patients with the ability to give consent
Page 4 of 6
In some situations, the study doctor or the company paying for the study may decide to stop the study. If this happens, the study doctor will talk to you about what will happen next.

If you become ill or are harmed because of study participation, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The staff of the study, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

You may be involved in, or in the future be asked to be involved in other research studies, either with us or with other investigators. Your decisions about this study do not affect any other studies.

The results of this study will be presented annually at the Family Day meetings hosted by the endocrinology department. At the conclusion of the study we will write to you describing our findings.

Sponsorship:

The sponsor of this study is Dr. Carol Westall and The Hospital for Sick Children. This study is being funded by the Canadian Institute for Health Research (CIHR).

Conflict of Interest:

The principal investigator, Dr. Carol Westall and the other research team members have no conflict of interest to declare.
Consent:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.

2) You have explained the possible harms and benefits (if any) of this study.

3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at Sick Kids.

4) I am free now, and in the future, to ask questions about the study.

5) I have been told that my medical records will be kept private except as described to me.

6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.

I have read and understood pages 1-6 of this consent form. I agree, or consent, to take part in this study.

Printed Name of Subject & Age

Subject’s signature & date

Printed Name of person who explained consent

Signature of Person who explained consent & date

Printed Witness’ name (if the subject/legal guardian does not read English)

Witness’ signature & date

If you have any questions about this study, please call Aparna Bhan at 416 813 7654 ext. 204170 or Carol Westall at 416 813 6516.

If you have question about your rights as a subject in a study or injuries during a study, please call the Research Ethics Manager at 416 813 5718.

Consent Form 9th May 2012

Page 6 of 6

Patients with the ability to give consent
Appendix J. Research Consent Form (Control).

Research Consent Form (Control subjects with ability to give consent)

Structural and functional markers of neuro-retinal complications in adolescents with early Type 1 diabetes

Investigators:

Principal and Qualified Investigator
Dr. Carol Westall  Principal Investigator

Co-/Sub-Investigator
Dr. Melanie Campbell  Professor, University of Waterloo

Other Study Team Members
Thomas Wright  Research Manager
Yaiza Garcia-Sanchez  Research Technologist II
Aparna Bhan  Research Coordinator  416 813 7654 x 204170
Alan Poon  Graduate Student

Purpose of the research:
The purpose of this study is to help us understand the earliest changes that occur in the eyes of young people with Type 1 diabetes. Current tests for detecting diabetic eye disease look for damage to the blood vessels in the retina (the back of the eye). Based on this, treatment is often given late in the disease when there is a risk of vision loss.

You do not have diabetes, we will use the images collected from your eye to compare with patients who do.

Recent evidence indicates that there may be changes to the retina that occur before the blood vessels become damaged. The adaptive optics corrected imaging system will allow us to see the retina in much more detail than is normally possible. This will allow us to see any changes that are happening to the retina before diabetic retinopathy has begun.

We will also evaluate three nerve function tests together with the retinal images to see how well they detect diabetic damage, and how well they can follow changes over time. These tests are the...
multifocal electrotoretinogram, the slow-flash multifocal electrotoretinogram and the full field electrotoretinogram. This has not been done before.

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This study does not take the place of a clinical exam. The clinical guideline is that persons with diabetes have an eye exam at least once per year or more often if recommended by their eye care practitioner.

Description of the research:

To take part in this study we will need you to visit the hospital once every year for approximately 3 hours. The study may continue for up to 5 years.

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2) We will measure your blood pressure.
3) Vision: We will examine your vision by asking you to read some letters on a regular vision chart to measure what you can see. We will also test how well you can see colour and read fading letters.
4) We will dilate your eye. This involves putting drops into your eye which will make your pupil bigger. This will take about 20 minutes to take effect and may last for 4-8 hours afterwards. While your eye is dilated your vision may be blurred.
5) We will ask you to complete a questionnaire about your dietary habits.
6) Electrotetinoigraphy. Three different tests will look at the nerve function in your eye. For all these tests we will place a small contact lens sensor (electrode) on the surface of your eye. We will use an anesthetic drop to numb the front of your eye.
a. Multifocal Electroretinogram (mfERG) and Slow-Flash Electroretinogram (sfERG). For both of these tests we will ask you to watch a pattern of flashing hexagons while we record the electrical responses that your eye makes. Each test will take about 10 minutes.

b. Photopic Negative Response (PhNR). This test will use a different machine to the mfERG and sfERG, however the contact lens sensor will be the same. You will be asked to look at a blue background light while we flash several dim red lights. Again this test will take about 10 minutes.

7) Fundus photography. We will take some pictures of the inside of your eye. These will be examined by a doctor to identify any problems that might already exist.

8) Refraction. We will test if you are near sighted (able to see things better up close than far away), far sighted (able to see things better far away than close), or if you have clear vision for both close and far. We will then shine a light into your eyes and hold lenses in front of your eyes.

9) Adaptive optics enhanced imaging. The imaging process is similar to having photographs taken of the back of your eye. You will be asked to sit and look at a small light in the machine. For some of the images the light may move and you will be asked to follow it with your eye. The testing will be painless and is not dangerous. It will not be possible to recognize you from the images we take. We expect the imaging to take about one hour, however there will be plenty of breaks during this time.

If you have taken part in any other studies with our group we will also compare your results from this study with that data.

For this study we will be testing 180 people with diabetes as well as 180 people without diabetes.

Potential Harms:

We know of no harms that this study could cause you. There may be harms we do not know about.

Potential Discomforts or Inconvenience:

Testing will take about 3 hours in total and you will need to come to the hospital.

Your eye may sting when we first put in the drops to dilate you. This is similar to getting grit in your eye but will only last for a few seconds.

While your eye is dilated your vision may be affected. This may stop you doing things like reading a book. You can also be sensitive to bright lights. These effects may last until the next day but usually your eye will return to normal after 4-8 hours.
Potential Benefits:

To you:

You will not benefit directly from participating in this study. If we identify any special problems with your eyes we will give you special help if needed.

To Society:

This research will help us to understand the earliest changes that happen to the eyes of people with diabetes. In the future this may help the development of new treatments for diabetic retinopathy.

Confidentiality:

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless required by law. For example, the law could make us give information about you if a child has been abused, if you have an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

Sick Kids Clinical Research Monitors, employees of the funder or sponsor [Canadian Institute of Health Research], or the regulator of the study may see your health record to check on the study. By signing this consent form, you agree to let these people look at your records. For example, people from Health Canada Health Products and Food Branch, if necessary, may look at your records. We will put a copy of this research consent form in your patient health record and give you a copy as well.

