Poor Glycemic Control is Associated with Neuroretinal Dysfunction in Short-Wavelength Cone Pathways of Adolescents with Type 1 Diabetes

by

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

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Master of Science
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Abstract

Studies demonstrate short-wavelength cone pathway dysfunction in patients with diabetes and no clinically visible DR. Poor glycemic control, as measured by hemoglobin A1c (HbA1c), is a strong risk factor for DR. We hypothesized that raised HbA1c was associated with short-wavelength cone sensitive visual evoked potential (S-VEP) and electroretinogram (sERG) dysfunction.

Forty adolescents with diabetes and 39 controls were tested using the S-VEP. Latencies to a short-wavelength stimulus were delayed in patients at low contrasts. Patient S-VEP latencies were not associated with HbA1c when controlling for age and time since diagnosis.

Twenty-one adolescents with diabetes and 19 controls were tested using the sERG. Implicit times of the b-wave were delayed but not associated with HbA1c when controlling for time since diagnosis. Patient PhNR amplitudes were reduced. A one-unit increase in HbA1c was associated with a 15% sERG PhNR amplitude reduction (p=0.004).

The sERG PhNR may be a potential biomarker for DR.
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List of Abbreviations

CIE 1931 Commission Internationale d’Eclairage
CDA Canadian Diabetes Association
CSME Clinically significant macular edema
CVDs Colour vision deficiencies
DCCT Diabetes Control and Complications Trial
DR Diabetic retinopathy
ERG Electroretinogram
ETDRS Early Treatment Diabetic Retinopathy Study (ETDRS)
GFAP Glial fibrillary acidic protein
HbA₁c Glycosylated hemoglobin
L-cone Long wavelength sensitive cone
L/M Long- and medium wavelength (red-green) sensitive cone pathway
L/M-VEP L/M-cone pathway visual evoked potential
M-cone Medium wavelength sensitive cone
NPDR Nonproliferative diabetic retinopathy
PhNR Photopic negative response
PDR Proliferative diabetic retinopathy
RGCs Retinal ganglion cells
S/(L+M) Short-wavelength sensitive (blue-yellow) cone pathway, S-cone pathway
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<td>sERG</td>
<td>Short-wavelength sensitive cone electroretinogram</td>
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<tr>
<td>S-cone</td>
<td>Short-wavelength sensitive cone</td>
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<td>S-VEP</td>
<td>S-cone pathway visual evoked potential</td>
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<td>T1D</td>
<td>Type 1 diabetes</td>
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<td>WDRS</td>
<td>Wisconsin Diabetes Registry Study</td>
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<tr>
<td>WESDR</td>
<td>Wisconsin Epidemiologic Study of Diabetic Retinopathy</td>
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<tr>
<td>V1</td>
<td>Primary visual cortex</td>
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<td>VEGF</td>
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<td>VEP</td>
<td>Visual evoked potential</td>
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1. Background

1.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder characterized by the presence of abnormally high blood glucose levels (hyperglycemia). It is caused by defective insulin secretion by pancreatic beta cells, defective insulin action or both (CDA, 2008a). Individuals with uncontrolled diabetes mellitus are unable to transport glucose into fat and muscle cells and are thus said to demonstrate glucose intolerance. To satisfy the energy needs of these cells, the breakdown of fat and protein is increased (Porth, Gaspard, & Matfin, 2006). The classic symptoms of diabetes are excessive urination (polyuria), thirst (polydipsia), hunger (polyphagia), and weight loss ("Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus," 2003)

Epidemiology and Economic Burden

An estimated 285 million people worldwide have diabetes (IDF, 2009). With a yearly increase of 7 million people worldwide diagnosed with diabetes, the predicted prevalence in 2030 is 438 million (IDF, 2009). Currently, over 3 million people in Canada have diabetes (CDA, 2010). This is more than doubled from an estimated 1.3 million in the past decade (CDA, 2009). The estimated economic burden of diabetes is $12.2 billion and will rise by another $4.7 billion by 2020 (CDA, 2009). Complications of diabetes such as heart disease, stroke, end-stage renal disease, and blindness incur increased costs to health care spending and increase wait times in hospitals (CDA, 2008a; CIHI, 2007). Rising obesity rates, sedentary lifestyles, and an aging population drive these increases (CDA, 2009). The estimated financial burden of vision loss in Canada is higher than that of cancer and cardiovascular disease (CNIB, 2009).
Types of Diabetes

Diabetes is classified into three major subtypes of disorders – type 1, type 2, and gestational diabetes. Other specific types of diabetes include diseases of the exocrine pancreas and endocrinopathies (CDA, 2008a).

Type 1 diabetes typically occurs in children and adolescents. It is primarily the result of an immune-mediated destruction of insulin-producing pancreatic beta-cells (CDA, 2008a). The vast majority of Canadians and individuals worldwide with diabetes (~90%) have Type 2 diabetes ("Diabetes in Canada," 2003; Porth, et al., 2006). It develops typically later in life and is associated with obesity. Type 2 diabetes may range from predominant insulin resistance with relative insulin deficiency to a predominant secretory defect with insulin resistance. Gestational diabetes mellitus refers to glucose intolerance with the onset or first recognition of pregnancy (CDA, 2008a).

Among the types of diabetes, there is increasing concern about the burden of type 1 diabetes on young populations. The clinical symptoms, etiology, epidemiology, management protocol, and complications of type 1 diabetes are further described.
1.2 Type 1 Diabetes Mellitus

1.2.1 Definition and Clinical Symptoms

Type 1 diabetes (T1D) is a chronic metabolic disorder characterized by low or absent levels of endogenously produced insulin. It is caused by the gradual and selective destruction of insulin-producing pancreatic beta-cells. In 95% of individuals with T1D, beta-cell destruction is autoimmune-mediated (Type 1A) (Porth, et al., 2006). This autoimmune destruction is thought to be caused by the dysregulation of autoaggressive T-cells and the subsequent infiltration of these cells as well as macrophages into pancreatic islets (Foulis, McGill, & Farquharson, 1991; Willcox, Richardson, Bone, Foulis, & Morgan, 2009). Autoantibodies to beta-cell antigens are present in plasma long before the onset of T1D and evidence islet reactivity (Porth, et al., 2006; Ziegler, Hummel, Schenker, & Bonifacio, 1999). The minority of T1D cases cannot be explained by autoimmunity and are classified as “idiopathic” (Type 1B) (Porth, et al., 2006).

Clinical symptoms of T1D are unlike the classic symptoms of diabetes, however patients are particularly prone to diabetic ketoacidosis. Diabetic ketoacidosis is a consequence of absolute or relative insulin deficiency resulting in hyperglycemia and an accumulation of ketone bodies in the blood with subsequent metabolic acidosis (Silverstein, et al., 2005). If left untreated, this condition can lead to circulatory failure and coma (Porth, et al., 2006). Symptoms of T1D become apparent when there is an 80-85% reduction in functional pancreatic beta-cell mass (Kaufman, 2003).
1.2.2 Etiology

The risk of T1D is modulated by genetic factors but environmental factors are thought to trigger and drive beta-cell destruction in genetically susceptible individuals (Kaufman, 2003).

Haplotypes DR4-DQ8 and/or DR3-DQ2 are in 95% of patients with T1D and provide the greatest risk for T1D development when inherited together (Caillat-Zucman, et al., 1992). In individuals with a genetic predisposition for T1D, the activation of naïve autoreactive T-cells occurs in pancreatic lymph nodes upon encountering an environmental trigger. Environmental factors implicated in the pathogenesis of T1D include viral exposure (most notably enteroviruses) in the perinatal period, toxins and nutritional factors (Kaufman, 2003; Knip, 2003).

1.2.3 Epidemiology

Type 1 diabetes occurs more commonly in children and adolescents, with males and females affected equally (Soltesz, Patterson, & Dahlquist, 2007). Peaks of presentation occur between 5-7 years of age and at puberty around 12 years of age. Girls are affected earlier than boys due to the associated gender effect of puberty (Soltesz, et al., 2007). Nearly 80% of individuals are diagnosed with T1D before 30 years of age but it can develop well into adulthood. Up to 10% of adults are described as having a latent form of T1D (Porth, et al., 2006).

Type 1 diabetes accounts for 5-10% of all case of diabetes. The prevalence is 15 million worldwide and 2 million in Europe and North America (Reimann, et al., 2009). There are temporal, seasonal, and geographical patterns of T1D incidence. Temporally, there is an increasing trend in the incidence of T1D in most regions of the world over the last few decades (Rosenbauer, Herzig, Von Kries, Neu, & Giani, 1999). The world-wide annual increase of T1D is 3.0% (IDF, 2009). Seasonally, there is a higher incidence of T1D in the winter in countries with marked differences in seasonal temperatures (Soltesz, et al., 2007). Lastly, geographically, the incidence of T1D appears to be highest in regions further away from the equator (Haller,
Atkinson, & Schatz, 2005). The annual incidence of T1D in Canada (21.7/100 000) and the United States (16.1/100 000) are among the highest rates world-wide (Soltesz, et al., 2007).

1.2.4 Management of T1D

Current standards of diabetes management recommend that patients achieve normal or near-normal glucose control while avoiding incidents of abnormally low blood glucose levels (hypoglycemia, plasma glucose <4.0mmol/L) (CDA, 2008b; Porth, et al., 2006). Severe hypoglycemia can result in confusion, seizure and/or coma. The most widely used clinical test to evaluate long-term glycemic control is glycated hemoglobin in the blood. Hemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}) values represent the mean glucose concentration in systemic circulation over the preceding 3-4 months (Porth, et al., 2006) (see section 1.3.5). All patients with T1D require exogenous insulin replacement (CDA, 2008b).

Age-specific glycemic goals have been suggested by the American Diabetes Association (Silverstein, et al., 2005). Since children and adolescents with T1D are at an increased risk for insulin-induced hypoglycemia, the intensity of glycemic control must be balanced against the risk and consequences of hypoglycemia (CDA, 2008b; Silverstein, et al., 2005). Physicians tailor treatment goals for each patient based on the patient’s ability to detect hypoglycemia, motivation for self-management, and lifestyle (Silverstein, et al., 2005).

The glycemic targets for most patients with T1D are an HbA\textsubscript{1c} value of $\leq 7.0\%$, fasting or preprandial plasma glucose between 4.0 and 7.0 mmol/L, and a 2-hour postprandial plasma glucose between 5 and 10 mmol/L (5.0-8.0 mmol/L if HbA\textsubscript{1c} targets are not met) (CDA, 2008b). Diabetes can be well-managed with education and support provided by an interdisciplinary team of diabetes health care professionals (CDA, 2008a).

The recommended management protocol for T1D includes three insulin injections daily or the use of an insulin pump, multiple blood glucose readings, HbA\textsubscript{1c} measurements every 3-4 months and an appropriate meal plan and exercise regime (CDA, 2008b; Silverstein, et al., 2005).
1.2.5 Complications

Chronic complications of T1D include nephropathy, dyslipidemia, neuropathy, hypertension, and retinopathy (CDA, 2008a).

Nephropathy is a renal disease characterized by an elevated albumin kidney excretion rate and gross proteinuria (Silverstein, et al., 2005). After 25 years of T1D, the incidence of nephropathy is approximately 9.0% (Bojestig, Arnqvist, Hermansson, Karlberg, & Ludvigsson, 1994).

Dyslipidemia is characterized by elevations in triglyceride and total cholesterol levels (Taskinen, 2002). Triglyceride and cholesterol components are artherogenic, leading to cardiovascular, cerebrovascular, and peripheral vascular disease (Kreisberg, 1998; Silverstein, et al., 2005). Children with T1D have a higher total cholesterol than the general population (Maahs, et al., 2005) and are more likely to develop early atherosclerotic changes (Järvisalo, et al., 2002; Parikh, et al., 2000).

Neuropathy results in a gradual loss of both large myelinated and unmyelinated nerve fibers that is characterized by progressive peripheral sensory loss. Symptomatic neuropathy is uncommon in children and adolescents with T1D, but sub-clinical impairment of neurologic function has been reported in up to 68% of pediatric patients (Hyllienmark, Brismar, & Ludvigsson, 1995; Karavanaki & Baum, 1999).

Hypertension or high blood pressure is a common problem of patients with diabetes. The incidence of hypertension in patients with T1D rises from 5% after 10 years of T1D to 33% after 20 years (Epstein & Sowers, 1992).

Lastly, diabetic retinopathy is a sight-threatening complication of diabetes characterized clinically by progressive alterations in the retinal vasculature (LM Aiello & Cavallerano, 1997). Diabetic retinopathy is the main topic of this thesis and will be discussed further in the following sections.
1.3 Diabetic Retinopathy

1.3.1 Epidemiology

Diabetic retinopathy (DR) is a chronic, progressive sight-threatening complication of diabetes. It is the leading cause of visual impairment and blindness among working age individuals in Canada ("CNIB Client Database," 2002) and the rest of the developed world (BEK Klein, 2007). In the United States, an estimated 86% of people with T1D have some degree of DR (Varma, 2008).

Studies conducted in the 1980s showed that the prevalence of DR in patients with T1D for 15 or more years, who were primarily of European origin, was over 90%. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) was a large-population based prospective study on the incidence and progression of DR. It included patients with T1D and concluded that DR was prevalent in nearly all patients with T1D (98%) for 20 or more years. In this population, 3.6% of patients were legally blind (R Klein, BE Klein, SE Moss, MD Davis, & DL DeMets, 1984a). Smaller prospective studies by Palmberg and co-workers (1981) and Burger et al. (1986) have come to similar conclusions. Palmberg et al. (1981) found that in a cohort of 461 patients with T1D, the prevalence of DR approached 90% after 17 years (Palmberg, et al., 1981). Similarly, Burger et al. (1986) found that in a cohort of 231 patients with T1D, the prevalence of DR was 92% after 15 years (Burger, Hövener, Düsterhus, Hartmann, & Weber, 1986).

There is a growing body of evidence which suggests that the prevalence of DR has since declined in recent years. This may be due to increased insulin use, glucose monitoring, and education programs which promote dilated eye examinations for earlier detection and treatment of DR (R. Klein, 2009). A population-based study by Le Claire et al. (2006) found that in a cohort of 474 patients with T1D, the prevalence of DR was 73% after 14 years of T1D. There was lower prevalence (10%) of moderate-severe NPDR compared to the WESDR study (35%). A lower prevalence of DR, ranging from 10% to 60%, has been reported by studies in Finland, Sweden, France, Denmark, and New Zealand (Falck, Kää, & Laatikainen, 1993; Henricsson, et
Less information is available regarding the incidence and progression of DR in population-based studies. According to the WESDR, the 10-year incidence and rate of progression of DR among patients with T1D was 89% and 76% respectively. The rate of progression to PDR in this population was 30% (R. Klein, Klein, Moss, & Cruickshanks, 1994).

1.3.2 Clinical Features

Diabetic retinopathy is commonly described as a progressive disease of the retinal vasculature (Antonetti, et al., 2006). It is diagnosed clinically by the presence of ophthalmoscopically visible lesions to the vascular structures of the retina and related deposits (ETDRS, 1985). The clinical features of DR are the result of increased vascular permeability, vascular closure, and retinal ischemia (Antonetti, et al., 2006).

The classification scale established by the Early Treatment Diabetic Retinopathy Study (ETDRS) is the gold standard for DR classification. The ETDRS broadly categorizes DR as either non-proliferative or proliferative (ETDRS, 1985).

Nonproliferative DR (NPDR) refers to the presence of clinically detectable microvascular abnormalities before the proliferation of new retinal blood vessels (neovascularization). NPDR can be subdivided into stages of severity: mild-to-moderate, moderate-to-severe, severe, and very severe. Proliferative DR (PDR) is the stage at which neovascularization is present (ETDRS, 1991b). As NPDR progresses to more severe stages, the risk of progression to PDR and diabetic macular edema increases (ETDRS, 1991a).
Nonproliferative Diabetic Retinopathy

Mild to moderate NPDR

Mild to moderate NPDR is characterized by the presence of retinal capillary microaneurysms, intraretinal hemorrhages, hard exudates, diabetic macular edema, and foveal avascular zone abnormalities (ETDRS, 1991b).

Retinal capillary microaneurysms are focal aborted new vessel growth or saccular outpouchings of the capillary wall that are typically located in the posterior pole of the retina (D. Lee & Higginbotham, 1999). They appear as small, round red dots about 125 um in diameter with sharp margins and a central light reflex. They tend to appear and disappear with time (ETDRS, 1985). Any red spot equal to or greater than 125 um in diameter with irregular margins, particularly if it surrounds a smaller central punctuate lesion, is a hemorrhage (ETDRS, 1985).

Hard exudates are serum lipoprotein deposits in the extracellular space of the retina from leaking retinal capillaries (Porte, Sherwin, Baron, Ellenberg, & Rifkin, 2003). They appear as white or yellowish-white deposits with sharp margins in the outer layers of the retina. Hard exudates may be dots, confluent patches, or complete rings of waxy appearance surrounding prominent microaneurysms or zones of retinal edema (ETDRS, 1985).

Diabetic macular edema (DME) is the accumulation of intraretinal fluid due to the breakdown of the blood retinal barrier that involves or threatens the center of the macula. It appears as retinal thickening, usually alongside hard exudates and microaneurysms (ETDRS, 1985). Clinically significant macular edema (CSME) can occur as early as NPDR (ETDRS, 1985). The ETDRS defines CSME as retinal thickening within 500 um of the fovea, hemorrhaging within 500 um of the fovea with adjacent retinal thickening, or one or more areas of retinal thickening that is within one disc diameter of the fovea (ETDRS, 1985).
The foveal avascular zone is the central part of the fovea that is devoid of capillaries. Non-perfused inner retinal capillaries surrounding the fovea can cause the margins of the foveal avascular zone to become irregular and the intercapillary spaces to become wider (D. Lee & Higginbotham, 1999). Foveal avascular zone abnormalities are only visible by fluorescein angiography (ETDRS, 1985).

**Moderate to severe NPDR**

Moderate to severe NPDR is characterized by the presence of cotton-wool spots, venous beading, intraretinal microvascular abnormalities, and as previously described, intraretinal hemorrhages (ETDRS, 1991b).

Cotton wool spots, also called soft exudates or nerve fiber infarcts, are localized areas of blocked axoplasmic flow in the retinal nerve fiber axons such that there is swelling and ultimately the atrophy of fibers. The swelling and atrophy of fibers are the result of occlusion and subsequent ischemia in the supplying arterioles of the nerve fiber layer (McLeod, Marshall, Kohner, & Bird, 1977). Cotton wool spots appear as round or oval striated spots with feathered borders. They are white, pale yellow-white, or grayish-white (ETDRS, 1985) and can occur transiently following the initiation of strict glycemic control (Hudson, 2008).

Venous beading is the thickening and hyaline degeneration of the venular wall adjacent to areas of capillary non-perfusion. It appears as the focal narrowing or dilatation of the retinal venules and is often associated with cotton-wool spots. Other venous abnormalities include the abrupt, curving deviation of a vein from its normal path (loops), the dilatation of a pre-existing channel or proliferation of a new channel (reduplication), white lines along one or both sides of the venous column (sheathing), and localized narrowing of venous calibre (focal narrowing) (ETDRS, 1985).

