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TABLE OF CONTENTS

DIABETES RESEARCH

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Research

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Use of High Resolution Digital Retinal Imaging in the Early Detection of Retinopathy in Type 1 Diabetes

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ABSTRACT

Background: Type 1 Diabetes Mellitus is one of the most common endocrine and metabolic conditions worldwide, affecting nearly half a million children under the age of 15 years with an anticipated rise in incidence of 4%. The ocular complications of type 1 diabetes can be blinding and thus, inflicting catastrophic consequences on quality of life. In this disease, the greatest impact in the prevention of vision loss comes from early detection and treatment.
Methods: Retinal screening was performed to capture both right and left eyes of children as young as 5 years of age. A Canon CR-2 Plus AF (Tokyo, Japan) non-mydriatic retinal camera with a CMOS chip, a resolution of 18 megapixel and an ISO setting of 400 (range available is from 200 to 6400 ISO) in sensitivity was used for these images. Image management used was *image SPECTRUM V5* (Canon USA, Irvine, CA), a postcapture imaging software that automatically separated a color image into three (3) monochromatic images; namely blue, green and red, to help visualize the nerve fiber layer, the retinal layers as well as the choroid, respectively.
Results: In subjects with positive findings (mild diabetic retinopathy), retinal pathology was noted on digital imaging and involved retinal hemorrhages and vascular changes consistent with microaneurysms. Severe nonproliferative diabetic retinopathy was detected in one 20-year-old subject. One subject presented with cataract. Subjects with a positive finding were counseled and a referral to an ophthalmologist was recommended.
Conclusions: Non-mydriatic retinal imaging used in mass screenings can help identify the early retinal changes and advance the management and care of patients with diabetic retinopathy. Improvements in digital imaging software and the ability to perform telemedicine from remote locations, can aid eye health care providers in the detection and isolation associated with various levels of retinopathy.

KEYWORDS: Retinopathy; Microaneurysms; Type 1 diabetes; Cataract.

ABBREVIATIONS: FFL: Friends for Life; RGB: Red, Green, and Blue.

INTRODUCTION

Type 1 diabetes may be considered one of the most common endocrine and metabolic conditions worldwide, affecting nearly half a million children under the age of 15 years.[1] Roughly 80,000 new cases involving patients younger than 15 years are added every year and an anticipated rise in incidence of 4% may be expected.[2-4] Best estimates are that between the ages of 15 to 25 years, another half a million young individuals are likely to have type 1 diabetes.

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The ocular complications of type 1 diabetes can be blinding. Blindness in the younger population inflicted with type 1 diabetes can have lifelong, catastrophic consequences on quality of life, ability to live independently, education, productivity and longevity. In considering a worldwide distribution, one quarter of children with type 1 diabetes live in Europe, while 23% live in Southeast Asia. North America and the Caribbean represent roughly 19% of cases.[5] China, with a population of 1.3 billion, is also experiencing a rise in type 1 diabetes with an incidence of 0.59 per 100,000 persons per year.[1] This number may appear deceivingly low when compared to that of the European countries; however, it is staggering when taking into consideration the total population of the country. In viewing mortality rates, children residing in high-income countries afflicted with the disease, have a mortality rate doubled than that of their counterparts without the disease.[6] In Sudan, the mortality rate is 42.6 deaths per 100,000[7] as compared to 0.63 deaths per 100,000 in the same age group of children living in the United States.[8]

Early detection of diabetic eye disease can lead to early treatment and prevention of this vision loss. The purpose of this study was to assess the feasibility of performing large-scale screenings of children with type 1 diabetes and their accompanying families using a non-mydriatic digital camera. In utilizing this technology, we integrated high resolution capturing devices; as well as, digital post-processing imaging as part of an efficient screening protocol. Further evaluations of this process included auto refraction, visual acuity, intraocular pressures and Spectral Domain-OCT analysis.

