A Proposal for Early and Personalized Treatment of Diabetic Retinopathy Based on Clinical Pathophysiology and Molecular Phenotyping

Thomas W Gardner, MD, MS[*] and

Kellogg Eye Center, University of Michigan Medical School, 1000 Wall Street, Ann Arbor, MI 48105

Jeffrey M. Sundstrom, MD, PhD

Penn State Hershey Eye Center, Penn State College of Medicine, 500 University Drive, HU19, Hershey, PA 17033

Abstract

This paper presents a new approach to the prevention and treatment of early stage diabetic retinopathy before vision is severely impaired. This approach includes two major steps. The first step is to understand the mechanisms of vision impairment and classify diabetic retinopathy on the basis of pathophysiologic adaptations, rather than on the presence of advanced pathologic lesions, as defined by current clinical practice conventions. The second step is to develop patient-specific molecular diagnoses of diabetic retinopathy so that patients can be treated based on their individual characteristics, a process analogous to the individualized diagnosis and treatment of cancer patients. This step is illustrated by proteomic analysis of vitreous fluid that reveals evidence of neuroretinal degeneration and inflammation, as well as vascular proliferation. Together, these steps may lead to improved means to preserve vision in the ever-increasing number of patients with diabetes worldwide.

Keywords

[*]Correspondence to: Dr. Gardner at: phone 734 232 8283, fax 734 232 8030, tomwgard@umich.edu or Dr. Jeffrey Sundstrom, Phone 717 350 4578, jsundstrom@hmc.psu.edu.

Clinical Characterization of Diabetic Retinopathy

Introduction

Professor Eva Kohner exemplifies a tradition of doing the right thing for patients with diabetes. She exemplified this approach whether in the context of clinical research trials such as the United Kingdom Prospective Diabetes Study, and in her discussions of individual patients. In addition, Professor Kohner has always supported the development of young investigators, so this lecture is presented jointly by a senior (TWG) and a younger investigator (JMS). Figure 1 shows Professor Kohner surrounded by a large group of male colleagues at the 1968 Airlie House Symposium. The Airlie House Classification of diabetic retinopathy was born at this symposium and became the forerunner of the Early Treatment Diabetic Retinopathy Study classification (1, 2) that has formed the basis for the important diagnostic and therapeutic trials over the last 50 years. While this classification scheme has been extremely helpful, it was designed in an era when the essential questions were about the benefit of pituitary ablation for proliferative diabetic retinopathy, and when the benefits and risks of panretinal laser therapy for proliferative diabetic retinopathy were still debated, a decade before the Diabetic Retinopathy Study results were published. Therefore, the context was the need for a structure by which to address severe blinding retinopathy. The success of that classification now brings us to the 21st century when there is the opportunity and the need for better intervention of early stage disease before patients are at risk for losing vision. The first section of this presentation focuses on the clinical characterization of diabetic retinopathy and the quantification of structural and functional changes in diabetic retinopathy. This work and the associated concepts are the product of many investigators, notably Drs. David Antonetti, Steven Abcouwer, Patrice Fort and Gregory Jackson, who are investigating distinct aspects of the neurovascular unit and its alterations in diabetes.

Classification of diabetic retinopathy—past and future

Current clinical teaching is that diabetes impairs vision because of visible features such as tractional retinal detachment, non-clearing vitreous hemorrhage, neovascular glaucoma, macular ischemia and/or diabetic macular edema. However, as shown in Figure 2, these events do not strictly correlate with the clinical classification of retinopathy as mild, moderate or severe, non-proliferative retinopathy or proliferative retinopathy. People with mild retinopathy and macular edema can have reduced vision and patients with advance proliferative retinopathy can have nearly normal vision, when vision is defined as visual acuity.

An alternative approach to this classification based on pathologic lesions is to consider the evolution of events in a continuum of pathophysiologic events from the onset of diabetes to clinical manifestations (3). Figure 3 illustrates a concept with at least 3 stages. In the first, multiple adaptive responses occur within weeks to months of the onset of diabetes. These responses, as measured primarily in animal models, include reduced electrical activity, reduced biosynthetic activity such as protein and lipid synthesis, autophagy and apoptosis (4–7). Likewise, patients with clinically intact retinas exhibit impaired autoregulation in which there are defective vascular responses to breathing 100% oxygen or to flickering light (8–10). In this first stage vision is intact and we consider that there is no diabetic retinopathy