Intraretinal microvascular abnormalities are tortuous, intraretinal blood vessels between arterioles and venules adjacent to areas of capillary non-perfusion (Hudson, 2008). They are only visible by fluorescein angiography and can be distinguished from normal retinal vessels by their
large branching angles and irregular calibre that varies from fine, thread-like vessels to dilated capillaries. Intraretinal microvascular abnormalities vary from barely visible to 31 um (ETDRS, 1985).

**Severe and very severe NPDR**

Severe and very severe NPDR are characterized by the increasing presence of the clinical features of mild to moderate NPDR. It represents progressive increases in the extent and severity of vascular closure and leakage (ETDRS, 1991a).

**Proliferative Diabetic Retinopathy**

In addition to the clinical features of NPDR, PDR is characterized by the presence new, fragile blood vessels that arise from the optic disc or elsewhere in the retina. They proliferate along the inner retinal surface (subhyaloid space) and into the vitreous with or without accompanying fibrous strands or sheets. Vascular proliferation into the vitreous can lead to subhyaloid or vitreous hemorrhage as well as tractional retinal detachment (ETDRS, 1985).
1.3.3 Primary Causes of Vision Loss

The major causes of vision loss in patients with DR are CSME and PDR (Olk RJ, 1993). Clinically significant macular edema is the most common cause of visual impairment among people with diabetes (R Klein, BEK Klein, SE Moss, MD Davis, & DL DeMets, 1984b; R. Klein, Moss, Klein, Davis, & DeMets, 1989). Clinically significant macular edema results in slowly developing moderate vision loss (DRS, 1981). The risk of vision loss after 3 years of CSME onset if left untreated is 33%. By contrast, PDR results in sudden-onset severe visual loss. The risk after 6 years if left untreated is 40% (DRS, 1981).
1.3.4 Pathogenesis of DR

Retinal vascular abnormalities visible via ophthalmoscopy are the basis of the clinical diagnosis for DR (section 1.3.3). There is a growing body of evidence, however, that supports the view of DR as both a vascular and neurodegenerative disease. The exact mechanism and/or cause of DR remain to be elucidated.

Metabolism and Blood Supply in the Healthy Retina

The retina is a metabolically active tissue consisting of neural, vascular, and glial cells. It uses a great deal of energy to support processes such as phototransduction, maintenance of ionic gradients, and synaptic activity. Its energy demands are normally met through the uptake of oxygen and glucose from the choroidal and retinal circulation (Lu & Adamis, 2006; Vijay & Harris, 2001).

The choroidal circulation supplies the outer retina and 90% of the retina’s oxygen supply (pO₂ ~ 80 mmHg) (Wangsawirawan & Linsenmeier, 2003). It is influenced to a lesser extent than the inner retinal vasculature by changing oxygen demands but it is more sensitive to carbon dioxide concentrations (Kisilevsky, et al., 2008). By contrast, the retinal circulation supplies the inner retina (except within the foveal avascular zone) and the remaining 10% of the retina’s oxygen supply (pO₂ ~ 25 mm Hg) (Ahmed, Braun, Dunn Jr, & Linsenmeier, 1993; Davidson, 2000; Wangsa-Wirawan & Linsenmeier, 2003). The degree to which in the inner retina is vascularized appears to correlate best with the degree of oxidative metabolic demand. Astrocytes, amacrine, Müller, and retinal ganglion cells are highly metabolic and very sensitive to hypoxia. They release vascular endothelial growth factors which help to secure a viable blood supply (Miller, Walsh, & Hoyt, 2005). Unlike the choroidal circulation, the retinal circulation responds to changes in oxygen tension through wide swings in flow rate (Davidson, 2000). The combination of high metabolic demand and a lower proportion of the overall vascular supply may limit the inner retina’s ability to adapt to metabolic stress (Antonetti, et al., 2006).
In the healthy retina, glycogen stores are inadequate to meet basal metabolic demands. Retinal neurons rely completely on the delivery of glucose from the circulation (Vijay & Harris, 2001). The retinal capillary endothelium of the inner blood-retinal barrier and the retinal pigment epithelium of the outer blood-retinal barrier prevent the passive diffusion of glucose and other blood-borne nutrients from circulation into the interstitial space surrounding neural cells. Glial cells are in close contact with retinal neurons and sense their energy needs through metabolic coupling (Vijay & Harris, 2001). Synaptically released glutamate, an excitatory neurotransmitter that is important for retinal function, triggers glial uptake of glucose from circulation via glucose transporters. Glucose is ultimately transported to neurons where it is broken down for energy. Some of the product (2-oxoglutarate) is also used for the production of glutamate for neurotransmission (Vijay & Harris, 2001).

Microvascular Abnormalities in DR

Retinal microaneurysms are one of the first visible signs of DR (Meyerle, Chew, & Ferris, 2009). In vitro studies which investigate the effect of diabetes on leukocyte function suggest that microaneurysms are the first signs of enhanced leukocyte-endothelial cell adhesion and entrapment (retinal leukostasis) early in the course of the disease (Chibber, Ben-Mahmud, Chibber, & Kohner, 2007). Leukocytes in patients with diabetes have been shown to be inflammatory and in turn more adherent to the vascular wall, suggesting a role in vascular occlusion. In response to this capillary non-perfusion, neighbouring capillaries dilate. Hyperglycemia has been shown to have a direct pro-inflammatory effect on leukocytes (Chibber, et al., 2007). Hyperglycemia-induced biochemical events are linked to mitochondria-driven oxidative stress (Balasubramanyam, Rema, & Premanand, 2002).

The resulting ischemia from vascular occlusion has been shown to increase the production of vascular permeability factors such as vascular endothelial growth factor (VEGF) (Skondra, et al., 2008). VEGF is normally expressed in astrocytes, amacrine, Müller, and retinal ganglion cells, as previously described (section 1.3.4.1), in addition to vascular endothelial cells (Kaur, Foulds, & Ling, 2008). The up-regulation of VEGF expression increases vascular permeability through the breakdown of the blood-retinal barrier (Skondra, et al., 2008). Leukocytes that are inflammatory
may also play a direct role in the breakdown of the blood retinal barrier through the release of proteolytic enzymes (Miyamoto & Ogura, 1999).

The breakdown of the blood retinal barrier involves the loss of pericytes (Cogan, Toussaint, & Kuwabara, 1961), the degeneration of endothelial cells (Kubabara & Cogan, 1965), and the thickening of the capillary basement membrane (Engerman, 1989). It is responsible for the leakage of fluid and lipoproteins into the retina that is visible via ophthalmoscopy. It also allows the extravasation of activated macrophages and neutrophils into the interstitial space surrounding neurons which may exacerbate an inflammatory process (Joussen, et al., 2004; Kaur, et al., 2008). As retinal ischemia progresses, VEGF induces the growth of new blood vessels. These new vessels are fragile and susceptible to hemorrhage, as they have incomplete tight junctions, a reduced number of pericytes and fenestrations (Lawrenson, 2000).

Neuronal Abnormalities in DR

Over 40 years ago, Bloodworth (1962) and Wolter (1961) observed neuronal cell loss in histological cross-sections of retinas from patients with diabetes (Bloodworth, 1962; Wolter, 1961). The involvement of retinal neurons in the pathogenesis of DR has since gained considerable attention. A number of animal and human studies have reported neuronal cell loss in addition to metabolic and morphological abnormalities both in the presence and absence of clinical DR. Neuronal abnormalities may be an early indicator of DR progression.

Retinal ganglion cells (RGCs) are among the most studied retinal neurons in diabetes. Barber et al. (1998, 2005) reported a reduction of cells of the RGC layer in streptozotocin-induced and Ins2Akita T1D rat models (Barber, et al., 2005; Barber, et al., 1998). Treatment with insulin largely prevented this neuronal cell death (Barber, et al., 1998). A number of other studies using the streptozotocin rat model of T1D have confirmed the loss of RGCs (Asnaghi, Gerhardinger, Hoehn, Adeboje, & Lorenzi, 2003; Chihara, Matsuoka, Ogura, & Matsumura, 1993; Lieth, Gardiner, Barber, & Antonetti, 2000; PM Martin, Roon, Van Ells, Ganapathy, & Smith, 2004; T. Scott, Foote, Peat, & Galway, 1986; Zhang, Ino-ue, Dong, & Yamamoto, 2000). In vitro studies have shown that the long-term administration of glutamate causes swelling, necrosis and the
death of RGCs (Lucas & Newhouse, 1957; Sisk & Kuwabara, 1985). This suggests that glutamate excitotoxicity may play a role in the death of RGCs (Vorwerk, et al., 1996). A decrease in the thickness of the RGC layer and the inner plexiform layer has been recently observed in patients with T1D and DR (Van Dijk, et al., 2009; Van Dijk, et al., 2010). The decrease in RGC thickness was associated with duration of diabetes (Van Dijk, et al., 2010).

Metabolic and morphological changes in RGCs have also been reported in diabetes. Impaired retrograde transport has been observed in large and medium-sized retinal ganglion cells in a rat model of T1D (Zhang, et al., 2000). Abnormal swellings of somas, axons, and dendrites in addition to an enlargement of dendritic field size have also been reported. The plasticity of the surviving RGCs may represent a compensatory response to a loss of RGCs in diabetes and/or a loss of upstream neural cells (Gastinger, Kunselman, Conboy, Bronson, & Barber, 2008; Qin, Xu, & Wang, 2006).

Bipolar, amacrine, and horizontal cells are also affected in diabetes. Bipolar cells have been shown to alter their expression of glutamate receptors in diabetes (Ng, Zeng, & Ling, 2004). Amacrine cells have been shown to display necrotic features and degenerate in diabetic rat retinas (Park, et al., 2003; Seki, et al., 2004). Lastly, decreased numbers of horizontal cells and decreased branching of their terminals in a T1D rat model has also been observed (Agardh, Bruun, & Agardh, 2001). In vitro studies investigating bipolar, amacrine, and horizontal cell loss and morphological changes have not been investigated extensively in DR (Smith, 2007).

A marked reduction in the thickness of the outer nuclear layer in a T1D rat model has been reported 24 weeks after the onset of diabetes. Apoptotic nuclei were present four weeks post onset of diabetes and this number increased as diabetes progressed (Park, et al., 2003). A selective loss of short-wavelength sensitive cones specifically has been found in histological cross-sections of patients with DR (Cho, Poulsen, Ver Hoeve, & Nork, 2000).

Lastly, glial cells are involved in metabolic coupling with neurons. It is expected that their function reflects neural integrity. In the healthy retina, glial fibrillary acidic protein (GFAP) is expressed in astrocytes but not in Müller cells (Bignami, Eng, Dahl, & Uyeda, 1972; Shaw &
Weber, 1983). The upregulated expression of GFAP in Müller cells has been observed in a number of studies using animal models of diabetes (Barber, Antonetti, & Gardner, 2000; Q. Li, Zemel, Miller, & Perlman, 2002; Lieth, et al., 1998b) and in histological cross-sections of human retinas with DR (Mizutani, Gerhardinger, & Lorenzi, 1998). Increased GFAP expression is a sign of severe Müller cell stress (Lieth, et al., 1998a). Significant dysfunction of the Müller cell glutamate transporter has been observed in an animal model of diabetes (Mysona, Rankin, Van Ells, Ganapathy, & Smith, 2005). Consistent with glial cell malfunction, a reduced ability of the retina to convert glutamate to glutamine has also been observed in a T1D rat model (Lieth, et al., 1998a). Taken together, these findings suggest that glial cell dysfunction underlies elevated extracellular glutamate in diabetes, perhaps contributing to glutamate excitotoxicity (Lieth, et al., 1998b).
1.3.5 Risk Factors

Glycemic control

Hemoglobin A₁c (HbA₁c), also known as glycosylated hemoglobin, is a minor subtype of hemoglobin A in erythrocytes composed of glucose bound irreversibly to hemoglobin A (Garel, Blouquit, Molko, & Rosa, 1979). It normally comprises approximately 4-6% of the total hemoglobin A concentration in a healthy individual (Porth, et al., 2006). Since this complex lasts the lifespan of an erythrocyte (90 to 120 days), HbA₁c % values represent the mean glucose concentration in systemic circulation over the preceding 3-4 months (Porth, et al., 2006). There is a strong association between HbA₁c and average glucose concentration (Nathan, et al., 2008). While there may be substantial variability in mean glucose concentrations for a given HbA₁c value, studies of patients with T1D suggest that glucose variability does not significantly change HbA₁c % values (Salardi, et al., 2002; Wilson, 2007).

A number of epidemiological studies have shown that poor glycemic control increases the incidence and progression of DR. In the WESDR study, an increased HbA₁c value at baseline was associated with an increased incidence and progression of DR over a 25 year period (R. Klein, Knudtson, Lee, Gangnon, & Klein, 2008). Smaller, cross-sectional prospective studies have also shown this relationship. Bojestig et al. (1998) found that in a population of patients with T1D, the risk of developing severe DR was significantly higher in patients with poor glycemic control (7.2 ≤ HbA₁c < 8.4 %) than in patients with good glycemic control (HbA₁c < 7.2%). In this study, only one patient with good glycemic control developed severe DR over a 25 year period (Bojestig, Arnqvist, Karlberg, & Ludvigsson, 1998). Goldstein et al. (1993) reported similar findings. In the Goldstein et al. study, patients with T1D who developed DR and required laser treatment had HbA₁c levels greater than 8.5% (Goldstein, et al., 1993). In addition, the Berlin Retinopathy Study found an association between HbA₁c and early background retinopathy, with a threshold HbA₁c value of > 9% (Danne, et al., 1994).

The Diabetes Control and Complications Trial (DCCT) is a large-scale study that has also demonstrated this relationship by demonstrating large declines in the incidence and progression
of DR with a reduction in HbA$_{1c}$ value (DCCT, 1993). The DCCT was designed to compare intensive and conventional diabetes therapy with regard to their effects on the development and progression of DR in patients with T1D. Patients with no DR and patients with mild to moderate DR at baseline were randomly assigned to an intensive or conventional insulin treatment group. The data reflects a mean follow-up period of 7.4 years. Strict glycemic control was highly effective in the primary prevention of DR. The incidence of retinopathy was reduced by 76% in the intensive treatment group when compared to the conventional treatment group. There was a 23% decrease in CSME in patients with DR as well as a 56% reduction in the number of patients requiring panretinal photocoagulation. Strict glycemic control was also highly effective at slowing the rate of DR progression in patients with mild to moderate NPDR at baseline. The rate of DR progression was reduced by 54% in the intensive treatment group when compared with the conventional treatment group. There was a 78% decline in the progression of DR in patients with no DR at baseline. A glycemic threshold was not found for the development of long-term complications (DCCT, 1996), however, the risk for DR progression was found to increase two to three fold between HbA$_{1c}$ values of 8% and >9% (DCCT, 1993).

**Duration of Diabetes**

A longer duration of diabetes has been implicated in the increased incidence and severity of DR. The WESDR reported that after the first 5 years of diabetes, 14% of patients in the younger-onset group (<30 years of age at diagnosis) developed some degree of DR. During this time, only 2% of patients in this group developed PDR. After 20 or more years of diabetes, 98% of patients in the younger-onset group developed some degree of DR and more than half (53%) developed PDR (R. Klein, et al., 1984a).

**Puberty**

Post-pubescence has been implicated in the increased prevalence of DR. Puberty has been shown to worsen metabolic control in adolescents with T1D as a result of both physiological and psychosocial factors (Hamilton & Daneman, 2002). Physiologically, the elevation in HbA$_{1c}$ at puberty is likely the result of some combination of insulin resistance, an increase in insulin-like
growth factor I, and activation of the hypothalamic-pituitary-gonadal axis which leads to the development of secondary sexual characteristics. Psychosocially, decreased compliance with insulin injections, glucose monitoring and regular eating patterns can occur as adolescents experience greater independence and assume self-care (Hamilton & Daneman, 2002).

The WESDR reported that post-pubertal individuals in the younger-onset group were 3.2 times more likely to have DR than pre-pubertal individuals irrespective of duration of diabetes and diastolic blood pressure (BE Klein, Moss, & Klein, 1990). Smaller, population-based studies have also reported these findings (Frank, et al., 1982; Malone, Grizzard, Espinoza, Achenbach, & Van Cader, 1984; Murphy, et al., 1990; Palmberg, et al., 1981; Rogers, et al., 1987).

Post-pubertal duration of diabetes

The duration of diabetes after puberty compared with before puberty has also been implicated in the increased prevalence of DR in a number of studies (Kostraba, et al., 1989; McNally, Raymond, Swift, Hearnshaw, & Burden, 1993; Olsen, et al., 2004; Vincze, Madácsy, Brooser, & Barkai, 1992).

Age

Increased age has been shown in some studies to be associated with an increased severity and prevalence of DR. The WESDR reported that for individuals with diabetes for 10 years or less, older age at examination was associated with an increased severity of DR. In addition, patients above 13 years of age were 5.2 times more likely to develop DR than patients younger than 13 years of age irrespective of duration of diabetes (R. Klein, et al., 1984a). These findings are consistent with a number of small, population-based studies (Burger, et al., 1986; Frank, et al., 1982; R. Klein, Klein, Moss, & Cruickshanks, 1998). It is important to note the possible overlap between an effect of age and that of puberty.
Age at diagnosis

The WESDR found no association between the age at diagnosis of diabetes and the four-year incidence or progression of DR in the younger or older onset groups (R. Klein, et al., 1984a; R. Klein, Klein, Moss, Davis, & DeMets, 1989).

Sex

A number of studies have demonstrated a greater prevalence of DR in males (Bodansky, et al., 1982; Dornan, et al., 1982; R. Klein, et al., 2008; Malone, et al., 1984). The WESDR reported that males had a 33% increased risk of DR progression (R. Klein, et al., 2008). A higher prevalence of DR has also been reported in females due to an earlier onset of puberty (Malone, et al., 1984) and greater insulin resistance (Moran, et al., 1999).
1.3.6 Problems with Current Treatment and Management of DR

Diabetic retinopathy is asymptomatic until the later stages of disease when the risk for acute, sudden vision loss is high (Fong, et al., 2003). The main treatments available for clinical DR are surgical interventions that arrest vision loss at best rather than recover lost vision. Serious complications may accompany these interventions (Hudson, 2008). Screening is one of the best strategies for preventing and slowing the progression of DR. Current methods, however, rely on clinically evident pathology. Early detection of DR before it is clinically visible may prevent vision loss in individuals with diabetes (L Aiello, et al., 1998).

Focal photocoagulation is recommended for patients with CSME and mild to moderate NPDR. It can be performed directly on microaneurysms or in areas of retinal edema without treatment of specific retinal vascular lesions (ETDRS, 1985). The ETDRS investigated the efficacy of focal photocoagulation in patients with CSME and mild to moderate NPDR. The risk of visual loss in patients with mild to moderate NPDR decreased by 50% (ETDRS, 1985). On the other hand, panretinal photocoagulation (PRP) is the primary treatment for high risk and severe PDR. Laser burns are placed on the retina in a grid pattern that covers areas of neovascularization but not the optic disk. The Diabetic Retinopathy Study (DRS) investigated the efficacy of PRP in patients with advanced DR. The DRS reported a regression of neovascularization in 30% to 55% of eyes after PRP. The cumulative risk of developing severe visual loss was reduced by more than 50% in the treated eyes (DRS, 1981). Serious complications of laser treatment include the loss of peripheral and parafoveal vision from PRP and focal photocoagulation treatments respectively (Hudson, 2008).