The data produced from this study will be stored in a secure, locked location. Only members of the research team [and maybe those individuals described above] will have access to the data. This could include external research team members. Following completion of the research study the data will be kept as long as required then destroyed as required by Sick Kids policy. Published study results will not reveal your identity.

Reimbursement:

Consent Form 9th May 2012
Page 4 of 6
Control subjects with the ability to give consent
We will reimburse you for all your reasonable out of pocket expenses for being in this study e.g., meals, babysitters, parking and getting you to and from Sick Kids. If you stop taking part in the study, we will pay you for your expenses for taking part in the study up until that point.

We will also provide you with some compensation, $10 / hour, in recognition of your time and effort.

**Participation:**

It is your choice to take part in this study. You can stop at any time. The care you get at Sick Kids will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study.

In some situations, the study doctor or the company paying for the study may decide to stop the study. If this happens, the study doctor will talk to you about what will happen next.

If you become ill or are harmed because of study participation, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The staff of the study, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

You may be involved in, or in the future be asked to be involved in other research studies, either with us or with other investigators. Your decisions about this study do not affect any other studies.

The results of this study will be presented annually at the Family Day meetings hosted by the endocrinology department. At the conclusion of the study we will write to you describing our findings.

**Sponsorship:**

The sponsor/funder of this study is Dr. Carol Westall and The Hospital for Sick Children. This study is being funded by the Canadian Institute for Health Research (CIHR).

**Conflict of Interest:**

The principal investigator, Dr. Carol Westall, and the other research team members have no conflict of interest to declare.
Consent:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.

I have read and understood pages 1-6 of this consent form. I agree, or consent, to take part in this study.

________________________________________  ________________________________________
Printed Name of Subject & Age                      Subject’s signature & date

________________________________________  ________________________________________
Printed Name of person who explained consent      Signature of Person who explained consent & date

________________________________________
Printed Witness’ name (if the subject/legal guardian does not read English)  Witness’ signature & date

If you have any questions about this study, please call Aparna Bhan at 416 813 7654 ext. 204170 or Carol Westall at 416 813 8516.

If you have question about your rights as a subject in a study or injuries during a study, please call the Research Ethics Manager at 416 813 5718.
Appendix K. Case Report Form.

**CASE REPORT FORM, REB#: 1000017047**

“Structural and functional markers of neuro-retinal complications in adolescents with early Type 1 diabetes” Dr. Carol Westall - Principal Investigator

### 1.0 Patient Information

- **Patient Number:**
- **Initials:**
- **Testing Session #:**
- **Date:** (dd/mm/yy)
- **Gender**: □ M □ F
- **Time/Date Booked:**
- **Time Arrived:**

### 2.0 Ophthalmic Examination

- **Tester’s Initial:**
- **Time Started**
- **Time Finished**

#### Visual Acuity (ETDRS)

- **UnCorrected**
- **Corrected**

#### Color Vision (with)

#### Color Vision (minimal)

- **Contrast Sensitivity (Pelli-Robson):**

---

### Ophthalmic Examination – intake scoring sheet

#### Visual Acuity

<table>
<thead>
<tr>
<th>ETD (Chart 1)</th>
<th>ETD (Chart 2)</th>
<th>ETD (Chart 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR KDC</td>
<td>1.0</td>
<td>RNOVS 1.0</td>
</tr>
<tr>
<td>DN CHV</td>
<td>0.9</td>
<td>ZCRDH 0.9</td>
</tr>
<tr>
<td>CDHNR</td>
<td>0.8</td>
<td>NVSOK 0.8</td>
</tr>
<tr>
<td>RVZOS</td>
<td>0.7</td>
<td>DRZKO 0.7</td>
</tr>
<tr>
<td>OSVDZ</td>
<td>0.6</td>
<td>SNHCV 0.6</td>
</tr>
<tr>
<td>NOZCD</td>
<td>0.5</td>
<td>CRVSZ 0.5</td>
</tr>
<tr>
<td>RDNSK</td>
<td>0.4</td>
<td>VKCCH 0.4</td>
</tr>
<tr>
<td>OKSVZ</td>
<td>0.3</td>
<td>SVKDN 0.3</td>
</tr>
<tr>
<td>KSNHO</td>
<td>0.2</td>
<td>KDHZC 0.2</td>
</tr>
<tr>
<td>HOVSN</td>
<td>0.1</td>
<td>HZCRO 0.1</td>
</tr>
<tr>
<td>VCSZH</td>
<td>0.0</td>
<td>OKDHN 0.0</td>
</tr>
<tr>
<td>CZDRV</td>
<td>-0.1</td>
<td>ZONKC -0.1</td>
</tr>
<tr>
<td>SHRZC</td>
<td>-0.2</td>
<td>RISVD -0.2</td>
</tr>
<tr>
<td>DNOKR</td>
<td>-0.3</td>
<td>DSORZ -0.3</td>
</tr>
</tbody>
</table>

#### Colour vision - Minimalist

P D T

#### Colour vision - HRR pseudoisochromatic plates

- **Demonstration Series**
- **Diagnostic Series**

---

**Contrast Sensitivity: Pelli-Robson Chart**

<table>
<thead>
<tr>
<th>Grade</th>
<th>0.00</th>
<th>0.30</th>
<th>0.60</th>
<th>0.90</th>
<th>1.20</th>
<th>1.50</th>
<th>1.80</th>
<th>2.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.45</td>
<td>0.75</td>
<td>1.05</td>
<td>1.35</td>
<td>1.65</td>
<td>1.95</td>
<td>2.25</td>
<td></td>
</tr>
</tbody>
</table>
## Case Report Form, REB# 100007647

"Structural and functional markers of neuro-retinal complications in adolescents with early Type 1 diabetes" Dr. Carol Westall: Principal Investigator

<table>
<thead>
<tr>
<th>Eye Drops</th>
<th>Tester’s Initial:</th>
<th>Fundus Photography</th>
<th>Tester’s Initial:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Pupil Diameter</td>
<td>Time Started</td>
</tr>
</tbody>
</table>

**Comments:**

<table>
<thead>
<tr>
<th>Eye Examination</th>
<th>Time Started</th>
<th>Time Finished</th>
<th>Tester’s Initial:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctiva</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior chamber</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refractive Error</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Comments:**

### 3.0 Experimental Tests

<table>
<thead>
<tr>
<th>AO Imaging</th>
<th>Tester’s Initial:</th>
<th>MFERG</th>
<th>Tester’s Initial:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Started</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time Finished</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ERG</th>
<th>Tester’s Initials:</th>
<th>A-Scan</th>
<th>Tester’s Initials:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Started</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time Finished</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 4.0 Blood Sugar Levels