because adaptive responses compensate for the metabolic insults. However, after approximately 5 to 10 years, a second stage can develop in which adaptive changes begin to decompensate and there are early clinical signs such as retinal hemorrhages, microaneurysms, macular edema, vascular non-perfusion and gliosis. At this point, subtle aspects of vision, such as visual field and contrast sensitivity, are impaired and this may be considered an early form of decompensation when non-proliferative retinopathy develops. A third stage occurs after additional time and suboptimal diabetes control, a state of aberrant repair may develop with neovascularization, gliosis, fibrosis, retinal neovascularization and frank loss of vision. This pathophysiologic stage is consistent with proliferative diabetic retinopathy. The current regulatory strategies for treating retinopathy are limited to the stages of adaptive decompensated and aberrant repair and have not yet been developed for patients that have mild retinopathy and good vision. However, a recent workshop sponsored jointly by the National Eye Institute and Food and Drug Administration strongly emphasized the need for the development of functional endpoints to facilitate the treatment of early stage diabetic retinopathy.

The clinical assessment of non-proliferative retinopathy is based on the Early Treatment Diabetic Retinopathy Study grading which is neither quantitative nor linear and evaluates pathologic features of retinopathy rather than pathophysiologic processes. This construct leads to uniform treatment of patients with diabetic macular edema or proliferative diabetic retinopathy without recognizing or quantifying the differences between patients. The heterogeneity is evident in the varied response of patients with DME to anti-VEGF therapy and the relationship between intraocular VEGF concentration and response to treatment remains unknown as VEGF is not measured prior to injection. As discussed recently by Gray (11), the stages of retinopathy called DME and PDR represent failure of the adaptive mechanisms within the retina and are consistent with a stage of disease that might be called retinal failure, analogous to renal failure in persons with diabetes. A forward-looking approach consistent with the concept of personalized medicine would be to develop *quantitative* assessment of retinal pathophysiology to enable early and individualized treatments.

Cellular features of diabetic retinopathy

The conventional clinical features of diabetic retinopathy certainly include a microvascular component, but numerous studies have shown that diabetes damages the entire retina involving retinal neurons, glial cells, microglial cells and the pigmented epithelium. More than 30 papers studying human retinal samples have shown that these changes all occur, along with macrophage infiltration and retinal pigment epithelial degeneration, as reviewed by Eisma et al (12).

The neurovascular unit represents the structural and functional integration between neurons, glial cells, microglial and blood vessels such that the metabolic and electrical functions of the retina are highly integrated, as emphasized by Eric Newman (13–15). Diabetes ultimately disrupts the entire neurovascular unit, such that it becomes "dis-integrated". The insidious, progressive functional disintegration occurs over time and likely accounts for the loss of contrast sensitivity, color perception, electroretinographic responses and impaired

autoregulation. At this point, it is not clear whether neuroglial cell dysfunction leads to vascular alterations or vice versa, or whether there is a synchronized loss of cellular integrity because of interdependence of blood vessels, neurons and glia in a given area of retina.

The interrelationship between blood vessels and retinal elements was first discussed by Bek (16), who examined dye cast perfusion preparations of post-mortem human eyes. All regions of non-perfusion exhibited extensive disorganization and loss of the retinal ganglion cells and the inner nuclear layer. More recently, Dodo and colleagues (17) showed thinning of the inner retina in regions of OCT sections in the areas of non-perfusion on fluorescein angiograms. This feature may represent a severe form of the disorganization and retinal inner layers ("DRIL") noted by Sun and colleagues (18, 19). The cellular basis for this OCT-based disorganization is not yet understood.

Clinical features of neurovascular disruption

The ultimate question relates to the visual consequences of retinal dysfunction in diabetes. Vision impairment in early diabetes has been noted for several decades, with defects in color vision contrast sensitivity, dark adaptation and electrophysiology responses. Yet, despite over 70 publications in this area, there have made little impact on clinical practice, and few studies have compared multiple aspects of visual function in the same subjects. However, Jackson et al (20) reported in a cohort patients with mild non-proliferative retinopathy and 20/30 acuity or better, about one-quarter had reduced dark adaptation responses as tested with the AdaptDx (MacuLogix, Hummelstown, PA) and contrast sensitivity by the Pelli-Robson method; one-third of patients had reduced photopic visual fields and 47% had reduced scotopic visual field sensitivity. However, 83% of patients had reduced visual field sensitivity as recorded by reduced sensitivity in the fovea using the 24-2 program of the Matrix frequency doubling perimeter (Zeiss-Meditech; Dublin, CA). Together, these data reveal defects in multiple layers of the retina from the inner retina (frequency doubling perimetry and contrast sensitivity) to the photoreceptor-RPE complex (dark adaptation and scotopic and photopic visual fields) and reveal some of the heterogeneity of cellular defects in diabetic retinopathy. Of note, these findings are not reflected by standard clinical examination or by photographic grading.