Vitreoretinal surgery is performed in patients with proliferative DR, non-clearing vitreous hemorrhage and/or traction retinal detachment. It involves the removal of media opacities such as vitreous hemorrhage (Michels, Rice, & Rice, 1991). The Diabetic Retinopathy Vitrectomy Study investigated the efficacy of early vitreoretinal surgery in patients with advanced PDR. This study reported that patients with T1D and severe PDR benefited most from early vitrectomy (DRVS, 1985, 1990). There are many possible post-operative complications including retinal detachment and iris and angle neovascularization (Schachat, Oyakawa, Michels, & Rice, 1983).
The Canadian Diabetes Association (CDA) recommends that the screening for DR commence five years after the onset of T1D in all patients 15 years of age and older. Screening should be performed annually if DR is not present (CDA, 2008a). The basis of this recommendation is the observation that there is a marked increase in the prevalence of DR in post-pubertal children after 5 years of T1D (R. Klein, et al., 1984a).

The current screening method for DR includes a dilated ophthalmic exam, indirect slit-lamp fundoscopy, or seven-fields 30° colour stereoscopic fundus photography which is the gold standard (CDA, 2008a). If DR is present, the CDA recommends the tight control of blood glucose (HbA1c ≤ 7.0%), blood pressure (≤ 130/80 mmHg), and cholesterol (≤2.0 mmol/L for low-density lipoproteins) levels. Surgery is only recommended for sight-threatening DR (CDA, 2008a; Hudson, 2008)

Diabetic retinopathy that is clinically evident progresses chronically. It can advance at a pace that is difficult to arrest with surgery or slow with tight blood glucose, blood pressure and lipid control (Hudson, 2008; Xu, 2008). Sensitive markers that can be used as an early warning sign of disease progression are needed to identify patients at risk of DR progression. This would provide a much needed window of opportunity for treatment while vision is intact (Antonetti, et al., 2006).
1.4 Short-wavelength Colour Vision Loss

A possible marker of early DR progression is short-wavelength colour vision loss. A number of studies have demonstrated this deficit in patients with T1D both with and without clinical DR. Short-wavelength cone pathway sensitivity loss may be one of the earliest cone pathway changes in the progression of DR, occurring even before it is clinically evident.

Spectral sensitivity loss in short-wavelength sensitive cone pathways has been reported in patients with T1D both with and without clinical DR through the measurement of detection thresholds. The flash-on-flash technique described by Hood and Greenstein (1982) and the two-colour increment technique developed by Stiles (1959) are psychophysical techniques which measure detection thresholds of specific cone pathways (VC Greenstein, Hood, & Campbell, 1982; Stiles, 1959). Both techniques require an observer to detect a small, brief coloured test light against an adapting background. In the flash-on-flash technique, the test light is presented simultaneously with a flash of the same wavelength and a specific intensity.

Terasaki et al. (1996) used the flash-on-flash technique and demonstrated that the detection threshold for a blue light against a yellow adapting background was elevated in patients with T1D with and without clinical DR. Detection thresholds were elevated in patients with no clinical DR and increased as the severity of DR worsened (Terasaki, Hirose, & Miyake, 1996). Similar results were reported in studies that used the two-colour increment technique (V Greenstein, Sarter, Hood, Noble, & Carr, 1990; VC Greenstein, Shapiro, Zaidi, & Hood, 1992).

The short-wavelength automated perimetry is the clinical application of the two-colour increment technique. It has gained considerable interest as a potential means for detecting the presence of visual field loss prior to that identified by the conventional white-on-white perimetry (Wild, 2001). Studies using the short-wavelength automated perimetry have shown sensitivity loss to a blue light in the central 30° of the visual field in patients with T1D and early DR (Nomura, Terasaki, Hirose, & Miyake, 2000) as well as without DR (Lobefalo, et al., 1998). The white-on-white perimetry did not find L- and M-cone pathway sensitivity loss in these studies. Similar findings have also been reported in patients with type 2 diabetes (Hudson, et al., 1998).
Spectral sensitivity loss in short-wavelength sensitive cone pathways has also been reported by studies which have used hue discrimination tests, another psychophysical technique. Many of these studies have used the Farnsworth-Munsell 100-Hue Test which requires an observer to arrange colour caps in order of hue. These studies have reported short-wavelength sensitivity loss in patients with T1D both with (V Greenstein, et al., 1990; A Kurtenbach, Schiefer, Neu, & Zrenner, 1999; Anne Kurtenbach, et al., 1994) and without DR (A Kurtenbach, et al., 1999; Anne Kurtenbach, et al., 1994; Muntoni, Serra, Mascia, & Songini, 1982).

Psychophysical evidence of short-wavelength colour vision loss has been supported by human and animal histological studies. A selective loss of short-wavelength sensitive (S-) cones has been shown in cross-sections of retinas from patients with DR (Cho, et al., 2000). In addition, a recent study of a mouse model with T1D has shown that within the first three months of diabetes, structural alterations of retinal ganglion cells are limited to small bistratified retinal ganglion cells (Gastinger, et al., 2008). Small bistratified retinal ganglion cells are involved specifically in short-wavelength visual processing (section 1.5.7).

A problem with psychophysical techniques is that they are subjective. Sensory precepts are based on the response of an observer and detection thresholds are defined by a predetermined criterion (H. Lee & Sharma, 2006b). Visual electrophysiology is a sensitive and specific tool that can be used to objectively assess the functional integrity of visual pathways.

A few studies using visual electrophysiology have reported a loss of functional integrity in short-wavelength sensitive cone pathways in patients with diabetes both with and without DR. A foundation in human colour vision, however, is first critical. This will be discussed in the next few sections.
1.5 Human Colour Vision

Spatial organization of cones in the retina

Human colour vision is mediated by the activity of cones. In the human retina, there is a total of 4-5 million cones packed in a hexagonal arrangement among approximately 120 million rods (PR Martin, 2004; Purves, Williams, White, & Mace, 1997). Cone density peaks to 200,000 cones/mm$^2$ in the foveola, a central 300μm region of the fovea that is avascular and rod-free (Purves, et al., 1997). Outside of the foveola, cone density falls sharply with increasing eccentricity (Shevell, 2003). Since there are approximately 25% more cones in the nasal than temporal retina, the cone distribution is described as nasotemporally asymmetric. This asymmetry is not apparent near the fovea but becomes more pronounced with increasing eccentricity (Shevell, 2003).

The spacing and packing arrangement of cones in the retina is known as the cone mosaic. This property is critical, as it affects the quality of the sampled retinal image (Shevell, 2003). The quality of this image can be described by the Nyquist sampling theorem (Olshausen, 2000).

The Nyquist sampling theorem states that to retain the quality of the original signal, the sampling frequency must be at least twice the highest frequency of the original signal (Olshausen, 2000). This is described mathematically as $f_s \geq 2 f_c$, where $f_s$ is the sampling frequency and $f_c$ is the highest frequency of the original signal. It follows then that the maximum spatial frequency that can be adequately sampled is half of the sampling frequency ($f_c \leq f_s/2$). This is known as the Nyquist limit.

In a normal human eye, the lens filters out any spatial variations finer than 60 cycles/degree – this is the optical cut-off. To adequately sample a retinal image, it follows that the photoreceptor sampling rate must be 120 cycles/degree and the Nyquist limit must be 60 cycles/degree. If one considers the cone-to-cone spacing in the retina, these requirements are met (Olshausen, 2000; Shevell, 2003). As will be further discussed, there are different types of cones. Each cone type
has a unique mosaic. A cone mosaic with a Nyquist limit lower than 60 cycles/degree undersamples the retinal image. This results in distortions caused by high frequencies that are misinterpreted as low frequencies (i.e. aliases) (Shevell, 2003).
Sensitivity and kinetics of cones to light

The cone threshold is approximately -2 log cd/m² in comparison with the rod threshold which is approximately -7 log cd/m² (Purves, et al., 1997). This marks the beginning of vision under starlight, twilight, and moonlight (mesopic) conditions where cones, as well as rods, function. A progressive increase in the intensity of illumination leads to progressive photoreceptor hyperpolarization (graded potentials) from the resting membrane potential of -40mV (Purves, et al., 1997).

In light, there is a reduction in the concentration of cyclic guanosine monophosphate (cGMP) in the outer cone segment. This leads to the closure of cGMP-gated channels in the outer segment membrane and consequently a reduction in the inward flow of Na⁺ and Ca²⁺ ions. The reduction in the inward flow of cations is offset by a constant K⁺ efflux via K⁺ leakage channels in the inner segment. Hyperpolarizing responses to light saturate when the membrane potential reaches about -65mV. Voltage-gated Ca²⁺ channels in the terminal membrane are closed when the photoreceptor is hyperpolarized. As a result, the rate of neurotransmitter release (i.e. glutamate) from the cone terminal is correspondingly low in light (Purves, et al., 1997).

More than 100 photons of light are needed to produce a response in a cone. The change in current produced by a single photon capture is small and difficult to distinguish from background noise. By contrast, rods produce a reliable response to a single photon of light (Purves, et al., 1997). For this reason, cones are 25-100 times less sensitive to light than rods (North, 1995).

At low light levels, the membrane potential of individual rods increases as illumination increases. Beyond 1 log cd/m², the membrane potential of individual rods begins to no longer vary as a function of illumination (saturate) and cones become increasingly dominant in determining what is seen. Rod saturation marks the beginning of vision under indoor lighting and sunlight (photopic) conditions. Approximately 50% of rods are saturated in sunlight (i.e. ~ 5 log cd/m²). Colour vision begins in mesopic conditions but it is best in photopic conditions (Purves, et al., 1997).
Cones respond faster to light than rods. The response of a cone recovers in about 200ms. This is more than four times faster than rods (Purves, et al., 1997). Due to the brevity of the cone response, cones can modulate their activity to a high temporal flicker (≥30 Hz). The slow recovery of rods to baseline limits their temporal resolution (Frishman, 2001).
Types of cones

There are three types of cone photoreceptors in the human retina: short (S-), medium (M-) and long (L-) wavelength sensitive cones, or more simply, blue, red and green cones respectively. Individuals with all three functioning cones types are called trichromats. Cones are named according to the region of the light spectrum to which a light-sensitive photopigment (iodopsin) found on the infoldings of the photoreceptor outer segment membrane demonstrates peak sensitivity or absorbance. There are three iodopsins with different sensitivities to different parts of the visible spectrum (Purves, et al., 1997). It is important to note that photopigments are “colour blind”. An appropriate combination of wavelength and intensity results in an identical photoreceptor response. Rushton described this concept as the Principle of Univariance (Rushton, 1972).

L- and M-cones

Long and medium-wavelength sensitive cones comprise more than 90% of all retinal cones. The cone mosaic is a nearly random distribution. The proportion of L- and M-cones vary between individuals, but large differences in the ratio of these cone types do not have a significant impact on colour perception (Purves, et al., 1997). The peak absorbance of L- and M-cones is 552-563 nm and 525-545 nm respectively, but there are some small variations amongst individuals due to alanine-serine polymorphisms at codon 180 (McKay, 2007; Sharpe, Stockman, Jagle, & Nathans, 1999). A possible reason for the close spacing of L- and M-cone absorption spectra is their genetic origin. The iodopsin amino acid sequences of L- and M-cones are 96% homologous (Nathans, Thomas, & Hogness, 1986). Both cone types are thought to have evolved recently (~35 million years ago), presumably from the duplication of a single ancestral gene (Gegenfurtner & Kiper, 2003). There is one gene for the L-cone iodopsin and 1-5 genes for the M-cone iodopsin. Since both of these iodopsin genes are found on the X-chromosome, there is a high proportion of congenital red-green colour deficiency among males (Shevell, 2003).
S-cones

Short-wavelength sensitive cones account for about 7% of all cones. The cone mosaic is sparser and more evenly spaced than L- and M-cones. There is also little inter-individual variation in the number of these cones compared to L- and M-cones (Shevell, 2003). S-cones absorb light maximally at 420 nm, demonstrating little overlap with the spectral sensitivities of L- and M-cones (McKay, 2007). S-cones, along with rods, presumably developed from an ancestral receptor before the development of L- and M-cones. The amino acid sequence of the S-cone iodopsin bears 42% homology to the sequences for the M- and L-cone iodopsins and rhodopsin (Nathans, et al., 1986; Shevell, 2003). The iodopsin gene for S-cones is autosomal. It is found on chromosome 7 (Calkins, 1999).
Retinal Circuitry of Cones

The retinal circuitry of cones specialize the cone system for fine discrimination vision. This includes high spatial resolution vision and specific to this thesis, colour vision. Fine discrimination vision in the cone system occurs at the expense of light sensitivity, a property of the rod system (Purves, et al., 1997).

In the rod system, several rods synapse on a single rod-bipolar cell to generate a large scale response. This pooling of signals is known as convergence. While convergence increases the sensitivity of the rod system to light, it decreases the ability of the system to sample high spatial resolution images, as the source of the retinal signal could originate anywhere within a large area of the retinal surface due to signal pooling. In the cone system, the ratio of cones to cone-bipolars in the fovea is nearly 1:1. This lesser degree of convergence specializes the cone system for high acuity vision while decreasing the sensitivity of the cone system to light (Purves, et al., 1997). Since cone density decreases with increasing eccentricity, fine discrimination vision is markedly reduced outside the fovea. For this reason, colour and high spatial resolution vision are foveal dominated properties of vision (Purves, et al., 1997).

Spectral opponency

Cone signals combine to form a spectrally non-opponent luminance channel and a spectrally opponent chromatic channel (Kaiser, 2009). Spectral opponency is an inhibitory mechanism through which signals from one or more cone types are subtracted from the signal of another cone type (cone antagonism). Since the spectral sensitivity of cone types is broad, cone antagonism is essential to avoid redundancy. It sharpens the spectral sensitivity of RGCs such that they respond to a narrower bandwidth of light. This avoids the additive effects of noise (PR Martin, 2004; McKay, 2007).

Most colour information is relayed to approximately four families of spectral neurons in the parvocellular layers (layers 3-6) of the lateral geniculate nucleus: wide-band long, wide-band short, narrow-band long and narrow-band short wavelength detectors. Each cell has a peak
response at a particular wavelength (McKay, 2007). It is important to note, however, that some colour information is relayed to the interlaminar koniocellular layers (McKay, 2007). This will be further discussed in a later section.

The presence of cone antagonism in the retina can be found in center-surround and spatially co-extensive (center only, no surround) single opponent receptive field organizations in spectrally sensitive ganglion cells. Bipolar cells also demonstrate cone antagonism, but typically with center-surround receptive fields (McKay, 2007). A receptive field is the region of the receptive surface, in this case the retina, to which a sensory nerve cell will respond (Purves, et al., 1997).

Center-surround receptive fields are a reflection of receptive field organization at the bipolar cell from select cone input. Spatially co-extensive single opponent receptive fields are assembled at the ganglion cell from the merging of cone inputs carried by bipolar cells. This is the effect of merging the receptive fields of bipolar cells with the same surround. The surrounds cancel and a center with cone antagonism remains (McKay, 2007).

The synaptic effect of cone input

The synaptic effect of cone input in light is either depolarizing (ON) or hyperpolarizing (OFF). ON-bipolars express metabotropic glutamate receptors (e.g. mGluR6, L-AP4). These receptors are G-protein coupled and when bound to glutamate, they activate a biochemical cascade via a second messenger that ultimately closes cGMP-gated Na\(^+\) channels due to the breakdown of cGMP. For this reason, the ON-bipolar-photoreceptor synapse is considered sign-inverting. In light, the hyperpolarization of a photoreceptor (-) leads to decreased glutamate release at the terminal. Decreased binding of glutamate to metabotropic receptors prevents the breakdown of cGMP. Consequently, cGMP-gated Na\(^+\) channels remain open, depolarizing the ON-bipolar cell (+) in light (Purves, et al., 1997). The axon terminals of ON-bipolar cells stratify in the ON(b)-sublamina of the inner plexiform layer (IPL) (Calkins, 1999).
OFF-bipolars express ionotropic glutamate receptors (e.g. AMPA or GluR$_{1-4}$, NMDA, kainate). These receptors are voltage-gated Na$^+$ channels which when bound to glutamate, increase the permeability of the bipolar cell to Na$^+$. For this reason, the OFF-bipolar-photoreceptor synapse is considered *sign-conserving*. As previously mentioned, in light, the hyperpolarization of a photoreceptor (-) leads to decreased glutamate release at the terminal. The reduced binding of neurotransmitter to ionotropic receptors prevents depolarization (-) (Purves, et al., 1997). OFF-bipolar cell axon terminals stratify superficial to ON-bipolar terminals in the OFF(a)-sublamina of the IPL (Calkins, 1999).

Graded potentials generated at the photoreceptor-bipolar cell synapse are passively conducted to the bipolar-ganglion cell synapse without action potential (spike) production (Purves, et al., 1997). The bipolar-ganglion cell synapse is sign-conserving, as retinal ganglion cells also express ionotropic glutamate receptors (Purves, et al., 1997).
The Chromatic Channel

The spectrally opponent chromatic channel has two major neural pathways - The L/M-(red/green) and S/(L+M)-(blue/yellow) cone pathways (Kaiser, 2009).

L/M-cone pathways

Retinal ganglion cells in L/M-cone pathways are midget ganglion cells. The majority (~80%) of midget ganglion cells are found within 7-10 ° eccentricity. They have single-opponent center-surround receptive fields such that L- or M-cone input drives either the center or surround (Dacey, 1996; McKay, 2007). The corresponding ON and OFF response is carried by midget bipolar cells (McKay, 2007; Purves, et al., 1997). The perception of red is signaled by a L-ON (center)/M-OFF (surround) or M-OFF/L-ON receptive field organization. Green is signaled by a L-OFF/M-ON or M-ON/L-OFF receptive field organization (McKay, 2007). Beyond 10° of eccentricity, cone opponency greatly diminishes until it is ultimately absent in the far periphery. Midget ganglion cells in the periphery have larger dendritic fields and do not make contact with a cone-specific subset of midget bipolar cells (Dacey, 1996). L/M-cone retinal pathways input to the parvocellular layers of the LGN (McKay, 2007).

S/(L+M) cone pathways

Retinal ganglion cells in S/(L+M) cone pathways have spatially co-extensive single opponent receptive fields. These ganglion cells are called small bistratified ganglion cells (Dacey & Lee, 1994). Most small bistratified ganglion cells identified have a mixed S-ON/(L+M)-OFF receptive field. The combined (L+M)-OFF cone input is from the luminance pathway and its spectral sensitivity is highest in the yellow range (McKay, 2007). This receptive field organization signals the perception of blue (Calkins, 2001). S-ON responses are predominantly but not completely segregated to the koniocellular layers (PR Martin, 2004). S/(L+M)-cone pathways are also called short-wavelength sensitive or simply S-cone pathways.
The perception of yellow may be signaled by a S-OFF/ (L+M)-ON receptive field. This has been described in retinal ganglion cell and LGN recordings, but these examples are relatively rare (De Monasterio & Gouras, 1975; Valberg, Lee, & Tigwell, 1986).

*Colour spaces*

The chromaticity and luminance of a stimulus can be described by a vector from the origin in a three-dimensional, spherical colour space. This space is referred to as the MBDKL colour space after its originators MacLeod, Boynton, Derrington, Krauskopf and Lennie (Schwartz, 2004).