**Notes:**

- Pre-Intake: _______________  HR: _______________
- Pre-Imaging: _______________  BP: _______________
- Pre-mFERG: _______________
- Post-mFERG: _______________

Most recent blood sugar levels (if known)

<table>
<thead>
<tr>
<th>Date: (dd/mm/yy)</th>
<th>Fasting:</th>
<th>P.P.:</th>
<th>HbA1c:</th>
</tr>
</thead>
</table>

Last Modified: June 13, 2012  VEU Lab  Page 2
Appendix L. Diabetes Screening Questionnaire.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. History of normal visual development before diabetes</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>2. LogMAR visual acuity of 0.3 or better</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>3. 5 years or more duration of diabetes</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hemoglobinopathy (when unable to obtain HbA1c reading)</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>2. Has background retinopathy more severe than minimal non proliferative diabetic retinopathy (diabetic retinopathy levels 10, 11, 14, 20 or broad grades 10.21(62))</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>3. Distance refractive error &gt; ± 5.08 &amp; / or &gt; ≈ 2.5 D</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>4. Other disease (inherited, inflammatory, infectious, etc.) including papillary opacity greater than NC-2, NO2, O22and or P1</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>5. CNS, psychiatric, neurological disorders, or disorders known to affect retinal/visual function</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>6. On systemic medication with known CNS effects</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

2.0 Diabetes Screening Questionnaire

In the past month, have you noticed any of the following changes or symptoms?

<table>
<thead>
<tr>
<th>Change or Symptom</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased frequency to urinate?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Waking up at night to urinate?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Increased thirst?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Drinking fluids more than usual?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Weight loss (when not planning to)?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Feeling more tired than usual?</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

3.0 Notes

September 13, 2012
Appendix M. Ophthalmic Imaging Form.

<table>
<thead>
<tr>
<th><strong>DATE OF VISIT</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>LAST NAME</td>
</tr>
<tr>
<td>DATE OF BIRTH</td>
</tr>
<tr>
<td>YY</td>
</tr>
<tr>
<td>ADDRESS</td>
</tr>
</tbody>
</table>

**IMPRINT OR ENTER DETAILS BY HAND**
- IN PATIENT
- OUT PATIENT
- EMERGENCY
- RESEARCH

**DIAGNOSIS/CLINICAL FINDINGS**
- After Imaging
- Patient may:
  - LEAVE
  - WAIT
- Please print clearly:
- Complete drawing on reverse side of return.

**Study:** Structural and functional markers of neuro-retinal complications in adolescents with early Type 1 diabetes

**Subject ID:** Test ID: Eye:

- Obtain consent for use of patient photography.

**FUNDUS IMAGING**
- Area of Interest:
  - Right Eye
  - Left Eye
  - Both Eyes
- Discus
- Macula
- Nerve Fibre Layer
- Choroidal Layer
- Periphery 7 Fields
- Stereo of macula
- Fundus Photography
- Fundus Autofluorescence
- Fluorescein Angiography
- Optical Coherence Tomography (OCT)
- Macular Cube 512 x 128

**ULTRASOUND**
- B-Scan
- A-Scan
- UBM

**ANTERIOR SEGMENT (AS), EXTERNAL IMAGING**
- Slit Lamp
- Gonioscopy
- External Photography
- External Video
- Corneal Topography
- Specular Microscopy
- AS-OCT
  - VDU
  - CDI
  - BMO

**Area of Interest:**
- Right Eye
- Left Eye
- Both Eyes
- Conjunctival surface
- Scleral

**Glasses:**
- Full Face
- Head posture
- Patient must look:
  - Head tilt 45°
  - Eye to View
  - Eyelid:
  - Corrected
  - Uncorrected
  - Cover test

**DILATION ORDERS**
- Before Imaging
- After Imaging

<table>
<thead>
<tr>
<th>Before Imaging</th>
<th>Administer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Eye</td>
<td>Penciclovezine 0.5%</td>
</tr>
<tr>
<td>Left Eye</td>
<td>Phenylephrine 2.5%</td>
</tr>
</tbody>
</table>

- Administered by: Date:
- Print Name: Time:

**Photographer’s Signature:** Date:
- Print Name: Time:

**Staff Physician:** (Print) Signature: Date/Time: Room #

**Examing Physician:** (Print) Signature: Date/Time: Room #

Form # 50233 (6/012) Chart Copy Page 1 of 2
Appendix N. Letter of Agreement from Doheny Eye Institute (UCLA).

**MUTUAL NONDISCLOSURE AGREEMENT**

This agreement is made this 1st day of November, 2014 by and between The Hospital for Sick Children, located at 555 University Avenue, Toronto, Ontario M5G 1X8 ("SickKids") and the Doheny Eye Institute with principal offices at 1450 San Pablo Street, Los Angeles, CA 90033, ("DEI").

1. **The Purpose.** DEI and SickKids (each a “Party” and collectively the “Parties”) wish to explore and possibly pursue a potential research relationship relating to automatic segmentation of Optical Coherence Tomography (OCT) or automated OCT segmentation, in connection with the exploration or actual pursuit of that research relationship (the “Relationship”), the Parties may disclose their respective Confidential Information (as defined below) to each other.

2. **Definition of Confidential Information.** Confidential Information means any information, technical data, or know-how, including but not limited to, that which relates to research, product plans, products, services, customers, markets, software, developments, inventions, processes, designs, drawings, engineering, hardware configuration information, marketing or finances of DEI or SickKids, which all shall be deemed as Confidential Information. Confidential Information does not include information, technical data or know how which (i) is in the possession of the receiving Party at the time of disclosure as shown by the receiving Party’s files and records immediately prior to the time of disclosure, or (ii) prior to or after the time of disclosure becomes part of the public knowledge or literature other than as a result of any improper inaction or action of the receiving Party, or (iii) is approved by the disclosing Party, in writing, for release.

3. **Nondisclosure of Confidential Information.** The receiving Party agrees not to use any Confidential Information disclosed to it for its own use or for any purpose other than to carry out discussions concerning, and the undertaking of the Relationship. The receiving Party will not disclose any Confidential Information belonging to the other Party to parties outside the Relationship or to its employees other than employees or agents under appropriate burden of confidentiality and who are required to have the information in order to carry out the discussions regarding the Relationship. The receiving Party agrees that it will take all reasonable measures to protect the secrecy of and avoid disclosure or use of Confidential Information of the other Party in order to prevent it from falling into the public domain or the possession of persons other than those persons authorized under this Agreement to have any such information. Such measures shall include the highest degree of care that the receiving Party utilizes to protect its own Confidential Information, and in no event less than objectively reasonable care. The receiving Party agrees to notify the other Party in writing of any misuse or misappropriation of Confidential Information of the other Party, which may come to receiving Party’s attention. If a court of competent jurisdiction orders a receiving Party to disclose a disclosing Party’s Confidential Information, that receiving Party will, to the extent legally permissible, (i) give the disclosing Party prior written notice sufficient to seek an appropriate protective order and (ii) cooperate with the disclosing Party (at the disclosing Party’s expense) in seeking that order.