Figure 4 compares retinal photographs, OCT thickness maps and corresponding frequency doubling perimetry fields in illustrative examples (3). The patient in the left column is a healthy normal person with 20/15 acuity at age 23; the thickness map shows a foveal thickness of approximately 300 microns and parafoveal thickness of approximately 375 microns and normal visual field sensitivity. By contrast, the 32-year-old patient in the middle column with 10 years of type 1 diabetes has 20/20 acuity and mild NPDR with only a few microaneurysms. This person's foveal thickness is reduced to less than approximately 250 microns and the parafoveal thickness is reduced to approximately 300 microns with 9 decibels of foveal sensitivity loss. The 19-year-old female in the right column has had type 1 diabetes since age three and has some neovascularization along the superotemporal arcade but 20/20 acuity and no subjective visual impairment. However, she exhibits diffuse thinning of her entire macula (250 microns) and has 15 decibels of foveal sensitivity loss. Figure 5 illustrates a 54-year-old male with 20/25 visual acuity who exhibits moderate capillary non-

perfusion in the midperipheral retina but slightly more in the inferior half of his eye. His Humphrey 60-4 field shows marked visual sensitivity loss of 17 to 18 decibels in the superior field and approximately 5 decibel sensitivity loss inferiorly. This case exemplifies close correspondence between retinal neurovascular damage and visual field sensitivity loss. These three cases show that standard clinical tools can reveal profound changes in retinal structure and function even in asymptomatic individuals. A further question is whether or not visual field alterations beyond visual acuity have any impact on patient's day to day living. Mazhar et al (21) reported that NEI-VFQ-25 scores for driving difficulty and the composite score fall dramatically beyond level 43 retinopathy. This finding suggests that diabetes and mild diabetic retinopathy impair multiple aspects of visual function beyond acuity.

In summary, the entire neurovascular unit with vascular damage, neuronal degeneration, glial cell and microglial cell dysfunction probably all contribute to impaired vision of persons with diabetes. Strategies to stabilize the neurovascular unit before loss of vision will be essential to enable early identification of patients at risk and treatment to maintain good vision. Clinical assessment and molecular assessment must be combined to provide more precise patient-specific therapy in the future.

Molecular Characterization of Diabetic Retinopathy

Introduction

A personalized approach to the treatment of diabetic retinopathy will require a molecular diagnosis to be paired with clinical findings so that optimal treatment can be provided for an individual patient. This approach has been successful in several areas of medicine. For example, prior to treatment, breast cancer tumors are stratified by tumor histology as well as at the molecular level. Tumors that express estrogen/progesterone receptors are treated with tamoxifen; similarly, tumors that express HER-2 are treated specifically with trastuzamab. If neither is present, then traditional non-specific chemotherapy is employed. This approach has had the benefited patients greatly by providing more specific treatments when possible with less side effects and better treatment outcomes (22–24). This concept continues to evolve with the development of prognostic multigene assays used to 'classify' breast cancer as the molecular level in order to predict progress and guide intervention (25). This approach has been successfully used in the eye for the prognosis and treatment of uveal melanoma (26).

At the present time, the treatment of diabetic retinopathy is primarily geared toward the treatment of DME and the treatment options are restricted to intravitreal anti-VEGF injections (28, 31) and intravitreal steroids (29, 30). In the coming years, it is clear that additional specific therapeutic agents will become available and these additional options will require the treating clinician to determine what specific therapeutic agent is going to be most beneficial to the current patient. In order to decide what specific therapeutic agent will work best, it is rational to conclude that the treating physician will need to classify diabetic retinopathy at the molecular level. In this regard, patients with elevated VEGF levels would be treated specifically with anti-VEGF agents. However, if testing revealed normal VEGF levels but elevated Factor X or Pathway X, then the patient could be treated with anti-Factor

X agents or Pathway X agents. While we recognize this approach will require new treatment options that are not currently available; the development of new therapies depends on new mechanistic and diagnostic insights.