The excitation of L-, M-, and S-cones corresponds to two orthogonal axes that form an isoluminant plane – the L/M- and S/(L+M)-axes. These axes are the two chromatic channels. At the extreme ends of the L/M-axis, cardinal red is at 0° and cardinal green is at 180°. At the extreme ends of the S/(L+M)-axis, cardinal blue is at 90° and cardinal yellow is at 270°. The 1931 Commission Internationale d’Eclairage (CIE) colour space further describes spectral hues and their saturation on this isoluminant plane using x’y’ coordinates. The x-axis indicates the proportion of red and the y-axis indicates the proportion of green. The proportion of blue is not indicated on an axis on the CIE chromaticity diagram but can be calculated by subtracting the sum of the proportions of red and green from 1.00 (Schanda, 2007). When all of the colours confused by dichromats are plotted on the CIE chromaticity diagram, the axes of which are generated are called confusion lines (Sharpe, et al., 1999). A third axis that is orthogonal to the isoluminant plane in the MBDKL colour space is known as the (L+M)-axis. This axis describes the luminance component of the stimulus (Rabin, Switkes, Crognale, Schneck, & Adams, 1994).
Congenital and Acquired Vision Deficiencies

There are two major types of colour vision deficiencies – congenital and acquired (Sharpe, et al., 1999).

Congenital colour vision deficiencies are the consequence of an inherited failure to make one or more of the cone pigments or an alteration in the absorbance spectrum of a cone pigment (Sharpe, et al., 1999). Congenital colour vision deficiencies are present from birth, are the same in both eyes, and are static in nature (Formankiewicz, 2009).

By contrast, acquired colour vision deficiencies appear during an individual’s lifetime, can occur in one eye only, be more severe in one eye than the other, and it can progress or regress depending on the disease state. Other signs, such as reduced visual acuity or visual field defects, may accompany acquired deficiencies (Formankiewicz, 2009). Acquired colour vision deficiencies arise secondary to retinal or systemic disease, or as a side effect of medication or chemical exposure. They reflect a problem that occurs anywhere along central visual pathways, from disorders of the pre-receptoral ocular media to cerebral achromatopsia or dyschromatopsia (Formankiewicz, 2009; Sharpe, et al., 1999). Verriest proposed a classification system that identifies three types of acquired colour vision deficiencies. Types 1 and 2 are red-green deficiencies while type 3 is a blue-yellow deficiency (Verriest, 1964).
Red-Green Deficiencies

Most congenital colour vision deficiencies affect colour discrimination along the red-green axis. Alterations in the L- and M- cone pigment genes can occur as a result of unequal chromosomal cross-over during meiosis (Sharpe, et al., 1999).

Unequal cross-over between L- and M-cone pigment genes in the intergenic region results in the loss of a pigment gene. The loss of a gene results in dichromatic colour vision such that only two bandwidths of light are needed to match all the colours that these individuals can perceive. The loss of the L-cone pigment gene is known as protanopia whereas the loss of the M-cone pigment gene is known as deutanopia. Protanopes and deuteranopes are both red-green blind. The perception of yellowish-green to red colours is dramatically reduced and they can only distinguish such colours on the basis of saturation and lightness variations. Protanopes tend to confuse reds, grays, and bluish blue-greens while deuteranopes tend to confuse purples, grays, and greenish blue-greens (Sharpe, et al., 1999).

Unequal chromosomal cross-over within L- and M-cone pigment genes in the intragenic region can also occur and results in the formation of hybrid genes coding for photopigments with abnormal absorption spectra. Protanomalous trichromats have a “green-like” red pigment. These individuals require more red than normal when matching a red-green mixture to a yellow primary (Verriest type 1). Deutanomalous trichromats have a “red-like” green. These individuals require more green that normal when matching (Verriest type 2). Many anomalous trichromats are unaware of their colour vision deficiency, but some with a severe deficit may have nearly as poor a colour discrimination as dichromats (Sharpe, et al., 1999).

Acquired red-green colour vision deficiencies are less common. Red-green colour vision is the longest seen unchanged, but when an acquired red-green colour vision deficiency is observed, it often occurs with an acquired blue-yellow colour vision deficiency (Formankiewicz, 2009). Conditions quoted as having an acquired red-green colour vision deficiency include Best’s disease, Stargardt’s disease, papillitis, diseases of the optic nerve such as optic neuritis and
Leber’s optic atrophy, and lesions of the optic nerve and posterior pathways (Miller, et al., 2004).

**Blue-Yellow Deficiencies**

Congenital blue-yellow colour vision deficiencies do not appear to be common. The loss of an S-cone pigment gene, known as tritanopia, often has an incomplete manifestation (incomplete tritanopia). In addition, the alteration of an S-cone pigment gene, known as tritanomaly, has not been satisfactorily documented. It may be mistaken for incomplete tritanopia or for acquired disorders such as dominant optic atrophy (Sharpe, et al., 1999).

Most acquired colour vision deficiencies affect colour discrimination along the blue-yellow axis (Verriest type 3). They appear before red-green colour vision deficiency and are often observed to occur early in the course of disease progression. For this reason, S-cone pathways are thought to be particularly susceptible to disease and insult (Formankiewicz, 2009). Conditions cited as having an early acquired blue-yellow colour vision deficiency include glaucoma, retinal detachment, pigmentary degeneration, age-related macular degeneration, myopic retinal degeneration, chorioretinitis, retinal vascular occlusion, papilledema (Miller, et al., 2004), retinal toxicity due to chloroquine (Razeghinejad, Torkaman, & Amini, 2005), diabetes and all stages of diabetic retinopathy as discussed (section 1.4).

Acquired blue-yellow colour vision deficiencies are proposed to stem from the vulnerability of S-cones. The following characteristics of S-cones suggest their unique susceptibility to disease and insult.

As previously described, there are only about 7% of S-cones in the human retina. A loss of a small percentage would cause considerable gaps in the S-cone matrix. This may result in a marked reduction in the sensitivity of the blue-yellow chromatic channel (Cho, et al., 2000).
Secondly, by virtue of the fact that the morphology and biochemistry of S-cones also differs greatly from L- and M-cones, which do not appear to be affected early in disease, these differences could contribute to susceptibility to disease and insult (Cho, et al., 2000).

The light funneling inner segment of the S-cone is longer and wider than that of M- or L-cones (Curcio, et al., 2004). The glutamate-releasing axon terminal of the S-cone is also smaller and penetrates the outer plexiform layer more deeply (Ahnelt, Keri, & Kolb, 2004). S-cones have more docking sites (ribbons) for glutamate than M- and L-cones. They are thus able to accommodate more bipolar cells at their terminal (Kouyama & Marshak, 1992). The membranes of S-cones also appear to be more permeable certain molecules such as chlorotriazinyl dyes which selectively stain these cones (De Monasterio, McCrane, Newlander, & Schein, 1985).

In terms of their biochemistry, carbonic anhydrase is present in L- and M- cones but not in S-cones (Nork, McCormick, Chao, & Odom, 1990). In addition, the calcium metabolism of S cones (Zrenner, Kramer, Bittner, Bopp, & Schlepper, 1982) and the amino acid sequence of arrestin, a molecule which plays a role in halting the phototransduction cascade, is substantially different in S-cones from that of L- and M-cones (Nir & Ransom, 1992; Purves, et al., 1997).
1.6 Short-wavelength Visual Processing

The perception of blue and yellow is made possible through the processing of short-wavelength light in S-cone pathways. S-cones, their retinal distribution, and circuitry are described in this section. Note that the lens, retinal vasculature, and macular pigment are known to absorb short-wavelengths very strongly. This pre-receptoral filtering is described below.

Pre-receptoral filtering of short-wavelength light

Pre-receptoral filtering of short-wavelength light affects the numbers of photons that are ultimately captured by S-cones in an observer. Ocular transmittance can be estimated by comparing measured scotopic spectral sensitivity to the absorption spectrum of rhodopsin (Pulos, 1989; Weale, 1954).

The effect of age on the lens is greatest at short-wavelengths. In the young adult, lens absorption is very high at wavelengths less than 390 nm and less than 10% between 450nm and 900nm. The human lens contains a short-wavelength absorbing pigment that is of high concentration at birth, decreases in concentration during the first five years of early childhood, and then plateaus in adulthood. An increase in lens density also contributes to lens absorption at short-wavelengths. Between 20 and 60 years old, the lens optical density (a measure of light attenuation) for 400nm increases 0.12 log units per decade on average. After age 60, the lens optical density accelerates to 0.4 log units per decade on average (Shevell, 2003). Brunescent cataracts, old vitreous and yellow subretinal fluid are also associated with age and strongly absorb short-wavelength light (Shevell, 2003; Zigler, 1981).

The retinal vasculature is a meshwork of capillaries between the cornea and the photoreceptors. The spectral properties of blood are dominated by hemoglobin which absorbs most strongly between 400nm and 450nm. Using the Beer-Lambert law, the transmittance of the vascular filter can be calculated from the hemoglobin spectrum, the effective concentration of hemoglobin in the blood, and the thickness of the blood layer (Shevell, 2003).
The final filter, the macular pigment, absorbs most strongly between 400-550nm with a peak near 458 nm. Macular pigment is a combination of the isomeric carotenoids zeaxanthin and lutein which are closely related to the xanthophylls pigments found in leaves (Shevell, 2003). It can be detected prenatally, increases in concentration during early life, and remains unchanged after 9 years of age (Werner, Donnelly, & Kliegl, 1987). The amount of macular pigment is highest in the center of the fovea and falls with increasing eccentricity (Shevell, 2003). As measured by the blue-on-yellow perimetry, macular pigment absorption has been shown to decrease the sensitivity of normal healthy subjects to short-wavelength light (Wild & Hudson, 1995)

Distribution of S-cones in the Human Retina

At fetal week 15.5, S-cones in the human retina distribute heavily throughout the fovea. These cones recede from the foveola as development progresses and an irregular “S-cone free” region 0.3-0.4° in diameter becomes increasingly distinct (Bumsted & Hendrickson, 1998; Shevell, 2003). S-cones reach a peak density of 2000 cells/mm² with a center to center spacing of 22μm at 1-2° eccentricity (Calkins, 2001; Shevell, 2003). Beyond this region, S-cones gradually decline in density and are sparsely distributed such that their center to center spacing nearly doubles (Calkins, 2001). Here, S-cones are more evenly spaced than would be expected from a random distribution and are thus described as having a “quasi-regular” or “quasi-hexagonal” packing arrangement (Calkins, 2001). The “quasi-regular” packing arrangement of S-cones contrasts the random distribution of the M- and L-cone mosaic (Calkins, 2001). S-cones are found mainly in the perifovea (Marc & Sperling, 1977).

The eye’s optics in typical viewing conditions may limit any evolutionary pressure to pack S-cones into the photoreceptor mosaic, particularly in the foveola (Shevell, 2003). In photopic conditions, the emmetropic eye is optimally in focus for middle wavelengths. The point of focus for short-wavelength light falls vitreal to the retina, creating a chromatic aberration of about -1 diopter (Calkins, 2001; Shevell, 2003). This results in a blurred retinal image of low contrast and spatial frequency (Calkins, 2001; Shevell, 2003). Most psychophysical data suggests that S-cone
Acuity is 10-15 cycles/degree. This is substantially lower than the 60 cycle/degree Nyquist limit in the human eye. When the S-cone mosaic functions in isolation from the other cones, it undersamples the retinal image (Olshausen, 2000; Shevell, 2003).

Tritanopia has been reported in the foveola using a number of psychophysical tests (Hartridge, 1945a, 1945b; Williams, MacLeod, & Hayhoe, 1981; Willmer & Wright, 1945). Williams et al. (1981) ruled out the possibility of Troxler fading or pre-retinal screening of short-wavelength light by the macular pigment (Williams, et al., 1981). The absence of S-cones in the foveola, however, is not subjectively obvious for objects larger than 0.3-0.4°. It renders individuals tritanopic within the most acute portion of the visual field (Shevell, 2003).

In the periphery, where S-cones are sparse, the perception of green-blue or red-blue colour variations for objects less than 0.3° are difficult to discriminate – this is known as “small field tritanopia” (H. Lee & Sharma, 2006a).

**S-cone retinal circuitry**

In S-cone retinal pathways, spectral opponency is created at two synaptic levels in the retina: the small-bistratified ganglion cell in which there is a convergence of ON- and OFF-bipolar cell inputs, and more recently identified, the S-cone synapse.

On average, 1-2 S-cones contact a single S-ON bipolar cell. The dendrites of S-ON bipolar cells stream underneath the cone terminals of L- and M-cones. They insert between a pair of horizontal cell processes (triad arrangement) to receive exclusive contact from S-cones at the cone terminal invagination. S-ON bipolar cells contact the inner dendritic tier of the small bistratified ganglion cell in the ON(b)-sublamina of the IPL (Calkins, 1999).

The dendrites of multiple types of diffuse-OFF bipolar cells (e.g. DB2, DB3) in the luminance pathway skip under the cone terminals of S-cones and non-selectively contact 5-7 L- and M-cones in the inside wall of cone terminal invaginations (triad associated basal sites). Diffuse-OFF
bipolar cells are known to contact parasol-OFF ganglion cells in the luminance pathway, but they also feed the S-cone pathway. Diffuse-OFF bipolar cells contact the outer dendritic tier of the small bistratified ganglion cell in the OFF(a)-sublamina of the IPL. As a result of luminance pathway input into the blue-yellow chromatic pathway, parasol-OFF ganglion cells should show some response to S-cone stimulation (Calkins, 1999).

In terms of the S-cone synapse, there is strong inhibitory feedback from L- and M- cones to S-cones via H2 horizontal cells. This is thought to contribute the S-ON/(L+M)-OFF center-surround receptive field organization that was recently identified in the S-cone (Packer, Verweij, Li, Schnapf, & Dacey, 2010). Diffuse bipolar cells in the luminance pathway have mixed L- and M-cone inputs to both the receptive field center and surround (McKay, 2007). Since both S-ON and diffuse (L+M)-OFF bipolar cells have opponent (L+M) surrounds, when signals from the S-ON and diffuse-OFF bipolar cells are combined at the small bistratified ganglion cell, the surrounds cancel, creating pure chromatic opponency with little spatial opponency (spatially co-extensive single opponent receptive field). These receptive field properties are retained by downstream neurons in the LGN (Packer, et al., 2010). Synaptic input from the S-ON bipolar cells to small bistratified ganglion cells (~30 synapses from 2-3 S-ON bipolar cells) is far greater than the input from the diffuse-OFF bipolar cells (~12 synapses from 2-3 diffuse-OFF bipolar cells) (Calkins, 1999).

Some preliminary data show that cones identified as S-cones based on their connections with S-ON bipolar cells also appear to contact a midget-OFF bipolar cell that in turn contacts a midget-OFF ganglion cell. This may correspond to an S-OFF/(L+M)-ON ganglion cell that is less often described in physiological recordings (Calkins, 1999). In addition, a recent study has shown that rods connect directly to ON-bipolars. This is thought to smooth out intensity changes at the scotopic-mesopic interface (Tsukamoto, et al., 2007).
Cortical projections of S-cone retinal pathways

The koniocellular layers of the LGN project directly into regions of increased cytochrome oxidase metabolism (blobs) of layer 3 of nine layers in the primary visual cortex (V1). This information is passed down the ventral (temporal) cortical pathway for higher-level visual processing where parvocellular information is also directed. The ventral cortical pathway encodes colour and fine form, specifically shape and texture. From V1, information is directed to extrastriate areas for higher level visual processing – sequentially, the thin and interstripe regions of V2, V4, and the posterior, central and anterior inferotemporal lobe regions (Purves, et al., 1997).
1.7 Visual Electrophysiology

Visual electrophysiology involves the objective measurement of neuro-functional changes in visual pathways. The visual evoked potential and the full-field electroretinogram are two visual electrophysiological tests used that can be modified to assess the integrity of specific cone pathways.
1.7.1 The Visual Evoked Potential

The visual evoked potential (VEP) is a non-invasive tool for studying the functional integrity of visual pathways, extending from the retina to the visual cortex, in vivo. It is a low voltage electrical response (1-20 µV) time-locked to a visual stimulus that is extracted, using signal averaging, from electroencephalographic activity (60-100 µV) (Odom, et al., 2004; Regan, 1989). The VEP is recorded using scalp electrodes that are placed over the primary visual cortex (V1). Since V1 is dominated by the central 10° of the visual field, the VEP is a foveal dominated response (Porth, et al., 2006; Regan, 1989).

The VEP response recorded is a radial extracellular potential change or field potential that is generated from conductance changes in the membranes of active neurons as well as glial cell buffering of extracellular potassium (Regan, 1989). Excitatory postsynaptic potentials generate a surface negativity while inhibitory postsynaptic potentials generate a surface positivity (Regan, 1989). Standard electrode placement allows for the comparison and analysis of waveforms (Odom, et al., 2010). The electrical response is a voltage change that varies over time and is plotted as a waveform (Regan, 1989). The amplitude and timing to the response (latency) elicited by a stimulus are the two major parameters used to evaluate the VEP (Regan, 1989). There is great inter-individual variation in the amplitudes of VEPs compared with latency of responses. The VEP amplitude can be modified by attention, cranial shape, distribution of sulci of the brain and size of the brain (Regan, 1989).

The visual stimulus for a VEP is a uniform flash of light (luminance), checks or stripes (patterned). Patterned stimuli are ideal for participants who are capable of fixating and concentrating on the visual stimulus. Most clinical studies use square wave checks; although the more appropriate sinusoidal gratings are used frequently for research studies. Square wave gratings produce more artifacts partially attributed to the multi-dimensional spatial frequency making up the square wave profile (Kulikowski, McKeefry, & Robson, 1997). Check stimuli are not selective at revealing responses from specific cone pathways (Regan, 1989).
The spatial frequency, contrast (luminance and colour), temporal frequency and mode of presentation of a stimulus are important determinants of visual pathway stimulation (Kulikowski, et al., 1997).

Spatial frequency refers to the number of cycles per degree (cpd) of visual angle or subtended to an angle of 1° in the eye (Regan, 1989). Luminance and colour contrast refer to the difference in brightness and chromaticity respectively between adjacent checks or stripes. It varies from 0-100% and can be defined by the Weber or Michelson contrast formulas (Regan, 1989). To generate chromatic VEPs, the luminance contrast of chromatic stimuli is as close to zero as possible (isoluminant) to avoid intrusion from achromatic pathways. The temporal frequency refers to the number of stimulus presentations per second (Regan, 1989). Lastly, the mode of presentation refers to whether the stimulus is presented in a pattern-reversal or onset-offset manner. Pattern-reversal refers to a repetitive pattern that abruptly reverses in luminance or colour contrast. Onset-offset refers to a pattern that appears and then disappears, to be replaced by a homogenous field of the same mean luminance and chromaticity (Regan, 1989).
1.7.1.1 Chromatic VEPs

Neurons in chromatic visual pathways have low chromatic thresholds and respond preferentially to chromatic stimuli of low temporal frequency since they respond in a sustained manner (Purves, et al., 1997). To generate chromatic pathway VEPs, colour stimuli are correspondingly of low colour contrast and temporal frequency (≤ 2 Hz) (Anderson, Holliday, Singh, & Harding, 1996; Purves, et al., 1997; Schiller & Logothetis, 1990). Chromatic VEPs are difficult to obtain at spatial frequencies greater than 7 cpd. Chromatic stimuli with ≤ 6 spatial cycles are recommended (Parry, Kulikowski, Murray, Kranda, & Ott, 1988).