4. **Publicity.** The receiving Party will not, without prior consent of the other Party, disclose to any other person, the fact that Confidential Information has been disclosed under this agreement, that discussions or negotiations are taking place between the Parties, or any of the terms conditions, status or other facts with respect thereto, except as required by law and then only with prior notice as soon as possible to the other Party.

5. **Return of Materials.** Any confidential materials or documents that have been furnished by one Party to the other in connection with the Relationship will be promptly returned by the receiving Party, accompanied by all copies of such documentation or certification of destruction, within (10) days after (i) the Relationship has been terminated or (ii) receipt of the written request of the disclosing Party.

6. **Intellectual Property.** Nothing in this agreement is intended to grant any rights to the receiving Party with regard to any Confidential Information disclosed by the disclosing Party, and each Party specifically reserves and retains all rights to the intellectual property and proprietary information and materials disclosed including but not limited to any rights to trademarks, patents, copyrights or trade secrets.
7. Term. The foregoing commitment of the Parties shall survive any termination of the Relationship between the Parties for a period of five years after application of Section 5 above.

8. Successors and Assigns. This agreement shall be binding upon and for the benefits of the undersigned Parties, their successors and assigns, provided that Confidential Information of the other Party may not be disclosed without the prior written consent of that Party. Failure to enforce any provision of this Agreement shall not constitute a waiver of any term hereof.

9. Governing Law. Any legal action, claim or other legal proceeding commenced by one Party hereto against another Party, arising out of this Agreement, shall be commenced in the courts of the jurisdiction in which the responding Party is situated; and for the purposes of such proceeding, this Agreement shall be governed by, and shall be interpreted, construed and enforced, in accordance with the laws of that same jurisdiction.

10. Remedies. The receiving Party agrees that any violation or threatened violation may cause irreparable injury, both financial and strategic, to the other and in addition to any and all remedies that may be available, in law, in equity or otherwise, the disclosing Party may seek injunctive relief against the threatened breach of this Agreement.

In witness whereof, this Mutual Nondisclosure Agreement is executed as of the date first above written:

The Donnelly Eye Institute:

By: __________________________
Name: Marissa Goldberg
Title: Executive Director, Chief Financial Officer

The Hospital for Sick Children:

By: __________________________
Name: Ramona Pears, Executive Director
Title: The Hospital for Sick Children

ACKNOWLEDGED AND AGREED TO:

By: __________________________
Name: Dr. Carol Westall, Ph.D.
Title: Vision Scientist, The Hospital for Sick Children
Appendix O. Letter of Agreement from Vision & Imaging Laboratory (Duke University).

From: Sina Farsiu, Ph.D. [sina.farsiu@duke.edu]
Sent: June 7, 2015 3:35 PM
To: Thomas Wright
Subject: RE: OCT segmentation and other stuff

Hi Tom,

I have been notified that I need to get an IRB exemption for my image segmentation collaborations with you. Our IRB manager needs an email from collaborators stating that their studies have IRB (at their home institute) and the images that they send me are deidentified (so I can pass them to my students without using Duke secure computers and you and me can pass them back and forth online with Dropbox, which Duke does not consider secure). She accepts a simple email acknowledgment (from whomever the IRB is attained by, if not you, then Carol?). Could you please send me an email like the following (or edit it as you wish):

Dear Sina,

Thank you for agreeing to collaborate with us on our ongoing project which requires segmentation of ophthalmic images including optical coherence tomography images by you. By this email, I acknowledge that our study was conducted under an approved IRB protocol from the Research Ethics Board at The Hospital for Sick Children. The ophthalmic images that we send you are deidentified. We do not require a contract or agreement with Duke. It is acceptable to transmit or store these deidentified images via potentially unsecure transfer/storage protocols such as Dropbox.

Tom Wright, PhD
Research Associate
The Hospital for Sick Children
University of Toronto

Thanks,
Sina

From: Carol Westall [mailto:carol.westall@sickkids.ca]
Sent: Monday, June 8, 2015 10:23 AM
To: Sina Farsiu, Ph.D.
Subject: IRB exemption for my image segmentation

Dear Sina,

Thank you for agreeing to collaborate with us on our ongoing project which requires segmentation of ophthalmic images including optical coherence tomography images by you. By this email, I acknowledge that our study was conducted under an approved IRB protocol from the Research Ethics Board at The Hospital for Sick Children. The ophthalmic images that we send you are deidentified. We do not require a contract or agreement with Duke. It is acceptable to transmit or store these deidentified images via potentially unsecure transfer/storage protocols such as Dropbox.

Kind Regards

Carol

Carol Westall PhD FARVO FAAO
Senior Scientist
The Hospital for Sick Children
Professor
Department of Ophthalmology and Vision Sciences
University of Toronto

The Hospital for Sick Children | 555 University Ave | Toronto, ON, Canada M5G 1X8
Phone: 416.813.7654 ext. 206516 | e-mail: carol.westall@sickkids.ca
http://www.sickkids.ca/Research/VEU/Research/index.html
Appendix P. Custom MATLAB® Scripts.

Note that training data may be referred to as “prelim” or “preliminary” and validation data may be referred to as “test”.