In order to treat patients based in this manner, the initial step to determine treatment will be to obtain intraocular fluids and measure the various targets of the specific treatments available. Therefore, we have started to define diabetic retinopathy at the molecular level in individual patients we have developed multiple methods to analyze the molecular components of vitreous. We have recently shown that office-based vitreous aspiration is safe and allow the treating physician to measure at least 42 different cytokines and growth factors from 75 μL of vitreous fluid (32). While aqueous fluid analysis would be technically easier, the data would be misleading as aqueous cytokine and growth factor levels do not reliably correlate with vitreous levels (33). We have also shown that office-based vitreous aspirates contain sufficient volume to be used to quantify 42 cytokines and growth factors in a multiplexed assay using a total 75 μL of fluid. While this approach works well if the specific target is detectable using ELISA, it is also possible that agents may be targeted to specific molecular pathways. To this end, we have developed an unbiased proteomics assay capable of detecting over 1000 proteins from 250 μL of vitreous fluid, the volume we are routinely able to obtain in clinical vitreous aspirates. Application of this method to larger cohorts of patients will ultimately lead to the identification biomarkers and novel treatment targets for diabetic retinopathy.

Methods—This study was approved by the University of Michigan Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. Vitreous was obtained from patients undergoing vitrectomy for macular hole and/or epiretinal membrane repair (Control; n=5; pooled; 50 μL each) or for removal of non-clearing vitreous hemorrhage (PDR; n=5; pooled; 50 μL each). The average age for Control group was 66.6 while the average age of the PDR group was 62.4. Two of the five patients in each group were males. Detailed demographics of the patients are shown in SD1. The vitreous samples were collected in the operating room before clinically indicated vitrectomy. Briefly, 250 μL of each pooled sample were depleted of abundant plasma proteins using the Multiple Affinity Removal System (MARS specific for the 14 most abundant human plasma proteins (Agilent (P/N5188-6560)) according to the manufacturers protocol. Following depletion, each sample was processed using SDS-PAGE with a 4-12% Bis-Tris homogenous gel (Invitrogen) using the MOPS buffer system. Each gel lane was excised into 40 equal sized segments and digested in-gel with trypsin. Trypsin digestion was performed using a ProGest robot (DigiLab). Fragments were washed with 25mM ammonium bicarbonate followed by acetonitrile, reduced with 10mM dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at RT, and digested with sequencing grade trypsin (Promega) at 37°C for 4h and then quenched with formic acid. The supernatant of each gel digest was analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive.

Data Analysis—Data were searched using the Mascot search engine with the SwissProt Human (forward and reverse appended with common contaminant proteins) database, carbamidomethyl (C) fixed modification. Variable modification parameters were set to

oxidation (M, Acetyl (N-term), Pyro-Glu (N-term Q), and deamidation (N,Q). The peptide mass tolerance was set to 10ppm and the fragment mass tolerance was set to 0.02 Da. A maximum of 2 missed cleavages at least two unique peptides per protein. The resulting mass spectra were searched against the SwissProt database using Mascot (SwissProt_Human; forward and reverse) and the resultant Mascot DAT files were parsed into Scaffold (Proteome Software; Portland OR) for validation, filtering and generation of non-redundant identifications. Pathway enrichment analysis was conducted using Ingenuity Pathway Analysis (Qiagen). Spectral counts were increased by one for all proteins to include proteins observed in only one condition. Proteins of low abundance were removed (sum of the spectral counts <10). The ration of PDR to Control was then calculated and loaded into IPA. The complete list of all proteins is available in Supplemental Document 1 (SD1).

Results—Vitreous was digested and subjected to ESI-MS/MS as described above. A total of 12,515 peptides and 929 proteins were identified in MH/ERM vitreous while 15,279 peptides and 1,213 proteins were identified in PDR vitreous. The overall FDR was 0.9% at the peptide level and 0.22% at the protein level. A complete list of the identified proteins is available for download (SD1). Pathway enrichment analysis was conducted using a right tailed Fisher's exact test. The top 50 signaling pathways present in vitreous fluid are shown in Table 1 and a complete listing is available in SD1. The operational definition of each pathway is provided by IPA and is available online. The activation state of each pathway is predicted by IPA and presented as the z-score where positive z-scores suggest pathway activation and negative z-scores represent pathway inhibition. The number of upregulated, downregulated and unchanged proteins in each pathway is also shown. The list of each protein identified within each pathway is available in SD1. The results of this analysis must be interpreted conservatively and with caution given the small sample size.