The chromatic VEP elicited to the described stimulus parameters is a largely biphasic or sometimes a monophasic waveform (M. Crognale, 2002). The major component is a large negative peak which is the response to the chromatic stimulus (M. Crognale, 2002). A stimulus on the L/M colour axis produces a long- and medium wavelength cone VEP (L/M-VEP). Likewise, a stimulus on the S/(L+M) axis produces a short-wavelength cone pathway VEP (S-VEP). The L/M- and S-VEP responses to their corresponding stimulus is shown in Figure 1.1.

![Figure 1.1 L/M-VEP (black) and S-VEP (grey) in a control subject. Response to chromatic stimulus (arrow)]
The major component of chromatic VEPs is of positive polarity and longer latency until 12-14 years of age when the polarity of the chromatic response reverses to negative and the latency decreases to adult ranges (M. Crognale, 2002). The latency of the S-VEP is longer than that of the L/M-VEP in infants and adults (Rabin, et al., 1994; Till, Rovet, Koren, & Westall, 2003).

A dysfunction in S-cone pathways in patients with T1D without DR has been shown with the use of chromatic VEPs. Elia et al. (2005) showed that S-VEP responses were delayed in post-pubertal adolescents with T1D. This delay was not associated with duration of diabetes, HbA$_1c$, or ambient blood glucose levels. A delay was not observed in the L/M-VEP (Elia, et al., 2005).
1.7.1.2 Achromatic VEPs

Neurons in achromatic visual pathways have low spatial resolution, low luminance thresholds, and unlike neurons in chromatic pathways, high temporal resolution since they respond in a transient manner (Purves, et al., 1997). To generate achromatic pathway VEPs, achromatic stimuli are correspondingly of low spatial frequency, low luminance contrast and either appear to move or they are of high temporal frequency (Kulikowski, et al., 1997).

The achromatic VEP elicited to the described stimulus parameters is a triphasic waveform. There is an initial prominent positive deflection occurring at approximately 100 ms which is the response to the stimulus followed by a negative and positive deflection (M. Crognale, 2002).

![Figure 1.2 Achromatic VEP. Response to an achromatic stimulus (arrow)](image)

The latency of achromatic VEP responses decreases to mature values within the first 12-15 weeks of life (M. A. Crognale, 2002).

Elia et al. (2005) did not find any delays in the achromatic pathway VEP in a cohort of adolescents with T1D and no ophthalmoscopic evidence of DR (Elia, et al., 2005).
1.7.2 The Full-Field Electroretinogram

The full-field electroretinogram (ERG) is a non-invasive tool for studying retinal function specifically in vivo. It records an aggregate electrical response from all cells in the retina to a spatially extended, diffuse flash of light using a corneal electrode (Frishman, 2006). Like the VEP, the electrical response recorded is a field potential that is generated from conductance changes in the membranes of active neurons as well as glial cell buffering of extracellular potassium, but in the retina specifically. The electrical response, which is plotted as waveform, is a voltage change that varies over time (Frishman, 2006). The amplitude and timing to the response (implicit time) are the two major parameters used to evaluate an ERG (Miller, et al., 2004).

Full-field ERGs are recorded under dark (scotopic) or light-adapted (photopic) conditions (Frishman, 2006). Cone system driven ERGs are obtained under photopic conditions using either a 30Hz flicker or single flash upon a rod-saturating background (Marmor, et al., 2009). At 30 Hz, the poor temporal resolution of the rod system, in addition to the presence of a rod-suppressing background, enables a cone-system response to be recorded. The cone system response to a 30 Hz flicker is thought to reflect inner retina activity (Bush & Sieving, 1996). A single flash in photopic conditions, however, records the activity of cell types in all retinal layers in the cone system (Frishman, 2006). This allows for better localization of dysfunction within cone pathways.
1.7.2.1 Long- and Medium-Wavelength Sensitive Cone Electroretinogram

The retinal response to white flash on a white rod-suppressing background is driven by L- and M-cone pathways. The components of the L- and M-cone electroretinogram (LM-ERG) include the a-wave, b-wave, oscillatory potentials (OPs), i-wave (short-duration flash of <5 ms) or d-wave (long-duration flash of approximately 200 ms) and the photopic negative response (PhNR) (Frishman, 2006; Rangaswamy, et al., 2007).

For the photopic ERG, the a-wave is the initial negative deflection of the waveform. Its slope is related to the kinetics of cone phototransduction (Hood & Birch, 1995).

The large, positive component following the a-wave is the b-wave. It is generated primarily from the activity of depolarizing ON-bipolar cells (Gurevich & Slaughter, 1993; Robson & Frishman, 2009; Stockton & Slaughter, 1989). Hyperpolarizing OFF-bipolar cells may limit the amplitude and control the shape of the photopic b-wave (Sieving, Murayama, & Naarendorp, 1994). There has been experimental support for Müller cell contribution to the photopic b-wave. Stronger support suggests that the Muller cell contribution is smaller than that of ON-bipolar cells (Karwoski & Xu, 1999; Robson, Maeda, Saszik, & Frishman, 2004; Xu & Karwoski, 1994).

Oscillatory potentials are a series of high-frequency wavelets of low-amplitude found on the ascending limb of the b-wave. Most studies demonstrate that they originate in the inner plexiform layer (Heynen, Wachtmeister, & van Norren, 1985; Ogden, 1973). Oscillatory potentials may originate from a negative feedback mechanism between amacrine cells and RGCs (Kenyon, et al., 2004).

For short-duration flashes, on the falling phase of the b-wave or just after it is a low-voltage positive component termed the i-wave. It is thought to be generated by RGCs (Rosolen, et al., 2004). For long-duration flashes, a positive-going deflection known as the d-wave follows the b-wave. It is thought to be generated by the depolarization of OFF-bipolar cells at light offset (Sieving, et al., 1994).
The photopic negative response is a slow developing negative-going wave after the b-wave. A second PhNR termed the PhNR_{OFF} is found after the d-wave for long duration stimuli. The PhNR may originate from the spiking activity of amacrine and/or ganglion cells, with some possible involvement of glial cells through a feed-forward mechanism to explain its extended time course (Viswanathan, Frishman, Robson, Harwerth, & Smith, 1999).

Figure 1.3. Cellular origins of the ERG (http://webvision.med.utah.edu, Appendix F)
Figure 1.4. L- and M-cone dominated ERG waveform in a healthy subject to a short duration flash

In the photopic cone ERG, reduced OP amplitudes have been reported in patients with diabetes both with (Vadalà, Anastasi, Lodato, & Cillino, 2002) and without DR (X. Li, Sun, Hu, Huang, & Zhang, 1992). Patients with reduced OP amplitudes have a greater chance of developing DR (Vadalà, et al., 2002) and a greater risk of progressing to more severe stages of DR (Bresnick, Korth, Groo, & Palta, 1984; Bresnick & Palta, 1987; Simonsen, 1980). Reduced b-wave and PhNR amplitudes in the photopic cone ERG have been reported in patients with early stages of DR (Kizawa, Machida, Kobayashi, Gotoh, & Kurosaka, 2006).
1.7.2.2. Short-wavelength Cone Sensitive Electroretinogram

The short-wavelength cone sensitive electroretinogram (sERG) is a modification of the full-field ERG used to preferentially stimulate S-cone retinal pathways. Most single-flash S-cone retinal responses use a short-wavelength (blue) flash of varying wavelength and intensity on a high intensity rod and L/M-cone-suppressing background (Marmor, Cabael, Shukla, Hwang, & Marcus, 2004). Depending on the wavelength characteristics of the stimulus and the background, the response may be either an S-cone dominated ERG or a composite waveform in which a late S-cone driven response can be recognized after the L- and M-cone driven response (Marmor, et al., 2004).

The components of the S-cone dominated ERG include an a-wave, a prominent b-wave (no recordable OPs), and if recorded at low stimulus frequencies (≤ 2Hz), the PhNR (Drasdo, et al., 2001; Marmor, et al., 2004; Mortlock, Chiti, Drasdo, Owens, & North, 2005). These components are thought to have corresponding neural origins to that of the L- and M-cone dominated ERG (Drasdo, et al., 2001).

![Figure 1.5 S-cone dominated ERG waveform in a healthy subject to a short duration flash](image_url)

Figure 1.5  S-cone dominated ERG waveform in a healthy subject to a short duration flash
A few electrophysiological studies using the full-field ERG have demonstrated a dysfunction in S-cone retinal pathways in patients with diabetes both with and without clinically evident DR.

Yamamoto (1996, 1997) obtained composite waveforms using a single flash method from patients with diabetes and reported a dysfunction in ON-bipolar cells in S-cone retinal pathways. Reduced b-wave amplitudes were demonstrated in patients both with and without clinically evident DR. For the L/M-cone b-wave, there were no significant differences in the amplitude and implicit times between patients and controls (Yamamoto, Kamiyama, Nitta, Yamada, & Hayasaka, 1996; Yamamoto, Takeuchi, & Kamiyama, 1997). Yamamoto (1997) found no association between the S-cone b-wave and HbA1c.

Mortlock et al. (2005) obtained S-cone dominated ERGs from patients with diabetes through a silent substitution method (Mortlock, et al., 2005). Some patients had no clinically evident DR while others had minimal (mild to moderate) DR. There was a delay in the implicit time of the b-wave in the minimal DR group compared with controls who did not have diabetes (Mortlock, et al., 2005).

The S-cone PhNR has not yet been investigated in patients with diabetes.
2. Purpose and Rationale

The purpose of this study was to investigate if glycemic control, as measured using HbA$_{1c}$, is associated with electrophysiological measures of retina-to-striate and/or neuroretinal function in short-wavelength sensitive cone pathways. The population tested was adolescents with T1D and no clinically visible DR.

Studies have demonstrated short-wavelength colour vision loss in patients with diabetes. The short-wavelength deficit may be one of the earliest functional cone pathway changes in diabetes, as it has been shown to occur before long- and medium-wavelength sensitivity loss. Short-wavelength colour vision loss has been shown to worsen with the increasing severity of clinically detectable DR (Section 1.4).

Electrophysiological measures of retina-to-striate and neuroretinal function in short-wavelength sensitive cone pathways have been studied in patients with diabetes. The potential of these measures to function as biomarkers for DR, however, is largely unknown.

Poor glycemic control is a strong risk factor for the incidence and progression of DR. The WESDR has shown that raised HbA$_{1c}$ is associated with an increased progression of DR (R. Klein, et al., 2008). A large-scale randomized control trial known as the DCCT has also demonstrated this relationship by showing that strict glycemic control (HbA$_{1c}$ < 6.0%) is associated with large declines in the incidence (53%) and progression (70%) of DR. The risk for DR progression was found to increase two to three-fold between HbA$_{1c}$ values of 8% and >9% (DCCT, 1993).

It is of interest to examine the association between glycemic control and electrophysiological measures of short-wavelength cone pathway function. An association would support the application of short-wavelength electrophysiological measures as a practical means for identifying patients who are at higher risk of later developing clinically visible DR. Sensitive
markers of early DR progression would provide a much needed window of opportunity for treatment while vision is intact. The identification of a biomarker would also help to elucidate early pathogenic mechanisms of DR.

The population of the current study was ideal in order to investigate electrophysiological markers that can identify early DR progression. In an adolescent population with diabetes, the time since diagnosis is likely shorter on average than an adult population. This is critical considering that the ultimate goal of a marker for DR is early detection.

Two electrophysiological studies have been conducted previously that assessed the association of HbA$_{1c}$ with short-wavelength cone pathway function. Elia et al. (2005) studied the integrity of retina-to-striate pathways using the short-wavelength cone pathway visual evoked potential (S-VEP). Yamamoto et al. (1997) studied the integrity of short-wavelength pathways in the retina using the short-wavelength sensitive cone electroretinogram (sERG). Both studies did not find an association with HbA$_{1c}$, but a few key factors were not taken into consideration.

Firstly, age is a risk factor for DR and a confounder in the interpretation of short-wavelength electrophysiological responses. The WESDR and the more recent WDRS have shown that older age at examination is associated with an increased prevalence of DR (R. Klein, et al., 1984a; LeCaire, et al., 2006). Short-wavelength VEP and ERG responses have also been shown to change with age. S-VEP latencies are longer than those of adults until 12-14 years of age (M. Crognale, 2002). Increased lens density and yellowing after 30 years of age may also contribute to longer sERG b-wave implicit times and decreased amplitudes (Gouras, MacKay, & Yamamoto, 1993). In a linear regression which included S-VEP latencies as the dependent variable and HbA$_{1c}$ as the independent variable, age was not included as a possible covariate in the Elia et al. (2005) study which included children aged 6-13 years. The association of age with S-VEP latencies in the age-matched control group was not assessed. Similarly, in the Yamamoto et al. (1997) study which included adults aged 23-60 years, age was not considered as a covariate with HbA$_{1c}$. The association of age with sERG b-wave implicit times and amplitudes in the control group were not assessed.
Secondly, a longer duration of diabetes is associated with the increased incidence and severity of DR. The WESDR shown that after 20 years of diabetes in the younger onset group, nearly all patients (98%) developed some degree of DR (R. Klein, et al., 1984a). The Yamamoto et al. (1997) study in particular did not include duration of diabetes as a potential covariate in its analysis with HbA$_{1c}$.

Finally, to our knowledge, no study to date has assessed the association between HbA$_{1c}$ and inner retinal function in short-wavelength cone pathways in patients with diabetes. This is an area of interest considering that inner retina functional and structural changes have been reported by a number of studies early in the course of diabetes and DR.

The following questions are posed for the current study:

1. What is the association of HbA$_{1c}$ to the latency of the S-VEP in adolescents with T1D and no clinically evident DR?

2. What is the association of HbA$_{1c}$ to the sERG b-wave amplitude and implicit time and the sERG PhNR amplitude in adolescents with T1D and no clinically evident DR?

In considering both questions, co-variates age and duration of diabetes were taken into account.
3. Hypotheses

We hypothesized that:

1. Raised HbA1c is associated with increased S-VEP latencies.

2. Raised HbA1c is associated with sERG dysfunction of the middle retina b-wave and inner retina PhNR.
4. Methods

4.1 Study Design

This is a cross-sectional, prospective study.

4.2 Recruitment

Participants with Type 1 Diabetes

Adolescents with type 1 diabetes were recruited from The Hospital for Sick Children’s (SickKids) Diabetes Clinic in collaboration with Dr. Farid Mahmud, a staff endocrinologist. A pamphlet explaining the nature of the study, its relevance, and the electrophysiological tests involved were provided to eligible participants.

Control Participants

Participants who did not have diabetes were recruited using the following modes of advertising:

a. Friends and siblings of participants with type 1 diabetes were invited to participate in the study
b. Posters were displayed in the community
c. An advertisement was placed in the SickKids newsletter *This Week*
4.3 Inclusion Criteria

Participants with Type 1 Diabetes

a. Duration of disease ≥ 5 years
b. Aged 12 to 20 years, inclusive
c. Normal visual development before the diagnosis of diabetes

Control Participants

a. Aged 12 to 29 years
b. Normal visual development

4.4 Exclusion Criteria

Participants with Type 1 Diabetes

a. Any severity of diabetic retinopathy
b. Participants with hemoglobinopathy (i.e. those in whom we cannot obtain reliable HbA1c readings)

All Participants

a. Distance refractive error worse than ± 5 diopters
b. LogMAR visual acuity worse than 0.3
c. Any other eye disease (inherited, inflammatory, infectious, etc.) including lenticular opacity greater than NC2, NO2, C2 and/or P1 according to the Lens Opacities Classification System II
d. Psychiatric or neurological disorders known to affect retinal/visual function
e. Systemic medication with known central nervous system effects
4.5 Patient Information

The SickKids patient information database (KidCare) was used to obtain glycated hemoglobin (HbA\(_1c\)) levels, dates of diagnosis, information on whether on the patient has any psychiatric or neurological disorders and systemic medications that the patient may be taking.

4.6 Research Ethics Board Approval

This study underwent a scientific peer review and was approved by members of the Research Ethics Board of the Hospital of Sick Children in Toronto, Ontario, Canada (see Appendix A for letter of approval).

4.7 Consent

This research was conducted according to the tenets of the Declaration of Helsinki.

All participants had the capacity to give consent. Consent forms for patients and controls listed the names of the investigators involved along with their respective roles, the purpose of the study, a clear description of the protocol, the potential harms and benefits of the study, the participant’s right to confidentiality as well as the right to refuse or withdraw from the study at any time (see Appendix B for the consent form).

The details of the consent form were carefully and thoroughly explained to all participants and/or their family members. All participants were given sufficient time to read the form. Any questions or concerns were fully addressed by research staff before the consent form was signed.
4.8 Study Protocol

All participants were tested once in the Visual Electrophysiology Unit at SickKids. A coin was tossed to randomly assign one eye for the study. All clinical, psychophysical, and electrophysiological testing was monocular. The eye not selected for testing was occluded with an eye patch.

4.8.1 Clinical Examination and Psychophysical Testing

An intake examination was performed on control and patient participants prior to electrophysiological testing. This included visual acuity, contrast sensitivity, and colour vision testing.

Visual Acuity

Visual acuity tests measure one’s ability to discriminate two objects of high contrast separated at a specific distance relative to a background (Kniestedt & Stamper, 2003). The smallest angle (subtended at the nodal point of the eye by two points) at which two objects can be resolved as separate is a measure of the eye’s ability to discriminate detail. This angle is known as the minimum angle of resolution (MAR) (Keirl & Christie, 2007).

Visual acuity was determined using the Logarithmic ETDRS Visual Acuity Chart (Precision Vision; Villa Park, IL). Participants were asked to read the letters on the chart while sitting at a distance of 4 meters. Each letter that was correctly identified was given a score of 0.02 logMAR (Kniestedt & Stamper, 2003).
Contrast Sensitivity

Contrast sensitivity is a measure of the threshold contrast or lowest contrast that can be detected (Keirl & Christie, 2007).

Contrast sensitivity was determined using the Pelli-Robson Contrast Sensitivity Chart (Clement Clarke International; Harlow, UK). The chart consists of 8 lines of letters of shades of grey against a white background. Each line has six letters - the first triplet of letters is always of higher contrast than the second triplet of letters and each triplet is of the same contrast. Contrast decreases downward from line to line. Participants were asked to read the letters on the chart while sitting at a distance of 1 meter. Contrast sensitivity was reported in log (1/contrast) units. Each letter that was correctly identified was given a score of 0.05 log units. Normal contrast sensitivity scores are between 1.65-1.95 (Pelli, Robson, & Wilkins, 1988).

H.R.R (Hardy Rand & Rittler) Pseudoisochromatic Plates

The H.R.R. Pseudoisochromatic Plates (Richmond Products; Boca Raton, FL) is a clinical colour vision test used to detect and estimate the severity of blue-yellow and red-green colour vision deficiencies (Hardy, Rand, & Ritter, 1957).

This test is comprised of demonstration, screening, and grading plates. Demonstration plates (plates 1-4) are used to explain how the test works to the participant. Screening plates are used to identify blue-yellow (plates 5 and 6) and red-green (plates 7-10) colour vision deficiencies. Lastly, grading plates (plates 11-24) are used to classify the identified colour defect as mild, medium, or strong. Each plate includes one or two coloured symbols (‘X’, ‘O’ or ‘Δ’) against a background of grey circles (Hardy, et al., 1957). The colours used in this test are well aligned with the dichromatic confusion lines (Dain, 2005).