### Import and generate all 11 retinal surfaces (training set)

```matlab
% Create pathlist
prelim_participant = {'204', '304', '310', '346', '451', '497', '498', ...
  '499', '504', '507', '508', '521', '522'};

% Create empty list to store all prelim_participant surfaces
prelim_group_s = {};

% Read each participant's .xml file, format to a 128x512x11 matrix, and ...
for iparticipant = 1:length(prelim_participant);
  dat = processXmlSurfaceFile(...
    sprintf('%s%d%s', 'C:\Users\alan poon\Documents\OCTTest\...
    'Surfaces by OCTExplorer - Prelim\',...
    (prelim_participant{iparticipant}),...
    '_Sequence_Surfaces_Iowa.xml'));
  %eval(['prelim_participant_' prelim_participantID{iparticipant} '=dat;']);
  prelim_group_s{iparticipant} = dat;
end

% Convert all prelim_group_s surfaces to the same configuration
% For prelim_group_s, convert OD to OS
for iparticipant = [3 4 5 9 10 12 13];
  prelim_group_s{iparticipant} = fliplr(prelim_group_s{iparticipant});
end
% For prelim_group_s, invert up/down
for iparticipant = 1:length(prelim_participant);
  prelim_group_s{iparticipant} = flipud(prelim_group_s{iparticipant});
end
```

### Import and generate all 11 retinal surfaces (validation set)

```matlab
% Create pathlist
test_participant = {'304_OS', '310_OD', '316_OD', '346_OD', '347_OD', ...
  '384_OD', '461_OS', '490_OD', '491_OS', '496_OD', '500_OD', ...
  '515_OD', '523_OD', '525_OS', '526_OD', '527_OD', '528_OD', ...
  '529_OS', '530_OS', '531_OD', '532_OS', '535_os', '538_od', ...
  '539_od', '540_os', '544_os', '545_os', '546_os', '547_os', ...
  '548_od', '549_od', '550_os', '552_os', '553_od', '556_os', ...
  '557_od', '558_os', '559_os', '560_os', '561_os'};

% Create empty list to store all test_participant surfaces
test_group_s = {};
```
% Read each participant's .xml file, format to a 128x512x11 matrix, and
% add to participants list
for iparticipant = 1:length(test_participant);
    dat = processXmlSurfaceFile...
    (sprintf('C:\Users\alan poon\Documents\OCTTest\...
        'Surfaces by OCTExplorer - Test\',(test_participant{iparticipant}),...%
        '_512x128_cube_z_sequence_Surfaces_Iowa.xml'));
    %eval([['participant_' test_participant{iparticipant} ']=dat;']);
    test_group_s{iparticipant} = dat;
end

% Convert all test_group_s surfaces to the same configuration
% For test_group_s, convert OD to OS
for iparticipant = [2 3 4 5 6 8 10 11 12 13 15 16 17 20 23 24 30 31 34 36];
    test_group_s{iparticipant} = fliplr(test_group_s{iparticipant});
end

Generate retinal layer thicknesses from surfaces (training set)
Note: 1 to 10 are individual retinal layer thicknesses; 11 is total retinal layer thickness
% Create empty list to store all retinal layer thicknesses
prelim_group_t = {};

% Calculate retinal layer thicknesses
for iparticipant = 1:length(prelim_participant);
    dat = zeros(128,512,11);
    % Calculate individual retinal layer thicknesses
    for iSurface = 2:11;
        dat(:,iSurface - 1) = prelim_group_s{iparticipant}(:,iSurface)...% - prelim_group_s{iparticipant}(:,iSurface - 1);
    end
    % Calculate total retinal layer thickness
    dat(:,11) = prelim_group_s{iparticipant}(:,11)...% - prelim_group_s{iparticipant}(:,1);
    prelim_group_t{iparticipant} = dat;
end

Generate retinal layer thicknesses from surfaces (validation set)
Note: 1 to 10 are individual retinal layer thicknesses; 11 is total retinal layer thickness
% Create empty list to store all retinal layer thicknesses
test_group_t = {};

% Calculate retinal layer thicknesses
for iparticipant = 1:length(test_participant);
    dat = zeros(128,512,11);
    % Calculate individual retinal layer thicknesses
for iSurface = 2:11;
    dat(:,:,iSurface - 1) = test_group_s{iparticipant}(:,:,iSurface)...
    - test_group_s{iparticipant}(:,:,iSurface - 1);
end
% Calculate total retinal layer thickness
dat(:,:,11) = test_group_s{iparticipant}(:,:,11)...
    - test_group_s{iparticipant}(:,:,1);
test_group_t{iparticipant} = dat;

Calculate between-group differences by subtraction

% Training set
prelim_mean_diff_retlayer = zeros(128,512,10);
preflat_mean_diff_retlayer(:,:,1) = prelim_mean_retlayer_T1D(:,:,1) -
    prelim_mean_retlayer_con(:,:,1);
preflat_mean_diff_retlayer(:,:,2) = prelim_mean_retlayer_T1D(:,:,2) -
    prelim_mean_retlayer_con(:,:,2);
preflat_mean_diff_retlayer(:,:,3) = prelim_mean_retlayer_T1D(:,:,3) -
    prelim_mean_retlayer_con(:,:,3);
preflat_mean_diff_retlayer(:,:,4) = prelim_mean_retlayer_T1D(:,:,4) -
    prelim_mean_retlayer_con(:,:,4);
preflat_mean_diff_retlayer(:,:,5) = prelim_mean_retlayer_T1D(:,:,5) -
    prelim_mean_retlayer_con(:,:,5);
preflat_mean_diff_retlayer(:,:,6) = prelim_mean_retlayer_T1D(:,:,6) -
    prelim_mean_retlayer_con(:,:,6);
preflat_mean_diff_retlayer(:,:,7) = prelim_mean_retlayer_T1D(:,:,7) -
    prelim_mean_retlayer_con(:,:,7);
preflat_mean_diff_retlayer(:,:,8) = prelim_mean_retlayer_T1D(:,:,8) -
    prelim_mean_retlayer_con(:,:,8);
preflat_mean_diff_retlayer(:,:,9) = prelim_mean_retlayer_T1D(:,:,9) -
preflat_mean_retlayer_con(:,:,9);
preflat_mean_diff_retlayer(:,:,10) = prelim_mean_retlayer_T1D(:,:,10) -
preflat_mean_retlayer_con(:,:,10);

% Validation set
test_mean_diff_retlayer = zeros(128,512,10);
test_mean_diff_retlayer(:,:,1) = test_mean_retlayer_T1D(:,:,1) -
    test_mean_retlayer_con(:,:,1);
test_mean_diff_retlayer(:,:,2) = test_mean_retlayer_T1D(:,:,2) -
    test_mean_retlayer_con(:,:,2);
test_mean_diff_retlayer(:,:,3) = test_mean_retlayer_T1D(:,:,3) -
    test_mean_retlayer_con(:,:,3);
test_mean_diff_retlayer(:,:,4) = test_mean_retlayer_T1D(:,:,4) -
    test_mean_retlayer_con(:,:,4);
test_mean_diff_retlayer(:,:,5) = test_mean_retlayer_T1D(:,:,5) -
    test_mean_retlayer_con(:,:,5);
test_mean_diff_retlayer(:,:,6) = test_mean_retlayer_T1D(:,:,6) -
test_mean_retlayer_con(:,:,6);
test_mean_diff_retlayer(:,:,7) = test_mean_retlayer_T1D(:,:,7) - test_mean_retlayer_con(:,:,7);
test_mean_diff_retlayer(:,:,8) = test_mean_retlayer_T1D(:,:,8) - test_mean_retlayer_con(:,:,8);
test_mean_diff_retlayer(:,:,9) = test_mean_retlayer_T1D(:,:,9) - test_mean_retlayer_con(:,:,9);
test_mean_diff_retlayer(:,:,10) = test_mean_retlayer_T1D(:,:,10) - test_mean_retlayer_con(:,:,10);