As expected, extracellular signaling pathways present in blood, such as acute phase response signaling, coagulation system and complement system, were present in vitreous obtained from in patients with non-clearing vitreous hemorrhage, though only the acute phase response was significantly activated (z-score = 1.77). The neuroprotective HIPPO signaling pathway was inhibited in PDR vitreous (z-score = −1.77) and THOP1, a neuroprotective peptidase that is upregulated in response to Aβ peptides in Alzheimer's disease, was found to be increased PDR vitreous compared to controls. These data are consistent with prior studies suggesting a neurodegenerative state is present in pre-clinical models of diabetic retinopathy as well as recent studies revealing early thinning of the retina in patients with diabetes. Also consistent with preclinical studies (41, 42), the NRF2-mediated oxidative stress response was activated in PDR vitreous (z-score = 3.74). It is interesting that pathways known to be associated with angiogenesis, including the Wnt/Beta-catenin and VEGF pathways were present, though insufficient information was present to determine activation pathway status. As shown in Table 2, further analysis of the data using the ontology and function algorithm in IPA (Disease and Function) did reveal significant activation in proteins involved in the Proliferation of Cells (z-score = 0.764) and Cell Proliferation of Vascular Endothelial Cells (z-score = 1.23). This ontology analysis also confirmed the pathway results above and revealed the IPA curated functions Growth of Neurites (z-score = −1.61),

Developmental of Neurons (z-score = −1.85), Developmental Process of Synapse (z-score = −2.31) and Neuritogenesis (z-score = −1.40) are inhibited in PDR vitreous.

Conclusions—Analysis of 250 μL of vitreous fluid is sufficient for MS-based proteomics to pathways involved in the pathophysiology of PDR. This amount of fluid is obtainable from patients in the clinical setting and suggests that it is feasible to characterize patients at the molecular level using unbiased proteomics approaches and/or ELISA-based methods. Future work analyzing individual samples in large cohorts will be required to better define the variation in molecular phenotypes among patients with diabetic retinopathy. Ultimately, a personalized approach to the treatment of diabetic retinopathy will emerge when clinical and molecular phenotypes are interpreted together in order to determine the optimal treatment for individual patients.

Summary

Thanks to the efforts of pioneers such as Professor Eva Kohner, diabetic retinopathy is no longer treated by pituitary ablation and many people with diabetes can retain good vision lifelong. The work of our predecessors now enables the opportunity to prevent late stage diabetic retinopathy with new clinical and molecular diagnostic tools that will lead to patient-specific treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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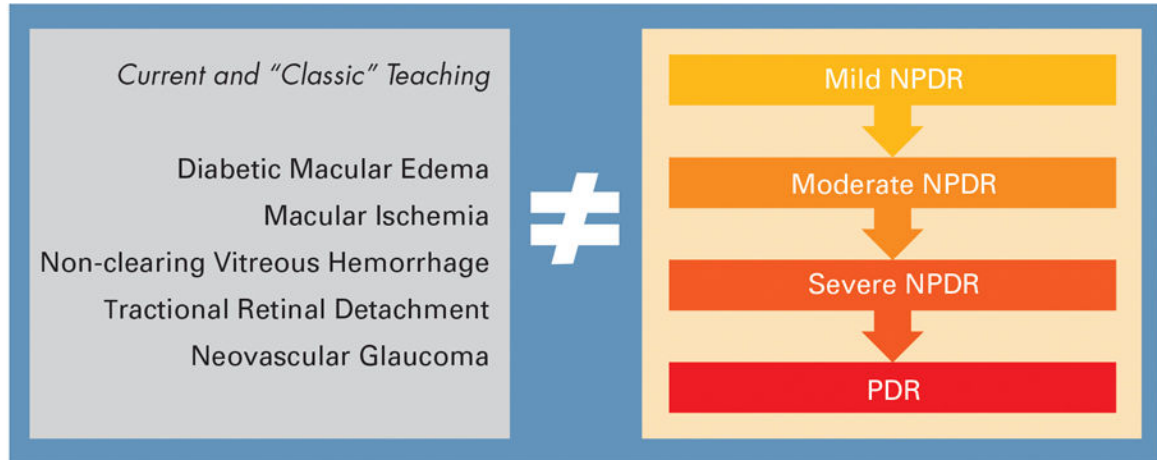
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Figure 1.
Attendees at the 1968 Airlie House Symposium with Professor Eva Kohner front and center (44).