The HRR was administered under standard illumination (C.I.E. source C). The participant was asked to verbally identify the symbol(s) on each plate. A criterion of two or more errors on the
screening plates was used to identify a colour deficiency. This criterion value has been reported to be 100% sensitive and 96% specific (Cole, Lian, & Lakkis, 2006).

**Mollon-Reffin Minimalist Test**

The Mollon-Reffin Minimalist test is a clinical colour vision test that, like the HRR, detects and estimates the severity of blue-yellow and red-green colour vision deficiencies (Mollon, Astell, & Reffin, 1991).

This test contains a set of coloured chips with hues of decreasing saturation that coincide with the protan (green), deutan (red) and tritan (blue-yellow) axes. It also includes a set of grey chips of varying saturation and an orange demonstration chip (Mollon, et al., 1991). The Mollon-Reffin test was administered under standard illumination (C.I.E. source C).

To demonstrate the task of the observer, the examiner placed five of the grey chips randomly on black Plexiglass. The orange demonstration chip, which does not lie on any confusion line, was then shuffled among the grey chips. The examiner invited the observer to identify the coloured chip by touching it with a pointer. If the observer successfully identified the orange chip, the examiner withdrew a coloured chip from the middle of the protan series and repeated the task. The examiner moved inwards along the confusion line and presented the least saturated chip if the initial protan chip was identified correctly. If, on the other hand, the response to the first protan chip was incorrect, the examiner moved outwards to the most saturated chip. This process was repeated for the tritan and deutan axes chips (Mollon, 1994).

This test was scored by noting the number (corresponds to the saturation) of the least saturated chip that was correctly identified in each of the three colour series (Mollon, 1994).
4.8.2 Blood Glucose Level Monitoring

A Registered Nurse from the SickKids Endocrinology Department monitored patients’ blood glucose levels periodically during their visit to ensure their safety. Ambient glucose levels have also been shown to affect the S-VEP (Schneck, Fortune, Switkes, Crognaile, & Adams, 1997) and the ERG (Klemp, et al., 2004). Ambient blood glucose readings were taken before, during, and after electrophysiological testing (Figure 4.1).

Blood glucose readings were required to be within the range of 4-10mmol/L. If patient’s blood glucose readings were over 10mmol/L during the initial reading, the nurse suggested an appropriate dose of insulin or moderate exercise such as a brief walk. If blood glucose readings were too low, the nurse provided a light snack until ambient blood glucose levels returned within a safe range.

Blood glucose readings were obtained from all patients using a calibrated One Touch Ultra Blood Glucose Meter (LifeScan; Toronto, ON). One Touch UltraSoft Lancets and Glucose Test Strips were also provided to patients (LifeScan; Toronto, ON).
4.8.3 Visual Evoked Potential

Stimuli

Vertical sinusoidal wave gratings were produced using the Neurosequence program (Vision Research Graphics; Durham, NH). The gratings were 1 cycle/degree. They were presented on circular field of the same mean luminance and chromaticity as the pattern. The diameter of the circular field was 7° of the visual angle. A stable red fixation point was placed in the middle of the circular field. Chromatic gratings were presented in an onset (100 ms)-offset (400ms) mode while achromatic gratings were presented in a pattern-reversal mode. The temporal frequency of all gratings was 1Hz.

Chromatic and achromatic stimuli were presented at 10, 20, and 40% chromatic and luminance contrast respectively. Chromatic stimuli were on the red-green (L/M) and blue-yellow (S/(L+M) CIE axes and had zero luminance contrast (isoluminant). They passed through white (CIE xy coordinates, 0.33, 0.33). The coordinates for the stimulus on the red-green axis were (red; 0.373, 0.299) and (green; 0.293, 0.364). The coordinates for the stimulus on the blue-yellow axis were (greenish-yellow; 0.371, 0.405) and (purple; 0.299, 0.261).

Display

A 55 cm x 50 cm FlexScan F930 Colour Display Monitor (EIZO; Ishikawa, Japan), with a screen size of 35 cm x 27 cm, was used to display the stimulus. The graphics processing unit was a NVIDIA GeForce2. The RASTER size was 1024 x 768 pixels and the refresh rate was 60 Hz.

Testing distance

The distance required to view the 1 cycle/degree gratings was 75 cm. Observers sat with their eyes 75 cm from the display.
Electrode placement

In accordance with the International Society for Clinical Electrophysiology of Vision (ISCEV) standards, sintered silver-silver chloride electrodes were used (Odom, et al., 2010). Five electrodes were placed on the scalp of participants according to the International 10-20 System of Electrode Placement (Jasper, 1958). Three active electrodes were placed over the occipital cortex. The term “active” refers to the electrodes placed over the occipital cortex that are recording electrical potentials elicited to a visual stimulus (Regan, 1989).

The distance from the nasion (nasal structure between the eyes) to the inion (bony structure at the base of the skull) was measured. The location of the first active electrode (OZ) was marked with a non-toxic removable marker 10% of the nasion-inion distance above the inion. The location of the ground electrode was marked 20% of the nasion-inion distance above OZ. A subsequent 20% above the ground electrode was used to mark the reference electrode location. The hemi-lateral circumference, from the nasion to OZ, was then measured. Ten percent of this circumference was used to mark the locations of active electrodes O1 and O2 on the left and right respectively of OZ. All marked electrode locations on the scalp were cleaned with Q-tips using NuPrep, a mild abrasive water-soluble detergent. Cleaning of the specified scalp locations was important to remove dead skin cells and oils which can impede the scalp-electrode interface and weaken the obtained signal.

To apply the electrodes, electrode paste was placed on the marked electrode locations. Each electrode was then pressed firmly into the paste onto the scalp. Coban, a self-adherent elastic tape, was wrapped around the hemi-lateral circumference to stabilize the active electrodes. Another piece of Coban was cut and placed over the reference and ground electrodes.

The electrodes were connected to a NuAmps differential amplifier (Compumedics; Charlotte, NC) via a headbox. To ensure a strong stable scalp-electrode signal, recording was not initiated until an impedance of 5kOhms or less was achieved.
**EEG acquisition and amplifier parameters**

Approximately 100 segments of EEG recordings (epochs), consisting of a 20 ms baseline before stimulus onset and 330ms of EEG post-stimulus onset, were recorded from O₁, O₂, and O₃ using the NeuroScan program (Compumedics). An analog bandpass filter of 1-100Hz was used to filter the signal before digitization. These limits were chosen, as the inclusion of frequencies below 1 Hz, such as movement artifacts, can saturate the amplifier as well as result in low amplitude artifactual responses resembling evoked potentials (Regan, 1989). If frequencies over 100Hz are permitted, the waveform may erroneously appear delayed (Regan, 1989).

The signal was amplified by x 19 000. It was digitized by a 16-bit analog-to-digital converter which resolves $2^{16}$ different levels of the total voltage range accepted by the converter. The sampling rate was 1000 Hz (Regan, 1989). This ensures that there are no frequencies above half the sampling rate that would be represented as low frequency components not present in the original EEG signal (i.e. aliasing) (Regan, 1989).

**Generating average waveforms**

Average waveforms from O₁, O₂, and O₃ at 10, 20 and 40% contrast were generated for repeatability purposes. A bandpass digital filter of 0.1-40 Hz was used to filter average waveforms.
**Analysis**

For each patient, average waveforms were placed on three waveboards using the NeuroScan program. Each waveboard displayed waveforms for a single stimulus condition (i.e. S/(L+M), L/M or achromatic). Nine waveforms were displayed on each waveboard - average responses were displayed at each contrast level (i.e.10, 20, 40%) from O₁, O₂, and O₂.

Latencies were scored for O₁, O₂, and O₂. The analysis of VEP waveforms was blinded as to whether the participant was a patient or control. Files labeled with subject IDs were replaced with a randomly assigned ID.

The outcome measure was the latency of the VEP response. The VEP amplitude was not used as an outcome measure due to greater inter-individual variation (Regan, 1989).

The achromatic response was scored at the first major positive peak occurring at approximately 100ms after stimulus onset. Table 4.1 lists the response ranges to chromatic stimuli that were used to score chromatic VEP waveforms. These ranges were estimated from Rabin et al. (1994) who used similar stimulus parameters.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>40%</th>
<th>20%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/M</td>
<td>110-160</td>
<td>130-180</td>
<td>130-240</td>
</tr>
<tr>
<td>S/(L+M)</td>
<td>130-210</td>
<td>150-230</td>
<td>198-280</td>
</tr>
</tbody>
</table>

**Table 4.1** Estimated response ranges (in milliseconds) to L/M and S/(L+M) chromatic stimuli

Provided that at least two channels were functioning, the latencies of O₁, O₂, and O₂ were averaged. If only one channel was functioning, the recording was labeled as “non-repeatable”.

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4.8.4 Full-Field Electroretinogram

**Stimuli**

For the sERG, 4 ms low intensity blue (420nm, 1.60 cd·s/m²) flashes were presented at 2Hz for 20 seconds on a rod and L- and M-cone suppressing amber (594nm, 11.05 cd/m²) background. The sERG was followed by the L- and M-wavelength sensitive cone ERG. The stimulus was a 4 ms high intensity broadband white flash (10 cd·s/m²) presented at 0.5 Hz for 20 seconds on a high intensity broadband rod-suppressing white background (30 cd·s/m²).

**System and set-up**

The Espion ColorDome Full-Field Desktop Ganzfeld was used to provide all stimulus flashes and background luminance (Diagnosys; Lowell, MA). A 2 x 1 x 1/8 inch external Wratten 47B filter (Kodak; Rochester, NY) was placed in front of the ColorDome Xenon (white) flash to obtain blue (420 nm) flashes.

In accordance with ISCEV standards, a gold-plated cup electrode, which served as ground, was placed on each participant’s forehead with electrode paste. A drop of topical corneal anesthetic (0.5% proparacaine) was instilled in the participant’s eye followed by mydriatic eye drops (2.5% phenylephrine and 1% tropicamide). A drop of 0.5% carboxymethylcellulose sodium solution was placed onto the surface of a bipolar Burian-Allen lens (Hansen Ophthalmic Development Laboratory; Iowa City, IA) and the lens was carefully placed on the cornea. The Burian-Allen lens is comprised of a reference electrode and a circular wire that surrounds the lens which is the active electrode. Participants were seated in front of the Ganzfeld with his/her head stabilized using a chin rest.

Prior to stimulus flashes, participants were light adapted for 10 minutes using a high intensity broadband rod-suppressing white background (29 cd/m²). This was followed by 30 seconds of a rod and L/M-cone suppressing amber (594nm, 11.05 cd/ m²) background.
ERG acquisition and amplifier parameters

Forty segments of sERG recordings and 10 segments of long- and medium-wavelength sensitive cone ERG recordings, consisting of a 10 ms baseline before stimulus onset and 300 ms of ERG post-stimulus onset, were recorded using the Espion V5 program (Diagnosys). An analog bandpass filter of 0.312-300Hz was used to filter the signal. The signal was amplified x 10 680 and the sampling rate was 1000 Hz to avoid aliasing.

Generating average waveforms

Segments containing obvious and/or large artifacts were manually identified and excluded. The remaining segments were used to generate an average waveform.

Analysis

The outcome measures were the implicit time and amplitude of the b-wave and the amplitude of the PhNR.

The amplitude of the b-wave was measured from the a-wave. The sERG PhNR amplitude was measured from baseline at 100 ms. A preliminary analysis of PhNR amplitudes from 90-120ms in control waveforms showed that PhNR amplitudes varied the least at 100ms. Further, the use of a fixed timing has been previously reported (Rangaswamy, et al., 2007). The LM-ERG PhNR amplitude was measured from baseline between the b-wave and the i-wave as previously reported (Kizawa, et al., 2006; Machida, Gotoh, Tanaka, & Tazawa, 2004).
4.8.5 Cycloplegic Refraction and Fundus Exam

All participants had a dilated cycloplegic refraction and fundus exam performed by qualified personnel (Ophthalmologist, Optometrist or Orthoptist). This ensured that participants did not have any pre-existing eye diseases and that refractive errors were within the range specified in the inclusion criteria.

A topical corneal anesthetic (0.5% proparacaine) followed by mydriatic eye drops (2.5% phenylephrine and 1% tropicamide) were instilled into the eye. Pupil size post dilation was 8-9 mm. A retinoscope was used to assess refractive error and an ophthalmoscope was used to examine the ocular media and the posterior pole of the fundus.

4.8.6 Stereoscopic Fundus Photography

Patients had stereoscopic fundus photographs taken with a Zeiss Digital FF 450plus Fundus Camera by a retinal imaging specialist at SickKids to identify clinical signs of DR. Seven fields of the fundus were photographed including Field 1 (centered on the optic disk) and Field 2 (centered on the macula) of the modified Airlie House classification (ETDRS, 1991b). The photographs were graded based on the Airlie House classification of DR and analyzed for the presence or absence of DR by retinal specialists Drs. Shelley Boyd or Wai Ching Lam.

4.8.7 Statistical Analysis

Descriptive statistics were performed on SPSS version 15.0. Repeated measures analyses were performed using the Mixed model procedure on SAS version 9.2. A multiple regression analysis was performed using R version 2.8.1.
4.8.7.1 VEP analysis

To assess the contribution of HbA1c to the S-VEP latency while taking into account age, time since diagnosis, and measurements at more than one contrast, a type of repeated measures analysis known as the linear mixed model was conducted.

Sample Size Calculation

The sample size calculation was conducted on a preliminary subset of data using the Overall and Doyle method. A two sample t-test power analysis was multiplied by 1- the baseline to endpoint correlation to take into account co-variance in a repeated measures design (Overall & Doyle, 1994). The alpha level of significance was 0.05 and the specified level of power was 0.80. The clinically significant difference between group means was 10ms and the standard deviation was 30ms. The estimated baseline to endpoint correlation was 0.70. The sample size calculation showed that approximately 40 patients and 40 controls were needed.

Linear Mixed Model

The term “repeated measures” refers to subject data that is measured multiple times, either under different conditions, at different times, or both (Littell, Pendergast, & Natarajan, 2000). The linear mixed model is commonly used to analyze repeated measures data, particularly if there are missing values at random that create an unbalanced data set (Cnaan, Laird, & Slasor, 1997). This type of model was applied to the VEP data set in order to include all observations, as waveforms were sometimes non-recordable due to noise experienced with the equipment. The linear mixed model is an extension of the classical linear regression model (Cnaan, et al., 1997).

The general equation of the classical linear regression model can be written as $y = \beta_0 + \beta_1X_{1i} + \beta_2X_{2i} + \ldots + \beta_jX_{ji} + \varepsilon$, where $y$ is the dependent variable, $\beta_0$ is the intercept, $\varepsilon$ is a normally distributed error term, and the $\beta$s are parameter estimates. Parameter estimates are an index of the amount of variation in the dependent variable explained by the associated independent variable (Dunteman & Ho, 2006). In the classical linear regression model, parameter estimates
are also known as fixed effects, as they define the expected values of the observations (Dunteman & Ho, 2006; Littell, et al., 2000).

The linear mixed model includes fixed effects, but it also includes random effects which define the variation between subjects (variance) and within subjects (covariance). It is for this reason that this model is termed “mixed”. The general equation of the linear mixed model can be written as $y = X\beta + ZU + e$, where $\beta$ is the parameter estimate for fixed effects, $Z$ is the coefficient for random effects, and $e$ is an error term (Littell, et al., 2000).

The inclusion of covariance in the linear mixed model is the basis of the repeated measures analysis. Multiple observations from any given subject are likely correlated. Ignoring the covariance between repeated observations may result in erroneous inferences (Littell, et al., 2000).

Note that in all repeated measures analyses conducted in this thesis, the covariance structure was specified as “unstructured”. This means that the variances and covariances were freely estimated from the data. The “unstructured” covariance pattern was determined to fit the data best, as it resulted in the model with the lowest Aaike Information Criterion value. The Aaike Information Criterion value is a measure of goodness-of-fit (Motulsky & Christopoulos, 2004). It was calculated using the restricted maximum likelihood method, a common method for estimating variance in linear mixed models (Yafune, Funatogawa, & Ishiguro, 2005).

**Between-Group Comparisons**

Before regression modeling, between-group comparisons were conducted using the linear mixed model approach. This was done to assess if there was indeed a significant difference in the latencies of the S-VEP in patients when compared to controls. A simple t-test could not be conducted due to repeated measurements (i.e. recordings at 10, 20 and 40% contrast for each stimulus condition). Patient and control VEP latencies were compared at each stimulus condition (i.e. L/M, S, and achromatic).
The fixed effect of interest was “group”, specifying that there are patient and control groups. The random effect was “contrast” to take into consideration the repeated measurements. A group by contrast interaction term, which is also a fixed effect, was added to see if there is a difference in the manner by which VEP latencies change across contrast conditions for patients and controls. A Šidák post-hoc pair wise comparison test was used to identify the exact contrast levels where the significant differences were found. The Šidák test is less conservative than Bonferroni, as it reduces the chances of missing real differences (Type II error) (Abdi, 2007).

**Regression Modeling and Model Selection**

A linear mixed model that included fixed effects HbA₁c, time since diagnosis, and age at testing, was conducted using VEP patient data. The random effect, as previously described, was contrast. The dependent variable of interest was the latency of the S-VEP response.

A backwards selection procedure was used to arrive at the final model. This procedure began with the full model, which includes all the predictor variables or fixed effects. The aim of the backwards selection procedure is to reduce the full model to the fewest number of predictor variables that fits the data best. The backwards selection procedure is described below.

Considering that this is not a classical linear model, a p-value greater than 0.05 was chosen as the criterion for removing predictor variables from the model. The likelihood ratio statistic was then used to compare the reduced model with the previous full model. If significant, it demonstrated that the removed variable made an important contribution to the model. The likelihood ratio statistic is equal to the difference in deviances of the two models being compared. It has a chi-square distribution with degrees of freedom equal to the difference in the number of parameters included in the two models (Dunteman & Ho, 2006). The goodness-of-fit of each model was determined using the Aikke Information Criterion value calculated using the restricted maximum likelihood method which is used to estimate variance in linear mixed models.
4.8.7.2 ERG analysis

To assess the contribution of HbA$_1c$ to the ERG outcome measures while taking into account time since diagnosis, multiple regressions were conducted.

Sample Size Calculation

It was estimated that there would be 20-30 participants after a 15% recruitment drop-out rate. The drop-out rate was estimated from a previous diabetes study in the lab involving adolescents. Since 10-15 participants are needed per covariate included in a linear regression model, all regressions including ERG data as the dependent variable were restricted to two independent variables (i.e. HbA$_1c$ and time since diagnosis). Non-significant correlations (p>0.05) between age and each ERG outcome measure in the control group supported the exclusion of this covariate.

Multiple Regression

Multiple regressions are classical linear regression models with two or more independent (predictor) variables. It is used to estimate the effects that each predictor variable has on the dependent variable while adjusting for the other predictor variables (Wall & Li, 2003).

Between-Group Comparisons

One-tailed t-tests comparing patient and control ERG outcome measures were conducted. This was done to assess if there was a significant difference in ERG b-wave implicit times, PhNR amplitudes and/or b-wave amplitudes in patients when compared to controls that could be further investigated in a regression analysis. To account for multiple comparisons, a Šidák adjusted p-value of less than 0.012 was used as the level of significance.
Regression Modeling and Model Selection

Multiple regression models that included independent variables HbA1c and time since diagnosis were conducted using ERG patient data.