**Calculate between-group differences by t-statistic (training set)**

prelim_group_t_tstat = zeros(128,512,11);

% Reshape each preliminary T1D participant into rows
tmp1 = zeros(5,65536,11);
for iparticipant = 1:5; % Participants 1 to 5 in prelim_group_t are T1D
    for iLayer = 1:11;
        dat = zeros(1,65526);
        dat = reshape(prelim_group_t{iparticipant}(:,:,iLayer),[1,65536]);
        tmp1(iparticipant,:,iLayer) = dat;
    end
end

% Reshape each preliminary control participant into rows
tmp2 = zeros(8,65536,11);
for iparticipant = 6:13; % Participants 6 to 13 in prelim_group_t are controls
    for iLayer = 1:11;
        dat = zeros(1,65526);
        dat = reshape(prelim_group_t{iparticipant}(:,:,iLayer),[1,65536]);
        tmp2(iparticipant - 5,:,iLayer) = dat;
    end
end

% Calculate the T-statistic
[h,p,ci,stat] = ttest2(tmp1,tmp2);
for iLayer = 1:11;
    prelim_group_t_tstat(:,:,iLayer) = reshape(stat.tstat(:,:,iLayer),[128 512]);
end

% Where the T-statistic is NaN, change to 0
prelim_group_t_tstat(isnan(prelim_group_t_tstat)) = 0;
% Where the T-statistic is Inf or -Inf, change to 0
prelim_group_t_tstat(isinf(prelim_group_t_tstat)) = 0;

**Calculate between-group differences by t-statistic (validation set)**

test_group_t_tstat = zeros(128,512,11);
% Reshape each test T1D participant into rows
for iparticipant = [3 6 7 19 20 23 26 31 36 37 40];
    for iLayer = 1:11;
        dat = zeros(1,65526);
        dat = reshape(test_group_t{iparticipant}(:,:,iLayer),[1,65536]);
        tmp3(iparticipant,:,iLayer) = dat;
    end
end

% Reshape to remove rows of zeros
for iparticipant = [8 9 10 11 12 13 14 15 16 17 18 21 24 27 28 29 30 32 33 34 35 38 39];
    tmp4(iparticipant,:,iLayer) = dat;
end

% Reshape each test control into rows
for iparticipant = [8 9 10 11 12 13 14 15 16 17 18 21 24 27 28 29 30 32 33 34 35 38 39];
    for iLayer = 1:11;
        dat = zeros(1,65526);
        dat = reshape(test_group_t{iparticipant}(:,:,iLayer),[1,65536]);
        tmp4(iparticipant,:,iLayer) = dat;
    end
end

% Reshape to remove rows of zeros
for iparticipant = [8 9 10 11 12 13 14 15 16 17 18 21 24 27 28 29 30 32 33 34 35 38 39];
    tmp4A(iparticipant,:,iLayer) = dat;
end
tmp4A(11,:,:) = tmp4(18,:,:);
tmp4A(12,:,:) = tmp4(21,:,:);
tmp4A(13,:,:) = tmp4(24,:,:);
tmp4A(14,:,:) = tmp4(27,:,:);
tmp4A(15,:,:) = tmp4(28,:,:);
tmp4A(16,:,:) = tmp4(30,:,:);
tmp4A(17,:,:) = tmp4(32,:,:);
tmp4A(18,:,:) = tmp4(33,:,:);
tmp4A(19,:,:) = tmp4(34,:,:);
tmp4A(20,:,:) = tmp4(35,:,:);
tmp4A(21,:,:) = tmp4(38,:,:);
tmp4A(22,:,:) = tmp4(39,:,:);
tmp4A(23,:,:) = tmp4(40,:,:);

% Calculate the T-statistic
[h,p,ci,stat] = ttest2(tmp3A,tmp4A);
for iLayer = 1:11;
    test_group_t_tstat(:,:,iLayer) = reshape(stat.tstat(:,:,iLayer),[128 512]);
end
% Where the T-statistic is NaN, change to 0
test_group_t_tstat(isnan(test_group_t_tstat)) = 0;

Create the ETDRS region divisions
Note: Layers 1 to 10 are individual retinal layer thicknesses; layer 11 is total retinal layer thickness

% Column 1: Si
% Column 2: Ti
% Column 3: Ii
% Column 4: Ni
% Column 5: So
% Column 6: To
% Column 7: Io
% Column 8: No
% Column 9: Fovea
% Column 10: InnerRing
% Column 11: OuterRing
% Column 12: OuterRegion (whole ETDRS)

% Create ETDRS regions
% triUR (upper right triangle)
    triUR=zeros(128,512,1);
square=ones(128,128);
square=triu(square,1);
idx=repmat(1:128,128,1);
triUR=square(:,idx(:));
% triLL (lower left triangle)
    triLL=zeros(128,512,1);
square=ones(128,128);
square=tril(square,-1);
idx=repmat(1:128,4,1);
triLL=square(:,idx(:));
% triUL (lower right triangle)
triUL=fliplr(triLL);
% triS (superior triangle)
triS=(triUL==triUR);
triS(64:128,:,1)=0;
% triI (inferior triangle)
triI=rot90(triS,2);

% triLL (lower left triangle)
triLL=zeros(128,512,1);
square=ones(128,128);
square=tril(square,0);
idx=repmat(1:128,4,1);
triLL=square(:,idx(:));
% triUR (upper right triangle)
triUR=zeros(128,512,1);
square=ones(128,128);
square=triu(square,0);
idx=repmat(1:128,4,1);
triUR=square(:,idx(:));
% triUL (lower right triangle)
triUL=fliplr(triUR);
% triN (nasal triangle)
triN=(triUL==triLL);
triN(:,256:512,1)=0;
% triT (temporal triangle)
triT=rot90(triN,2);

% Fovea
\[
\begin{align*}
t & = 0:\pi/20:2\pi; \\
R1 & = 42.66666666666667; \ x0 = 256; \ y0 = 64; \\
xi & = R1^\circ \cos(t)+x0; \\
yi & = R1^\circ \sin(t)/4+y0;
\end{align*}
\]

\%LineHandler = line(xi,yi,'LineWidth',1,'Color',[0 0 0]);
Fovea = poly2mask(xi, yi, 128, 512);