Mechanisms of Vision Loss

Current and "Classic" Teaching

Diabetic Macular Edema
Macular Ischemia
Non-clearing Vitreous Hemorrhage
Tractional Retinal Detachment
Neovascular Glaucoma

≠

Mild NPDR
Moderate NPDR
Severe NPDR
PDR
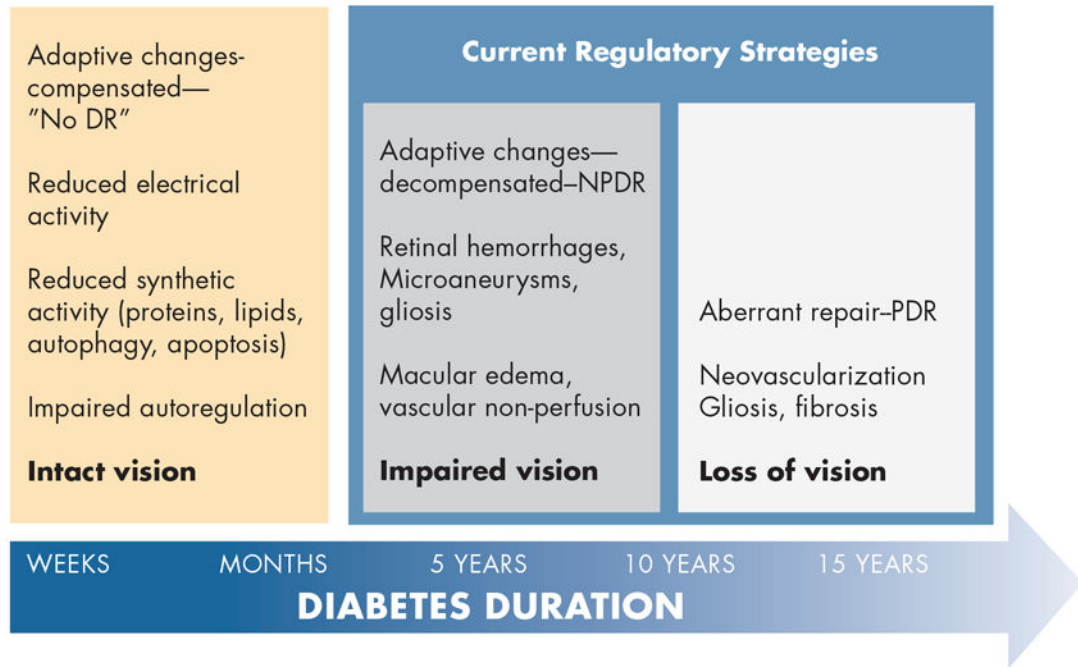
**Figure 2. Classification of mechanisms of vision loss in diabetic retinopathy**
The clinical features of diabetic retinopathy that are associated with vision loss (left) do not coincide directly with the severity scale (right).