A backwards selection procedure was used to arrive at the final model. An Aaike Criterion p-value of greater than 0.157 was used as the criterion for removing predictor variables. This value is traditionally used in classical linear modeling (Dunteman & Ho, 2006). A model was deemed significant if the overall p-value was less than 0.05. The goodness-of-fit was determined using the Aaike Information Criterion value. It was calculated using the maximum likelihood method which is used to fit classical linear models (Dunteman & Ho, 2006).
5. Results

5.1 Participants

Eighty-three patients and 52 control subjects were recruited and scheduled for testing between September 2006 and April 2010.

Of the 83 patients, 26 did not attend their scheduled testing sessions. Of the 57 patients who participated, 9 were excluded from the study for the following reasons: 5 patients were found to have retinal vascular lesions following stereoscopic fundus photography and/or a dilated fundus exam (Table 5.1), 2 had a refractive error greater than -5.00 dioptres, and 2 patients had abnormal colour vision (HRR). One patient requested to leave the study. The remaining 47 patients were included in the study (Figure 5.1a).

Forty patients participated in VEP testing (equipment malfunction was the reason from non-participation of 7 patients). sERG testing began in February 2008. Twenty one patients underwent the sERG protocol. The long- and medium-wavelength sensitive cone ERG protocol began in August 2008. Of the 21 who participated in the sERG protocol, 15 participated also in the long- and medium-wavelength sensitive cone ERG protocol.

Of the 52 control subjects, 2 did not attend their scheduled testing session. Three control subjects were excluded from the study - one participant had abnormal colour vision (HRR), another had a visual acuity worse than 0.3 log MAR, and one control had missing intake information. Two control subjects requested to leave the study. The remaining 45 control subjects were included (Figure 5.1b).

Thirty nine control subjects participated in VEP testing (equipment malfunction was the reason for non-participation of 6 control subjects). Nineteen controls underwent the sERG protocol and 12 underwent the long- and medium-wavelength sensitive cone ERG protocol.
**Figure 5.1** Summary of the proportion of a) patients and b) controls who were tested and the proportion who were not tested for the indicated reasons

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Airlie House Field - Fundus details</th>
</tr>
</thead>
<tbody>
<tr>
<td>307</td>
<td>F1,F2- Large nerve fiber layer hemorrhage at disc margin</td>
</tr>
<tr>
<td>346</td>
<td>F6- Peripheral retinal hemorrhage</td>
</tr>
<tr>
<td>350</td>
<td>F1 - small microaneurysm vs. dilated vessel below optic disk</td>
</tr>
<tr>
<td>380</td>
<td>F7 - Small retinal hemorrhage in the periphery</td>
</tr>
<tr>
<td>456*</td>
<td>Small microaneurysm</td>
</tr>
</tbody>
</table>

**Table 5.1** Fundus photography results for patients with retinal vascular lesions

---

* This patient was identified as having a small microaneurysm based on a dilated fundus examination only (a fundus photograph was not taken due to the extended leave of the imaging specialist).
5.2 Study Population Demographics

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 47)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (Male/Female)</strong></td>
<td>21/26</td>
<td>18/27</td>
</tr>
<tr>
<td><strong>Age at testing (years)</strong></td>
<td>15.67 ± 1.67 (11.95 to 18.82)</td>
<td>16.55 ± 3.82 (12.15 to 27.05)</td>
</tr>
<tr>
<td><strong>Time since diagnosis (years)</strong></td>
<td>9.64 ± 3.19 (4.90 to 15.87)</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>8.58 ± 1.22 (6.4 to 12.00)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 5.2 Patient and control demographics presented as mean ± SD (range), n/a – not applicable

On average, patients were nearly the same age as controls (Figure 5.2). The larger age range of controls tested can be attributed to its correspondingly wide age range in the inclusion criteria.

Histograms displaying the distribution of time since T1D diagnosis and HbA1c values are in Figures 5.3 and 5.4 respectively.
Figure 5.2 Boxplot describing the age range of patients and controls. The lower end of the box represents 25% of the distribution (1st quartile) and the top end of the box represents 75% of the distribution (3rd quartile). Taken together, the entire box represents the middle 50% of the distribution. The line in the middle of the box is the median and the whiskers are the 5-95% span of the distribution. Circles are outliers and stars are extreme outliers. A one-tailed t-test was conducted to compare the age ranges.
The time since diagnosis was between 6 and 12 years in 50% of patients (Figure 5.3). The longest time since diagnosis was nearly 16 years.

![Histogram displaying the distribution of the time since T1D diagnosis](image)

**Figure 5.3** Histogram displaying the distribution of the time since T1D diagnosis

Fifty percent of patients had an HbA$_{1c}$ value between 7.8 and 9.30% (Figure 5.4). This is higher than the 7.0% target recommended by the Canadian Diabetes Association. The highest HbA$_{1c}$ value was 12% and the lowest was 6.4%.

![Histogram displaying the distribution of patient HbA$_{1c}$ values closest to the day of testing](image)

**Figure 5.4** Histogram displaying the distribution of patient HbA$_{1c}$ values closest to the day of testing.
Of the 47 patients, 46 had visual acuity data, 43 had the refractive error assessed and 46 had contrast sensitivity assessment. Forty-six patients had colour vision as assessed by HRR and 42 had assessments using the Mollon-Reffin Minimalist test. Data from the remaining patients were missing.

Visual acuity, refractive error, and contrast sensitivity were within normal limits for patients and controls (Table 5.3). Fifty percent of patients had a visual acuity within -0.08 and 0.04logMAR, a refractive error within -2.19 and 0.00 (plano), and a contrast sensitivity within 1.65 and 1.80 (Figures 5.5-5.7, 1st and 3rd quartiles). Note that the median contrast sensitivity for both patients and controls was 1.65 (Figure 5.7). Fifty percent of controls had a visual acuity within -0.1 and 0.06logMAR, a refractive error within -0.75 and 0.75, and a contrast sensitivity within 1.65 and 1.75 (Figures 5.5-5.7, 1st and 3rd quartiles).

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visual acuity (logMAR)</strong></td>
<td>-0.02 ± 0.09 (-0.20 to 0.22)</td>
<td>-0.02 ± 0.13 (-0.20 to 0.28)</td>
</tr>
<tr>
<td><strong>Refractive error</strong></td>
<td>-1.02 ± 1.67 (-4.25 to 1.25)</td>
<td>-0.25 ± 1.76 (-0.20 to 4.25)</td>
</tr>
<tr>
<td><strong>Contrast sensitivity</strong></td>
<td>1.71 ± 0.10 (1.60 to 1.95)</td>
<td>1.72 ± 0.12 (1.45 to 1.95)</td>
</tr>
</tbody>
</table>

Table 5.3 Visual acuity, refractive error, and contrast sensitivity presented as mean ± SD (range)

Colour vision results for patients were within normal limits for both HRR and Mollon-Reffin Minimalist tests.

Of the 45 control subjects, all had visual acuity and contrast sensitivity values which fell within normal limits. Twenty four of the control subjects had missing refractive errors; however any myopic refractive error was ruled out because the visual acuities were within normal limits for all control subjects. HRR and Mollon-Reffin Minimalist scores were normal for all control subjects.
Figure 5.5 Boxplots comparing visual acuity ranges of controls with patients

Figure 5.6 Boxplots comparing refractive error ranges of controls with patients
Figure 5.7 Boxplots comparing contrast sensitivity ranges of controls with patients

\[ p = 0.33 \]
5.3 VEP analysis

Between-Group Comparisons

S-VEP latencies were compared in patients and controls using a linear mixed model.

Table 5.4 shows the results of three separate between-group comparisons that were conducted for each VEP stimulus condition. The fixed effects included were “group”, which was the parameter of interest, and a “group*contrast” interaction term. The random effect included was “contrast”. The Šidák adjusted p-value for multiple comparisons was p < 0.012.

<table>
<thead>
<tr>
<th>Effects</th>
<th>p-values for VEP stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Fixed: Group (Patients vs. Controls)</td>
<td>0.0085*</td>
</tr>
<tr>
<td>Fixed: Group*Contrast</td>
<td>0.20</td>
</tr>
<tr>
<td>Random: Contrast</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 5.4 Results of a VEP between-group comparison using a linear mixed model

*p<0.012
The results show that there is a significant difference in S-VEP latencies but not L/M- or achromatic-VEP latencies between patients and controls. This is displayed graphically in Figures 5.8-5.10. A Šidák post-hoc pair wise comparison test further demonstrated that the difference in S-VEP latencies was found specifically at 10 and 20% contrast (Figure 5.8).

Regardless of the stimulus condition, there was no difference in the manner by which the VEP latency changed across contrasts between patients and controls. The high significance level for contrast demonstrated that there was a significant difference in the S-VEP latency from one contrast level to the next.

![Figure 5.8 S-VEP latencies by contrast in controls and patients, mean ± SE](image)

*\(p<0.012\)
Figure 5.9 L/M-VEP latencies by contrast in controls and patients, mean ± SE

Figure 5.10 Achromatic-VEP latencies by contrast in controls and patients, mean ± SE
Regression Modeling and Model Selection

To assess the association of HbA1c to the S-VEP latencies while controlling for age, time since diagnosis, and repeated measurements, a linear mixed model was conducted. The dependent variable was S-VEP latencies in the patient group. The independent variables were HbA1c, time since diagnosis, age (fixed effects), and contrast (random effect).

Model 1 demonstrated that HbA1c, time since diagnosis and age did not contribute significantly to the variation in patient S-VEP latencies (Table 5.5). An attempt to remove any one of the variables from the model did not result in a model that was significantly different from the first according to the likelihood ratio test (p>0.05).

| Model 1: S-VEP latencies = β0 + HbA1c + time since diagnosis + age + [contrast] |
|---------------------------------|-------|------------------|-----------------|--------|
|                                 | β     | Standard error   | Confidence interval (95%) | p-value |
| Intercept (β0)                  | 310.18| 97.33            | -0.31-620.67 | 0.0034 |
| HbA1c (%)                       | 1.20  | 6.61             | 0.01-2.39   | 0.85   |
| Time since diagnosis (years)    | -4.40 | 2.31             | -8.79-0.00  | 0.07   |
| Age at testing (years)          | -3.36 | 3.89             | -6.71- 0.02 | 0.39   |
| Akaike Information Criterion    | 851.8 |                  |                |        |
| Null Model Likelihood Ratio Test| p < 0.001* |

Table 5.5 Results of a linear mixed model of S-VEP latencies including HbA1c, time since diagnosis and age as covariates

*p<0.05
Considering that the first model was indeed the final model, a likelihood ratio test was conducted against the null model which contains no fixed effects (all $\beta$s =0).

The results demonstrate that the variation in the S-VEP latencies in the patient group can be attributed to the fixed and random effects included in the model, albeit the contribution was not significant. S-VEP latencies were not significantly associated with HbA$_{1c}$, time since diagnosis, and age, after consideration of repeated measurements.
5.4 ERG analysis

**Between-Group Comparisons**

LM-ERG b-wave implicit times, PhNR amplitudes, and b-wave amplitudes were compared in patients and controls using a one-tailed t-test.

In the LM-ERG, fifty percent of patients had a b-wave implicit time within 34-36 ms while controls were within 34-35 ms (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles). The PhNR of half of patients peaked within 50-86 uv while controls were within 48-73 uv (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles). Fifty percent of patients had a b-wave amplitude within 147-183uv whereas controls were within 167-247uv (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles).

There were no abnormalities in patient LM-ERG b-wave implicit times (Figure 5.11) and PhNR amplitudes (Figure 5.12) when compared with controls, but there was a reduction in the b-wave amplitudes in the patient group (Figure 5.13). The Šidák adjusted p-value for multiple comparisons was \( p < 0.012 \).

![L- and M-cone ERG b-wave implicit time by Group](image)

**Figure 5.11** Boxplots of LM-ERG b-wave implicit times in controls and patients
**Figure 5.12** Boxplots of LM-ERG PhNR amplitudes in controls and patients

**Figure 5.13** Boxplots of LM-ERG b-wave amplitudes in controls and patients
sERG b-wave implicit times, PhNR amplitudes, and b-wave amplitudes were then compared in patients and controls using a one-tailed t-test.

In the sERG, fifty percent of patients had a b-wave implicit time within 44-49ms while controls were within 38-47ms (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles). The PhNR of half of patients peaked within 51-55uv while controls were within 51-91uv (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles). Fifty percent of patients had a b-wave amplitude within 52-75uv whereas controls were within 66-87uv (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles).

The sERG b-wave implicit times were delayed (Figure 5.14) and the PhNR amplitudes were reduced (Figure 5.15) in patients when compared with controls. The b-wave amplitudes however were not significantly different from controls (Figure 5.16). The Šidák adjusted p-value for multiple comparisons was $p < 0.012$.

![Boxplots of sERG b-wave implicit times in controls and patients](image)

\textbf{Figure 5.14} Boxplots of sERG b-wave implicit times in controls and patients
Figure 5.15 Boxplots of sERG PhNR amplitudes in controls and patients

*\( p = 0.0021 \)

Figure 5.16 Boxplots of sERG b-wave amplitudes in controls and patients

\( p = 0.0535 \)
Regression Modeling and Model Selection

Given that a significant reduction in the LM-ERG b-wave amplitude was found in patients, a linear regression model including HbA1c and time since diagnosis was conducted. A non-significant correlation between age and the LM-ERG b-wave amplitude supported the exclusion of this covariate (p>0.05).

Table 5.6 describes the results of the regression. On the basis of the Aaike Criterion p-value of p>0.157 for the removal of a covariate, both HbA1c and time since diagnosis were significant. Since the overall p-value of the model was greater than 0.05, however, this regression was not significant. The goodness-of-fit was 142.23 and Pearson’s r was 0.53.

<table>
<thead>
<tr>
<th>Model 1: LM-ERG b-wave amplitudes = ( \beta_0 + \text{HbA1c} + \text{time since diagnosis} )</th>
<th>( \beta )</th>
<th>Standard error</th>
<th>Confidence interval (95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (( \beta_0 ))</td>
<td>228.33</td>
<td>61.90</td>
<td>0.00 – 456.65</td>
<td>0.003</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>-12.89</td>
<td>6.35</td>
<td>-25.78 – 0.00</td>
<td>0.07*</td>
</tr>
<tr>
<td>Time since diagnosis (years)</td>
<td>4.58</td>
<td>2.68</td>
<td>0.00 – 9.16</td>
<td>0.12*</td>
</tr>
</tbody>
</table>

Table 5.6 Description and results of a linear regression model of sERG b-wave amplitudes including HbA1c and time since diagnosis as covariates

*\( p < 0.157 \)
The results demonstrate that the variation in LM-ERG amplitudes in the patient group is not significantly attributed to HbA\(_1c\) and time since diagnosis.
Given that a significant delay in sERG b-wave implicit times and a reduction in the sERG PhNR amplitudes were found, two linear regressions including HbA\textsubscript{1c} and time since diagnosis as covariates were conducted. Non-significant correlations between age and the sERG b-wave implicit times and PhNR amplitude supported the exclusion of this covariate (p>0.05).

Regression modeling with sERG PhNR amplitudes as the dependent variable and HbA\textsubscript{1c} and time since diagnosis as independent variables resulted in a significant association (Table 5.7-5.9). Regression modeling with sERG b-wave implicit times did not result in any significant associations.
Table 5.7 describes the results of a regression with sERG PhNR amplitudes as the dependent variable and HbA1c and time since diagnosis as the independent variables.

The goodness-of-fit of this model was 190.97. With the highest $\beta$ value and the lowest p-value, HbA1c was found to be significantly associated with the sERG PhNR amplitudes. The $\beta$ value associated with time since diagnosis was not significant, as it was greater than 0.157. According to the Aaike Information Criterion, this made this variable eligible for removable from the regression.

| Model 1: sERG PhNR amplitudes = $\beta_0 + \text{HbA1c} + \text{time since diagnosis}$ |
|-------------------------------------------------|---|-----------------|-----------------|-------|
|                                                        | $\beta$ | Standard error | Confidence interval (95%) | p-value |
| Intercept ($\beta_0$)                                 | 113.56 | 30.58          | 0.00 - 227.12      | 0.002  |
| HbA1c (%)                                              | -9.52 | 3.03           | -19.04 - 0.00      | 0.005* |
| Time since diagnosis (years)                           | 1.45  | 1.28           | 0.00 - 2.89        | 0.275  |
| Residual standard error                                |       |                | 20.38 on 18 degrees of freedom |       |
| Akaike Information Criterion                          |       |                | 190.97             |       |

*Table 5.7 Description and results of a linear regression model of sERG PhNR amplitudes including HbA1c and time since diagnosis as covariates

*p<0.157
Table 5.8 describes the results of the second regression of which time since diagnosis was removed. The goodness-of-fit of this regression has improved slightly from the first, as the Aaike Information Criterion value has decreased. The associated $\beta$ value for HbA$_{1c}$ remains significant.

<table>
<thead>
<tr>
<th>Model 2: sERG PhNR amplitudes $= \beta_0 + \text{HbA}_{1c}$</th>
<th>$\beta$</th>
<th>Standard error</th>
<th>Confidence interval (95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept ($\beta_0$)</td>
<td>130.93</td>
<td>26.59</td>
<td>0.00 – 261.85</td>
<td>9.44 x 10^{-5}</td>
</tr>
<tr>
<td>HbA$_{1c}$ (%)</td>
<td>-9.86</td>
<td>3.04</td>
<td>-19.72 – 0.00</td>
<td>0.004*</td>
</tr>
<tr>
<td>Residual standard error</td>
<td>20.53 on 19 degrees of freedom</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akaike Information Criterion</td>
<td>190.41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.8 Description and results of a linear regression model of sERG PhNR amplitudes excluding time since diagnosis

*P<0.157
To ensure that time since diagnosis did not contribute significantly to the original regression, a likelihood ratio test was conducted (Table 5.9). The non-significant results (p>0.05) of the likelihood ratio test demonstrate that time since diagnosis does not contribute significantly to the model. Model 2 therefore best explained the data.

<table>
<thead>
<tr>
<th>Regression</th>
<th>Log likelihood</th>
<th>Degrees of freedom</th>
<th>Chi-square</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-91.487</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-92.202</td>
<td>-1</td>
<td>1.431</td>
<td>0.232</td>
</tr>
</tbody>
</table>

**Table 5.9** Likelihood ratio test comparing Model 1 with Model 2

Referring back to Table 5.8, the results show that a one-unit increase in HbA\textsubscript{1c} is associated with a 9.86uv or approximately a 15% decrease in the sERG PhNR amplitude. Figure 5.15 displays graphically the association between HbA\textsubscript{1c} values and the sERG PhNR amplitudes in the patient group.
Figure 5.17 Final univariate regression of sERG PhNR amplitudes in the patient group including HbA$_{1c}$ values as a covariate
6. Discussion

Long-term glycemic control, as measured by hemoglobin A$_1c$ (HbA$_1c$), is a strong risk factor for DR. The WESDR, an observational epidemiologic study, has shown that patients with a higher HbA$_1c$ value at baseline have an increased incidence and progression of DR (R. Klein, et al., 2008). The DCCT has also demonstrated this relationship by showing that strict glycemic control (HbA$_1c$ <6.0%) is associated with large declines in the incidence (53%) and progression (70%) of DR (DCCT, 1993).