% InnerRegion
\[
\begin{align*}
t & = 0:\pi/20:2\pi; \\
R2 & = 128; \ x0 = 256; \ y0 = 64; \\
xi & = R2^\circ \cos(t)+x0; \\
yi & = R2^\circ \sin(t)/4+y0;
\end{align*}
\]

\%LineHandler = line(xi,yi,'LineWidth',1,'Color',[0 0 0]);
InnerRegion = poly2mask(xi, yi, 128, 512);

% OuterRegion
t = 0:pi/20:2*pi;
R3 = 256; x0 = 256; y0 = 64;
xi = R3*cos(t)+x0;
yi = R3*sin(t)/4+y0;
%LineHandler = line(xi,yi,'LineWidth',1,'Color',[0 0 0]);
OuterRegion = poly2mask(xi, yi, 128, 512);

% InnerRing
InnerRing = InnerRegion - Fovea;
% Si
Si=InnerRing;
Si=Si+triS;
Si(Si<=1)=0;
Si(Si==2)=1;
% Ti
Ti=InnerRing;
Ti=Ti+triT;
Ti(Ti<=1)=0;
Ti(Ti==2)=1;
% Ii
Ii=InnerRing;
Ii=Ii+triI;
Ii(Ii<=1)=0;
Ii(Ii==2)=1;
% Ni
Ni=InnerRing;
Ni=Ni+triN;
Ni(Ni<=1)=0;
Ni(Ni==2)=1;
% OuterRing
OuterRing = OuterRegion - InnerRegion;
% So
So=OuterRing;
So=So+triS;
So(So<=1)=0;
So(So==2)=1;
% To
To=OuterRing;
To=To+triT;
To(To<=1)=0;
To(To==2)=1;
% Io
Io=OuterRing;
Io=Io+triI;
Io(Io<=1)=0;
Io(Io==2)=1;
% No
% Create list of ETDRS regions
region = {Si Ti Ii Ni So To Io No Fovea InnerRing OuterRing OuterRegion};

Tabulate participant, retinal layer, region, location and thickness for LME model in -R-(training set)
Headings: Patient Layer Region Pixel Thickness

prelim_table = zeros(6669000,5);

% Create list of regions
region = {Si Ti Ii Ni So To Io No Fovea};

% Organize participant thicknesses into one list
prelim_group_list = {};
prefim_group_list = prelim_group_t;

% Fill in table
for iparticipant = 1:length(prelim_group_list);
    for iLayer = 1:10;
        dat1 = zeros(128,512);
        dat1 = prelim_group_list{iparticipant}(:,:,iLayer);
        for iRegion = 1:length(region);
            dat2 = zeros(1,1); % Reset dat2 since its length varies
            dat2 = dat1(region{iRegion}==1);
            i = nnz(prelim_table(:,1)) + 1;
            j = length(dat2) + i - 1;
            prelim_table(i:j,1) = iparticipant;
            prelim_table(i:j,2) = iLayer;
            prelim_table(i:j,3) = iRegion;
            prelim_table(i:j,4) = (1:length(dat2))';
            prelim_table(i:j,5) = dat2(1:length(dat2),1);
        end
    end
end
csvwrite('prelim_table.csv',prelim_table)

Tabulate participant, retinal layer, region, location and thickness for LME model in -R-(validation set)
Headings: Patient Layer Region Pixel Thickness

test_table = zeros(17955000,5,1);
% Organize participant thicknesses into one list

% Add test_group_t T1D to cells 1 to 12
% [3 6 7 19 20 22 23 26 31 36 37 40]
test_group_list{1} = test_group_t{3};
test_group_list{2} = test_group_t{6};
test_group_list{3} = test_group_t{7};
test_group_list{4} = test_group_t{19};
test_group_list{5} = test_group_t{20};
test_group_list{6} = test_group_t{22};
test_group_list{7} = test_group_t{23};
test_group_list{8} = test_group_t{26};
test_group_list{9} = test_group_t{31};
test_group_list{10} = test_group_t{36};
test_group_list{11} = test_group_t{37};
test_group_list{12} = test_group_t{40};

% Add test_group_t control to cells 13 to 35
% [8 9 10 11 12 13 14 15 16 17 18 21 24 27 28 29 30 32 33 34 35 38 39]
test_group_list{13} = test_group_t{8};
test_group_list{14} = test_group_t{9};
test_group_list{15} = test_group_t{10};
test_group_list{16} = test_group_t{11};
test_group_list{17} = test_group_t{12};
test_group_list{18} = test_group_t{13};
test_group_list{19} = test_group_t{14};
test_group_list{20} = test_group_t{15};
test_group_list{21} = test_group_t{16};
test_group_list{22} = test_group_t{17};
test_group_list{23} = test_group_t{18};
test_group_list{24} = test_group_t{21};
test_group_list{25} = test_group_t{24};
test_group_list{26} = test_group_t{27};
test_group_list{27} = test_group_t{28};
test_group_list{28} = test_group_t{29};
test_group_list{29} = test_group_t{30};
test_group_list{30} = test_group_t{32};
test_group_list{31} = test_group_t{33};
test_group_list{32} = test_group_t{34};
test_group_list{33} = test_group_t{35};
test_group_list{34} = test_group_t{38};
test_group_list{35} = test_group_t{39};

% Fill in table
for iparticipant = 1:length(test_group_list);
    for iLayer = 1:10;
        dat1 = zeros(128,512);
dat1 = test_group_list{iparticipant}(:,:,iLayer);
for iRegion = 1:length(region);
    dat2 = zeros(1,1); % Reset dat2 since its length varies
    dat2 = dat1(region{iRegion}==1);
    i = nnz(test_table(:,1)) + 1;
    j = length(dat2) + i - 1;
    test_table(i:j,1) = iparticipant;
    test_table(i:j,2) = iLayer;
    test_table(i:j,3) = iRegion;
    test_table(i:j,4) = (1:length(dat2))';
    test_table(i:j,5) = dat2(1:length(dat2),1);
end
end
csvwrite('test_table.csv', test_table)
Appendix Q. Custom ‘R’ Scripts.