Figure 3. A model of adaptive and maladaptive stages of diabetic retinopathy development
Within the first weeks to months of diabetes, the retina adapts to a lower metabolic steady-state with reduced electrical and biosynthetic activity, adaptive autophagy and apoptosis, and impaired autoregulation. Vision remains intact and there is no clinical evidence of diabetic retinopathy. After 5 - 10 years of diabetes, adaptive mechanisms begin to decompensate with the appearance of mild nonproliferative retinopathy, and early impairment of vision. Aberrant repair can ensue with the onset of proliferative retinopathy and loss of vision. Regulatory strategies are currently limited to the latter two stages (3). Artwork by David Murrel, MFA.
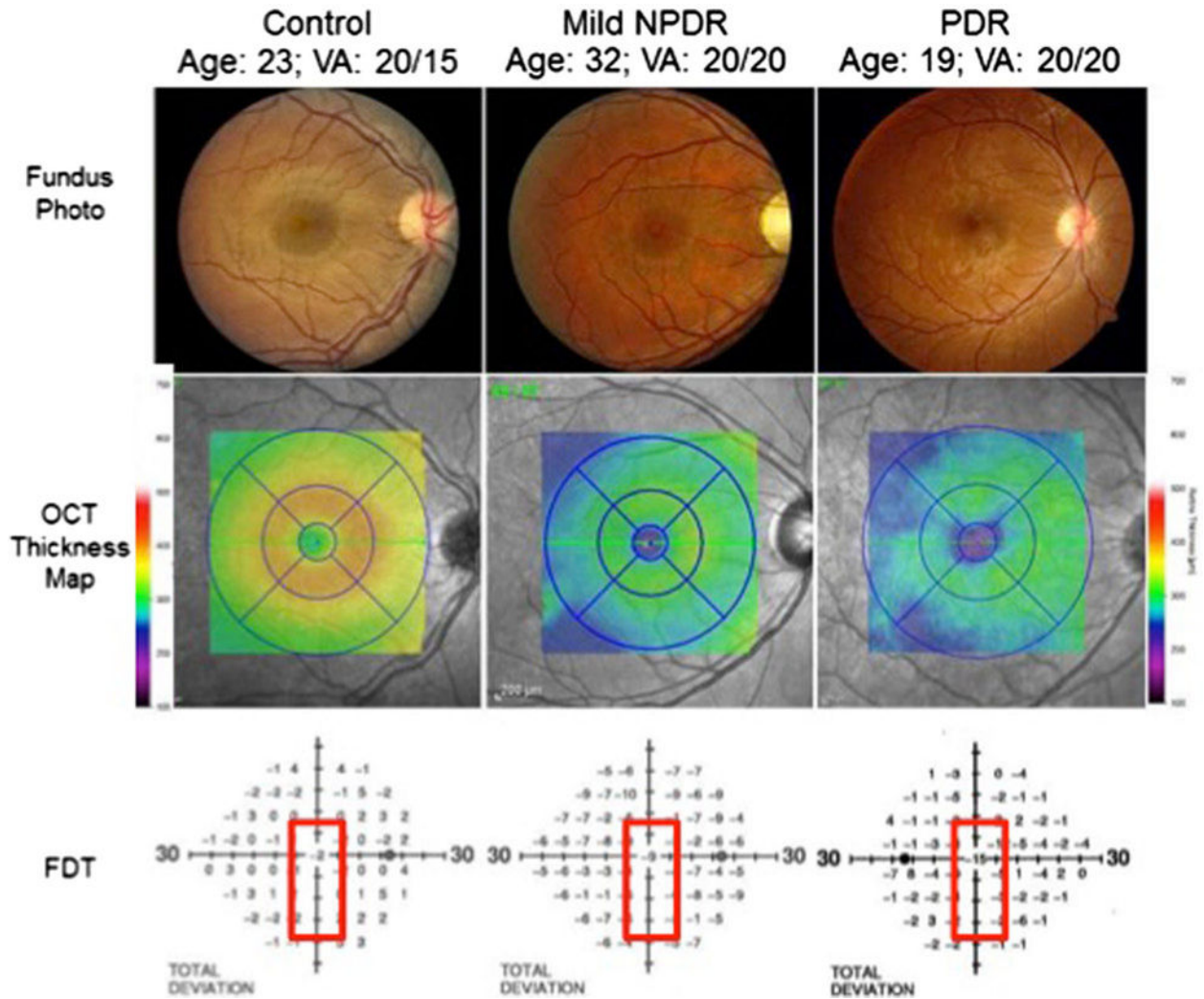
Figure 4.
Color fundus photographs (top panel) of a healthy person (left), a person with mild nonproliferative diabetic retinopathy (NPDR; middle), and a person with proliferative diabetic retinopathy (PDR; right) demonstrate preserved macular anatomy without obvious vascular compromise and visual acuities 20/20 or better. Spectral-domain optical coherence tomography (SD-OCT) macular thickness mapping (central panel) demonstrates foveal and parafoveal thicknesses reduced by between 50 and 75 μm comparing healthy eyes to those with mild NPDR or PDR. The lower panel demonstrates a concomitant decline in foveal sensitivities in the diabetic patients as measured by frequency-doubling perimetry, with a 9 decibel (dB) visual field sensitivity loss in the patient with mild NPDR and a 15 dB sensitivity loss in the patient with PDR.

Figure 5. Peripheral visual field sensitivity loss in a patient with moderate NPDR

This 76 year old female had 20/25 visual acuity and moderate reduction of capillary perfusion, especially in the inferior retina. Her 60-4 Humphrey visual field showed marked (17 - 18 dB) sensitivity loss in the superior hemifield, corresponding to the inferior vascular nonperfusion.