In the current study, the association of HbA$_1c$ was investigated with electrophysiological measures of retina-to-striate and neuroretinal function in short-wavelength sensitive cone pathways. An association would support the use of short-wavelength electrophysiological measures as a practical means for identifying patients who are at higher risk of later developing clinically visible DR.

The potential of short-wavelength electrophysiological measures to function as biomarkers for DR is largely unknown. Two electrophysiological studies have been conducted that assessed the association of HbA$_1c$ with short-wavelength cone pathway function. Elia et al. (2005) studied the integrity of retina-to-striate pathways using the S-VEP. Yamamoto et al. (1997) studied the integrity of short-wavelength pathways in the retina using the sERG. Both studies did not find an association with HbA$_1c$, but a few key factors were not taken into consideration.

Firstly, age is a risk factor for DR and a confounder in the interpretation of short-wavelength electrophysiological responses. The WESDR and the more recent WDRS have shown that older age at examination is associated with an increased prevalence of DR (R. Klein, et al., 1984a; LeCaire, et al., 2006). Short-wavelength VEP and ERG responses have also been shown to change with age (M. Crognale, 2002; Gouras, et al., 1993). In a linear regression which included S-VEP latencies as the dependent variable and HbA$_1c$ as the independent variable, age was not included as a possible covariate in the Elia et al. (2005) study which included children aged 6-13 years. The association of age with S-VEP latencies in the age-matched control group was also not assessed. Similarly, in the Yamamoto et al. (1997) study which included patients aged 23-60
years, age was not considered as a covariate with HbA$_{1c}$. The association of age with S-VEP b-wave implicit times and amplitudes in the control group was not assessed.

Secondly, a longer duration of diabetes is associated with the increased incidence and severity of DR. The WESDR showed that after 20 years of diabetes, nearly all patients (98%) in the younger-onset group developed some degree of DR (R. Klein, et al., 1984a). The Yamamoto et al. (1997) study did not include duration of diabetes as a potential covariate in its analysis with HbA$_{1c}$.

Finally, no study to date has assessed the association between HbA$_{1c}$ and inner retinal function in short-wavelength cone pathways of patients with diabetes. Van Dijk (2009, 2010) has recently reported thinning of the inner retinal layers in patients with T1D both with and without early DR (Van Dijk, et al., 2009; Van Dijk, et al., 2010). Functional studies by Kizawa et al. (2006) on the photopic negative response (PhNR) and Vadala et al. (2002) and Li et al (1992) on oscillatory potentials also demonstrate inner retinal dysfunction both in the presence and absence of clinical DR (X. Li, et al., 1992; Vadalà, et al., 2002). These studies have assessed the integrity of the inner retina in L- and M-cone pathways. Considering the reported vulnerability of short-wavelength cone pathways to disease and insult, it may be of interest to investigate inner retinal function in short-wavelength cone pathways specifically.

In this thesis, the association of HbA$_{1c}$ was investigated with the S-VEP and the sERG. The outcome measure of the S-VEP was latency. The outcome measures of the sERG were the implicit time and amplitude of the b-wave and the amplitude of the PhNR. In considering both questions, age and duration of diabetes were taken into account.

Linear regressions including a sERG outcome measure as the dependent variable were limited to two independent variables (i.e. HbA$_{1c}$ and duration of diabetes) due to a small sample size. Non-significant correlations between age and each sERG outcome measure in the control group supported the exclusion of this covariate from the regression. Age may contribute to abnormalities in the sERG after 30 years of age, as short-wavelength pre-receptoral filtering increases markedly due to increased lens density and yellowing (Gouras, et al., 1993).
In this thesis, S-VEPs were delayed in patients with T1D at lower chromatic contrast levels (10 and 20%). This was probably linked to the fact that the stimulus used approached the contrast threshold of short-wavelength cone pathways. Glycemic control was not associated with the S-VEP latencies when age and time since diagnosis were taken into account. A type of repeated measures regression known as the linear mixed model was used to take in account measurements at 10, 20 and 40% contrast. The advantage of including repeated measurements in the analysis is that it reduced the variability in the dependent variable so that more accurate estimates could be made for the covariates included.

Abnormalities were also found in the sERG. The middle retina b-wave showed increased implicit times and the inner retina PhNR was reduced in patients. A linear regression showed that HbA$_{1c}$ was associated significantly with sERG PhNR amplitudes but not the b-wave implicit times when duration of diabetes was taken into account. The significant association between HbA$_{1c}$ and the sERG PhNR was the main result of this study.

The sERG PhNR results in this study are supported by the S-VEP findings. Retinal ganglion cell axons in short-wavelength cone pathways project to the koniocellular layers of the lateral geniculate nucleus to comprise the retina-to-striate short-wavelength cone pathways. The lack of an association of HbA$_{1c}$ with the S-VEP, however, may be a reflection of the variability of the VEP. There may have been insufficient power in this study to reflect the association that was found in the sERG PhNR. This eliminates the S-VEP as a potential sensitive biomarker of early DR progression in patients with diabetes.

The reduction of sERG PhNR amplitudes in patients with T1D and no clinically visible DR may be related to a loss of integrity of small bistratified ganglion cells. These ganglion cells are found in short-wavelength cone pathways. Glycemic control may play a direct role in the loss of integrity of these cells.

Gastinger et al. (2008) reported the selective enlargement of the somas of small bistratified ganglion cells in an animal model of T1D after three months of uncontrolled hyperglycemia (Gastinger, et al., 2008). Soma swelling may be related to apoptosis (Weber, Kaufman, &
Hubbard, 1998). The mechanism by which hyperglycemia may induce cell death is glutamate excitotoxicity. Experimentally induced hyperglycemia in animal models has been shown to depress the function of glutamate transporters on glial cells through oxidation (Q. Li & Puro, 2002; Puro, 2002). Glial dysfunction may be linked with abnormal elevations of glutamate in the retina (Lieth, et al., 1998a). High levels of retinal glutamate are toxic to inner retinal cells, causing the degeneration of inner retinal layers (Lucas & Newhouse, 1957; Sisk & Kuwabara, 1985).

The reduction in the sERG PhNR amplitudes may also be related to decreased retinal blood flow in the perifoveal capillary network. Retinal blood flow supplies the metabolically active inner retina (Wangsa-Wirawan & Linsenmeier, 2003) and S-cones are found mainly in the perifovea (Marc & Sperling, 1977).

In the perifovea, abnormally reduced blood speeds have been demonstrated in patients with diabetes (Arend, et al., 1991). Progressive slowing of blood speeds in perifoveal capillaries has been shown in patients with DR over a mean follow-up time of approximately 2.5 years (Rimmer, Fallon, & Kohner, 1989). Retinal ganglion cells are particularly sensitive to a decrease in blood oxygen content. Adler et al. (1981) has shown that systemic hypoxia significantly decreased the basal spiking (action potential) rate of retinal ganglion cells (Alder & Constable, 1981). Hyperglycemia may contribute to decreased retinal blood flow, as strict glycemic control initiated immediately after the induction of experimental diabetes in an animal model has been shown to be sufficient to maintain normal retinal blood flow (Clermont, et al., 1994).

Increased implicit times of the sERG b-wave were found in this study reflecting middle dysfunction in short-wavelength pathways. The results suggest that neurotransmission signals from ON-bipolars and/or upstream S-cones are delayed. This dysfunction was not associated with HbA$_1c$ or duration of diabetes, suggesting that middle retina dysfunction in short-wavelength cone pathways is associated with other factors. Inner retinal disease may compromise outer and middle retina function due to a shift in oxygen tension (Flower & Patz, 1971).
Finally, in this thesis, cone pathway abnormalities were not restricted to short-wavelength pathways. The middle retina b-wave amplitude of the LM-ERG was found to be reduced in patients when compared to controls, albeit this reduction was not significantly associated with \( \text{HbA}_{1c} \) or duration of diabetes. The results suggest that there is a reduction in the number of functioning ON-bipolar cells in L/M-cone chromatic pathways and/or upstream L- and/or M-cones.

A loss of L- and M-cone pathway sensitivity may be more directly associated with other factors related to diabetes apart from \( \text{HbA}_{1c} \) and duration of diabetes. Lobefalo et al. (1998) tested patients with T1D with and without microalbuminuria. The sensitivity of both groups to an achromatic stimulus outside of the central 18° of the visual field was assessed using the achromatic perimetry which assesses the sensitivity of L- and M-cone pathways specifically. The authors reported a loss of sensitivity in the microalbuminuria group compared to the group of patients without microalbuminuria. A reduction in the sensitivity of L- and M-cone pathways was not found when the microalbuminuria group was compared with controls. The results of the Lobefalo et al. study suggest that there may be other factors related to diabetes that are associated with the loss of sensitivity in L-and M-cone pathways. High systolic or mean arterial blood pressure (Hovind, et al., 2004) and high triglyceride levels (Chaturvedi, et al., 2001) are risk factors for microalbuminuria. These factors may be involved in the loss of sensitivity of L- and M-cone pathways in patients with diabetes.

There are number of unique characteristics in this study that allowed for a better investigation of the association of \( \text{HbA}_{1c} \) with electrophysiological measures of retina-to-striate and neuroretinal function in short-wavelength sensitive cone pathways.

Testing adolescents reduced the chances of confounding factors in adults such as short-wavelength pre-receptoral filtering caused by increased lens density and lens yellowing. The duration of diabetes in an adolescent population is also likely not as long as in an adult population. This is critical considering that the ultimate goal of a marker for DR is detection early in the course of disease progression.
Ambient blood glucose levels were also monitored and maintained within 4-10 mmol/L before, during, and between electrophysiological tests in this study. Schneck et al. (1997) showed that S-VEP latencies follow acute increases and decreases in blood glucose levels. The 4-10 mmol/L range, as suggested by Dr. Denis Daneman (endocrinologist and the Chief of Pediatrics at SickKids), is broad enough to ensure the safety of patients and to adjust blood glucose levels within a reasonable time frame. While it is likely that blood glucose levels may have changed slightly during electrophysiological testing, ambient glucose levels were adjusted in consultation with a nurse such that any changes would be minor.

Finally, to our knowledge, this is the first study to date to investigate and find an association between HbA1c and the sERG PhNR in diabetes. This thesis reports that poor glycemic control is associated with neuroretinal dysfunction, specifically in the inner retina, of short-wavelength cone pathways in adolescents with T1D and no clinically evident DR.

The sERG PhNR may be a potential biomarker of DR.
7. Future Directions

To further strengthen and expand on the results of this current study, a key question that no study to date has definitively answered is whether neuroretinal dysfunction is predictive of vascular lesions that characterize DR. In order to address this question, a longitudinal study expanding on the design of this current study would need to be conducted. A cohort design employing the sERG PhNR as the outcome measure of interest may help to answer this question. Results of a longitudinal design may also help to further elucidate the mechanisms underlying the pathogenesis of DR.

In a cohort design, a group of patients without DR would be routinely followed-up for 5 years. Short-wavelength electroretinogram testing would also be conducted and the sERG PhNR amplitude would be assessed in these patients. All patients would undergo a dilated fundus exam and colour fundus photography to look for vascular lesions characteristic of DR. Patients not identified as having clinical DR during this longitudinal study would serve as an internal control group for the patients identified as having clinical DR.

A logistic regression model with DR/no DR as the dependent variable and sERG PhNR amplitudes, HbA$_1c$, duration of diabetes, and age as the independent variables would then be generated. The results of this regression would demonstrate the association of the sERG PhNR amplitude with the presence or absence of clinically visible DR while taking into account other known risk factors for DR. If there is an association between the sERG PhNR and clinical DR, the dependent variable could be further partitioned into varying severities of DR in order to assess the association of the sERG PhNR with DR progression. A positive association between the sERG PhNR and increasing DR severity would further support the sERG PhNR as a potential biomarker for DR. It would also provide a window of opportunity for a structural vs. functional association study, potentially using adaptive optics technology. Structural assessment of retinal areas such as the inner retina and correlations with sERG PhNR function in the same cohort of patients may help to elucidate early pathogenic mechanisms of DR that are not well understood.
References


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Appendix A: Research Ethics Board Approval

RESEARCH ETHICS BOARD

August 14, 2009

Dr. Carol Westall
Ophthalmology
The Hospital for Sick Children

Dear Dr. Westall:

Your study "Non-invasive Measures (biomarkers) of Eye Brain Function in Early Diabetes"

REB File No.: 1000007794

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 August 2010. The REB approved continuing review at level 3D. 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): Wal Ching Lam, Derek Stephens, Lawrence Leiter, Amir Hanna, Thomas Wright, Giuseppe Mirabella
Appendix B: Sample Consent Form

Department of Ophthalmology
Hospital for Sick Children (SickKids)

RESEARCH CONSENT FORM (For Subjects with diabetes, with capacity to consent)

TITLE OF STUDY:
Non invasive Measures (biomarkers) of Eye Brain Function in Early Diabetes.

INVESTIGATORS:

Director of Electrophysiology: Dr. Carol Westall (416) 813-6516
Responsible Individual: Dr. Carol Westall (416) 813-6516
Endocrinologist Dr. Lawrence Leiter (416) 867-7441
Endocrinologist Dr. Amir Hanna (416) 867-3721
Retinal Specialist: Dr. Wai-Ching Lam (416) 813-6525
Biostatistician: Mr. Derek Stephens MSc (416) 813-7377
Research technologist: Mr. Thomas Wright BSc (416) 813-7790
Research Fellow: Dr. Giuseppe Mirabella (416) 813-7654 x. 3606
Masters Candidate: Ekta Lakhani (416) 813-7654 x. 3606
Masters Candidate: Michelle McFarlane (416) 813-7654 x. 3606
Masters Candidate: Julianna Sienna (416) 813-7654 x. 3606

Purpose of the Research:

The purpose of this study is to help us understand more clearly if early eye and brain changes occur in children and young persons with type I diabetes. You do not have diabetes. Current tests for diagnosing diabetic eye disease are based on the assumption that the primary problem is damage to blood vessels of the retina. Based on this blood vessel damage, treatment is given late in the disease when there is high risk of sudden, severe visual loss. Recent evidence suggests that the nerve cells within the retina and the brain may be damaged before, or along with, blood vessels.
We will evaluate four nerve function tests together to see how well they detect diabetic damage, and how well they can follow changes over time. This has not been done before.

We will then check your vision with a visual acuity test (ability to see fine detail). The colour visual evoked potentials (colour VEP), colour electroretinogram (colour ERG) and multifocal electroretinogram (mfERG) will also be conducted. Lastly, we will complete the routine eye exam with refraction (whether you need glasses) and ophthalmoscopy (examine the back of the eye).

If the routine clinical examination (visual acuity, refraction, ophthalmoscopy) show any abnormalities we will discuss these findings with you and refer you for further testing if required.

**Description of the Research:**

Participation in this study will require you to visit the hospital once for approximately 2.5 hours. The order in which the tests will be administered is as follows:

1) Introduction to study by coordinator, discussion of inclusion criteria, consent form signed, questions answered.
2) Vision: We will examine your vision by asking you to read some letters on a regular vision chart to measure what you can see.

[* The above listed tests should take about 10mins.]*

3) Colour Visual Evoked Potentials (VEP):

We will place small gold cap electrodes (sensors) filled with electrode paste on you head after the scalp is cleaned with a very mild abrasive gel. A bandage will be wrapped around the head to keep the caps in place. Using a colour monitor we will then flash coloured lights at your eyes. The electrodes on the head will pick up signals from the brain responding to the flashing lights. The test, including explanation of the test, and sensor placement will take about 30 mins.
NOTE: To do the tests listed below we will need to put drops in your eyes for pupil dilation which will make your pupils bigger. After testing, your vision will be blurred, especially up close for about 4-8 hours and probably until the next day.

* Snack Break (during pupil dilation)

4) Colour Electroretinogram (ERG):

To do this colour test a local anaesthetic (numbing) eye drop and a drop for pupil dilation will be used. We will place a small piece of fiber across the front of each of your eyes. The fiber will be stuck by a piece of tape to the bridge of the nose and extend under the coloured part of the eye where it will stick to the face at the other side of the eye. This will be done for both eyes. We will then flash some lights at your eyes. The fiber will pick up electrical signals from the back of the eye. The colour ERG should take about 30mins.

5) Multifocal Electro-retinogram (mfERG):

While your pupils are still large we will place a small contact lens sensor (electrode) at the front of your eye after we have placed an anaesthetic drop in your eye, which will numb the front of your eye. The contact lens will pick up electrical signals from your retina. You will be asked to look at a target on a very small television, rather like looking through a telescope. The test is painless and will take approximately twenty minutes to complete.

6) Multifocal slow-flash electroretinogram.

The slow-flash flash ERG will seem almost identical to the multifocal electroretinogram. The difference is that bright flashes are inter-dispersed by more dark images. The data give us information about structures deeper in your retina. The test will take approximately twenty minutes to complete.

7) Ophthalmoscopy:

We will look at the inside of your eye with a bright light. This is a very routine clinical test.
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.

[* Ophthalmoscopy and Refraction should take about 10mins.*]

New information from this study or other studies may affect whether you want to continue to take part in the study. If this happens, we will tell you about this new information.

If the study involves taking photographs, videotaping or sound recordings, a separate consent form will need to be completed.

The entire examination will require about 2.5hrs.

**Potential Harms**

We know of no harm that taking part in this study could cause you.

**Potential Discomforts or Inconvenience**

The eye drops and anaesthetic drop may cause slight discomfort, but this resolves within 10 seconds. The drops, which we use to dilate your pupil, may cause your vision to be slightly poorer for 4-8 hours and pupils may remain dilated until the next day. During the colour visual evoked potential test, the scalp will be cleaned with a mild abrasive paste. This may cause mild discomfort to the areas of the scalp being cleaned. After the test, electrodes will be removed and the electrode paste cleaned off. Sometimes small amounts of paste remain in the hair, but easily removed when the hair is washed. Lastly, the testing will require about 2hrs and 30 mins so you may be tired at the end of the day from doing all the tests.
**Potential Benefits:**

You will not benefit directly from participating in this study. However, if you display problems with your vision then we can give you special help if needed.

**Confidentiality:**

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law requires us to do this. 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 of the study (Juvenile Diabetes Research Foundation), or the regulator of the study may see your health record to check on the study. For example, people from Health Canada Health Products and Food Branch, (or) U.S. National Institutes of Health, (or) U.S. Food and Drug Administration, if necessary, may look at your records.

By signing this consent form, you agree to let these people look at your records. We will put a copy of this research consent form in your patient health record. We will give you a copy for your files.

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.

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. This could happen even if the medicine [or treatment] given in the study is helping you. 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.
**Sponsorship**

The sponsor/funder of this research is the Juvenile Diabetes Research Foundation.

**Conflict of Interest**

Some of the people doing this study may have a conflict of interest. That means that they may benefit personally, financially, or in some other way from this study.

Dr. Westall (Principal Investigator) has received or may receive for research related to the present study (money, or one or more of the following other benefits: speaker's fees, travel assistance, industry-initiated research grants, investigator-initiated research grants, consultant fees, honoraria, gifts, intellectual property rights such as patents, etc.) from sponsor(s) that have activities related to the present study.
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 describe 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.
7. I have read and understood pages 1 to 5 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 & 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 ____________ at__________________

If you have questions 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 C: Patient Demographic Information

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Legend: “NR” – non-recordable
Appendix G: Copyright Permission

Dear Michelle,

This email serves as formal permission for you to use http://webvision.med.utah.edu/imageswv/DONFig3.jpg in your thesis.

Best regards,

Bryan