---
title: "OCT Region / Layer analysis"
author: "Tom Wright"
date: "October 7, 2015"
output: pdf_document
---

Analysis of OCT data by layer / region

```
require(nlme)
require(lsmeans)
options(contrasts = c(factor = "contr.treatment", ordered = "contr.poly"))
path = "/mnt/AO/Diabetes OCT Segmented data"
filename_prelim = 'prelim_table.csv'
filename_test = 'test_table.csv'
```

```
{r loadData, echo=FALSE, results='asis'}
data_test = read.csv(file.path(path,filename_test),
header=FALSE,
col.names = c('Subject','Layer','Region','Pixel','Thickness'),
colClasses = c(rep('factor',4),'numeric'))
data_prelim = read.csv(file.path(path,filename_prelim),
header=FALSE,
col.names = c('Subject','Layer','Region','Pixel','Thickness'),
colClasses = c(rep('factor',4),'numeric'))
```

data_test$Diabetic<-factor(rep('No',nrow(data_test)),levels = c('Yes','No'))
data_test$Diabetic[data_test$Subject %in% 1:12]<-'Yes'
data_prelim$Diabetic<-factor(rep('No',nrow(data_prelim)),levels = c('Yes','No'))
data_prelim$Diabetic[data_prelim$Subject %in% 1:5]<-'Yes'
contrasts(data_test$Diabetic)<-contr.sum
contrasts(data_prelim$Diabetic)<-contr.sum

...# Preliminary data
```
{r prelim_analysis,echo=FALSE}
model_lme.form <- formula(Thickness ~ Diabetic)

nLayers = length(levels(data_prelim$Layer))
nRegions = length(levels(data_prelim$Region))
#Preallocate the output
output = matrix(numeric(),
nrow=nLayers,
ncol=nRegions,
dimnames=list(1:nLayers,1:nRegions))
output_pvals = matrix(numeric(),
nrow=nLayers,
ncol=nRegions,
dimnames=list(1:nLayers,1:nRegions))
notes = matrix(character(),
nrow=nLayers,
ncol=nRegions)
ThicknssValues = matrix(numeric(),
   nrow=0,
   ncol=14)

curLayer = 0
for(layer in levels(data_prelim$Layer)){
   curRegion = 0
   curLayer = curLayer + 1
   row.names(output)[curLayer] = paste('Layer',layer)
   row.names(output_pvals)[curLayer] = paste('Layer',layer)
   for(region in levels(data_prelim$Region)){
      curRegion = curRegion + 1
      colnames(output)[curRegion] = paste('Region',region)
      colnames(output_pvals)[curRegion] = paste('Region',region)

      subData<-subset(data_prelim,Layer==layer & Region==region)
      fval=NA
      pval=NA
      tryCatch({
         fm1<-lme(model_lme.form,
            random =~1|Subject,
            data=subData,
            control=lmeControl(opt='optim'))
         x=lsmeans(fm1,~Diabetic)
         ThicknessValues <- rbind(ThicknessValues,
            c(layer,
            region,
            as.numeric(confint(x)[1,]),
            as.numeric(confint(x)[2,])))
         fval = anova(fm1)[2,3]
         pval = anova(fm1)[2,4]
      },warning = function(w){
         notes[curLayer,curRegion] = w
      },error = function(e){
         notes[curLayer,curRegion] = e$message
         fval = NA
         pval = NA
      })
      output[curLayer,curRegion] = fval
      output_pvals[curLayer,curRegion] = pval
   }
}
cat('F-Values
')
round(output,4)
cat('P-Values
')
round(output_pvals,4)
if (!all(is.na(notes))){
   cat('Notes
')
   print(notes)
}

ThicknessValues<-data.frame(ThicknessValues)
names(ThicknessValues)<-
   c('Layer','Region','Diabetic_Y','Thickness_Y','SE_Y','df_Y','Lower.CL_Y','Upper.CL_Y',  
   'Diabetic_N','Thickness_N','SE_N','df_N','Lower.CL_N','Upper.CL_N')
write.csv(ThicknessValues,file.path(path,'output_estimates_prelim.csv'))
write.csv(output,file.path(path,'output_fvals_prelim.csv'))
write.csv(output_pvals,file.path(path,'output_pvals_prelim.csv'))
```
#Validation data

```{r validation_analysis,echo=FALSE}
model_lme.form <- formula(Thickness ~ Diabetic)

nLayers = length(levels(data_test$Layer))
nRegions = length(levels(data_test$Region))

#Preallocate the output
output = matrix(numeric(),
    nrow=nLayers,
    ncol=nRegions,
    dimnames=list(1:nLayers,1:nRegions))

output_pvals = matrix(numeric(),
    nrow=nLayers,
    ncol=nRegions,
    dimnames=list(1:nLayers,1:nRegions))

notes = matrix(character(),
    nrow=nLayers,
    ncol=nRegions)

ThicknessValues = matrix(numeric(),
    nrow=0,
    ncol=14)

curLayer = 0
for(layer in levels(data_test$Layer)){
    curRegion = 0
    curLayer = curLayer + 1
    row.names(output)[curLayer] = paste('Layer',layer)
    row.names(output_pvals)[curLayer] = paste('Layer',layer)
    for(region in levels(data_test$Region)){
        curRegion = curRegion + 1
        colnames(output)[curRegion] = paste('Region',region)
        colnames(output_pvals)[curRegion] = paste('Region',region)
        subData<-subset(data_test,Layer==layer & Region==region)
        fval=NA
        pval=NA
        tryCatch({
            fm1<-lme(model_lme.form,
                random =~1|Subject,
                data=subData,
                control=lmeControl(opt='optim'))
            x=lsmeans(fm1,~Diabetic)
            ThicknessValues <- rbind(ThicknessValues,
                c(layer,
                region,
                as.numeric(confint(x)[1,]),
                as.numeric(confint(x)[2,])))
            fval = anova(fm1)[2,3]
            pval = anova(fm1)[2,4]
        },warning = function(w){
            notes[curLayer,curRegion] = w
        },error = function(e){
            notes[curLayer,curRegion] = e$message
            fval = NA
            pval = NA
        })
        output[curLayer,curRegion] = fval
        output_pvals[curLayer,curRegion] = pval
    }
}
```
cat('F-Values\n')
round(output,4)
cat('P-Values\n')
round(output_pvals,4)

if (!all(is.na(notes))){
  cat('Notes\n')
  print(notes)
}

ThicknessValues<-data.frame(ThicknessValues)
names(ThicknessValues)<-
c('Layer','Region','Diabetic_Y','Thickness_Y','SE_Y','df_Y','Lower.CL_Y','Upper.CL_Y',
  'Diabetic_N','Thickness_N','SE_N','df_N','Lower.CL_N','Upper.CL_N')

write.csv(ThicknessValues,file.path(path,'output_estimates_validation.csv'))
write.csv(output,file.path(path,'output_fvals_validation.csv'))
write.csv(output_pvals,file.path(path,'output_pvals_validation.csv'))

...
Appendix R. Estimates of Mean Retinal Thickness for Training Set.

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Appendix S. Estimates of Mean Retinal Thickness for Validation Set.

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