Table 1

Ingenuity Canonical Pathways	−log(p-value)	Ratio	z-score
Acute Phase Response Signaling	2.85E+01	3.00E-01	1.177
LXR/RXR Activation	2.42E+01	3.20E-01	−1.718
Coagulation System	2.28E+01	6.57E-01	−0.209
Intrinsic Prothrombin Activation Pathway	2.23E+01	7.24E-01	0.229
Complement System	2.20E+01	6.22E-01	−0.5
FXR/RXR Activation	1.92E+01	2.72E-01	NaN
Protein Ubiquitination Pathway	1.31E+01	1.63E-01	NaN
Extrinsic Prothrombin Activation Pathway	1.23E+01	6.67E-01	NaN
Autophagy	6.27E+00	2.32E-01	NaN
NRF2-mediated Oxidative Stress Response	5.64E+00	1.26E-01	3.742
Phagosome maturation	5.58E+00	1.42E-01	NaN
Clathrin-mediated Endocytosis Signaling	5.29E+00	1.21E-01	NaN
IL-12 Signaling and Production in Macrophages	5.25E+00	1.35E-01	NaN
IGF-1 Signaling	4.83E+00	1.48E-01	NaN
Inhibition of Matrix Metalloproteases	4.50E+00	2.31E-01	NaN
Production of Nitric Oxide and ROS in Macrophages	4.34E+00	1.11E-01	−1.279
HIPPO signaling	4.13E+00	1.51E-01	−3.464
Agranulocyte Adhesion and Diapedesis	3.67E+00	1.08E-01	NaN
RhoGDI Signaling	3.61E+00	1.07E-01	0.943
Hypoxia Signaling in the Cardiovascular System	3.48E+00	1.59E-01	NaN
Unfolded protein response	3.41E+00	1.70E-01	NaN
Myc Mediated Apoptosis Signaling	3.11E+00	1.43E-01	NaN
Antigen Presentation Pathway	3.06E+00	1.89E-01	NaN
Granulocyte Adhesion and Diapedesis	3.05E+00	1.02E-01	NaN
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	2.97E+00	1.63E-01	1.342
PI3K/AKT Signaling	2.95E+00	1.10E-01	1.604
Actin Cytoskeleton Signaling	2.73E+00	8.85E-02	0.728
Remodeling of Epithelial Adherens Junctions	2.70E+00	1.36E-01	2
p70S6K Signaling	2.66E+00	1.03E-01	−1.89
Regulation of Cellular Mechanics by Calpain Protease	2.59E+00	1.43E-01	1
Axonal Guidance Signaling	2.46E+00	7.16E-02	NaN
ILK Signaling	2.36E+00	8.76E-02	0
Leukocyte Extravasation Signaling	2.35E+00	8.53E-02	0.258
ERK5 Signaling	2.23E+00	1.25E-01	NaN
VDR/RXR Activation	2.21E+00	1.15E-01	NaN
14-3-3-mediated Signaling	1.96E+00	9.16E-02	2.646
Endoplasmic Reticulum Stress Pathway	1.95E+00	1.90E-01	NaN
Wnt/Beta-catenin Signaling	1.88E+00	8.38E-02	−0.707

Ingenuity Canonical Pathways	−log(p-value)	Ratio	z-score
Antioxidant Action of Vitamin C	1.83E+00	9.52E-02	0.378
Role of Tissue Factor in Cancer	1.82E+00	9.09E-02	NaN
RhoA Signaling	1.79E+00	9.02E-02	1
Mitotic Roles of Polo-Like Kinase	1.74E+00	1.11E-01	NaN
Lipid Antigen Presentation by CD1	1.74E+00	1.67E-01	NaN
Tumoricidal Function of Hepatic Natural Killer Cells	1.74E+00	1.67E-01	NaN
Aldosterone Signaling in Epithelial Cells	1.72E+00	8.00E-02	NaN
LPS/IL-1 Mediated Inhibition of RXR Function	1.66E+00	7.51E-02	−0.816
Epithelial Adherens Junction Signaling	1.64E+00	8.28E-02	NaN
Semaphorin Signaling in Neurons	1.63E+00	1.15E-01	NaN
Dendritic Cell Maturation	1.57E+00	7.65E-02	1.069

Table 2

Functional Ontology	p-Value	Activation z-score
Proliferation of cells	7.79E-40	7.64E-01
Cell proliferation of vascular endothelial cells	2.56E-13	1.23E+00
Growth of neurites	1.99E-15	−1.61E+00
Development of neurons	3.39E-13	−1.85E+00
Developmental process of synapse	3.87E-11	−2.31E+00
Neuritogenesis	8.58E-11	−1.40E+00

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