MATERIALS AND METHODS

Retinal screening was performed during the annual Friends for Life (FFL) Conference in Orlando, Florida between July 1 and July 6, 2014. Friends for Life is the largest association dedicated to educating children with type 1 diabetes and their families on all aspects of the disease. In addition to providing families with the most advanced knowledge in diabetes care, FFL offers education regarding eye health and a respective retinal screening to all children with type 1 diabetes.

The screening program captured both right and left eyes of children as young as 5 years of age without the use of mydriatic agents. All images were captured in a dimly lit room at a level of 125 candelas. Retinal screening was performed utilizing a Canon CR-2 Plus AF (Tokyo, Japan) non-mydriatic retinal camera with a CMOS chip, a resolution of 18 megapixel and an ISO setting of 400 (range available is from 200 to 6400 ISO). The CR-2 Plus AF has a 45-degree field of view and minimum pupilary dilation of 3.3 mm that enables multiple images to be stitched together to increase the field of view of the posterior pole from 45 degrees to 110 degrees. We used the autofocus function of the Canon CR-2 Plus AF; as well as, the auto capture (with blink detection preventing unintentional capture). To improve imaging quality, we made use of the auto exposure feature that resulted in consistent, good color (hue); as well as, good overall exposure and balance of all captured images. It has been well documented that a static image, such as a retinal photograph (Figure 1A), has a higher sensitivity in pick-up rate for evidence of retinopathy as seen in Figure 1B; as patients tolerate better a single flash than continuous bright light from an indirect ophthalmoscope.[9-11] Furthermore, with high-resolution digital imaging, it becomes possible to separate specific layers of the posterior pole.

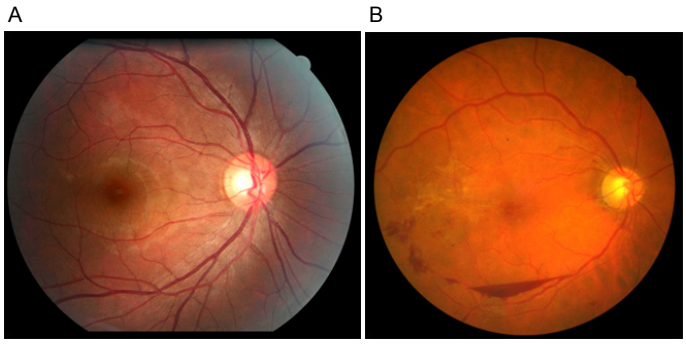

Figure 1: A. Healthy, retinal view at an angle of 45 degrees. **B.** View of the posterior pole demonstrating retinal hemorrhages of a subject with DM1.

A typical color image is composed of Red, Green, and Blue (RGB) light. All images were postcapture-processed using a propritary software, *image SPECTRUM V5* (Canon USA, Irvine, CA, USA) that met all FDA as well as HIPAA regulation standards. The postcapture imaging software automatically separates a color image into 3 monochromatic images highlighting the area of the retinal layers (green channel at about 550 nm) of a subject with diabetic retinopathy findings (Figures 2A, 2B and 2C). Figure 2D illustrates a fundus auto-fluorescence of the same eye, highlighting retinal hemorrhages and masking the exudates seen in other modalities as seen in Figures 2A, 2B and 2C.

In the search for evidence of diabetic retinopathy, using a green (red free image), monochromatic channel postcapture, increases the image contrast and can highlight small dot hemorrhages that can otherwise be missed. RGB allows a visual representation of where the event is happening; for example, if there is a retinal hemorrhage, it will be best seen in the G channel, thus confirming retinopathy. Another post-processing filter used in screening for retinopathy is called Emboss. This pre-set monochromatic filter allows us to observe the captured retinal image in 3D, topographical view of the green layer of the retina at 550 nm (Figure 2C). Retinal auto-fluorescence is another area of interest in ocular imaging that looks specifically for lipofuscin. We used auto-fluorescence imaging to determine if small changes associated with retinopathy could be visualized. Subjects with a positive finding (mild diabetic retinopathy) were counseled and a referral to an ophthalmologist was recommended.

RESULTS

One hundred and fifty eight children with type 1 diabetes underwent retinal screenings throughout the 2014 Annual

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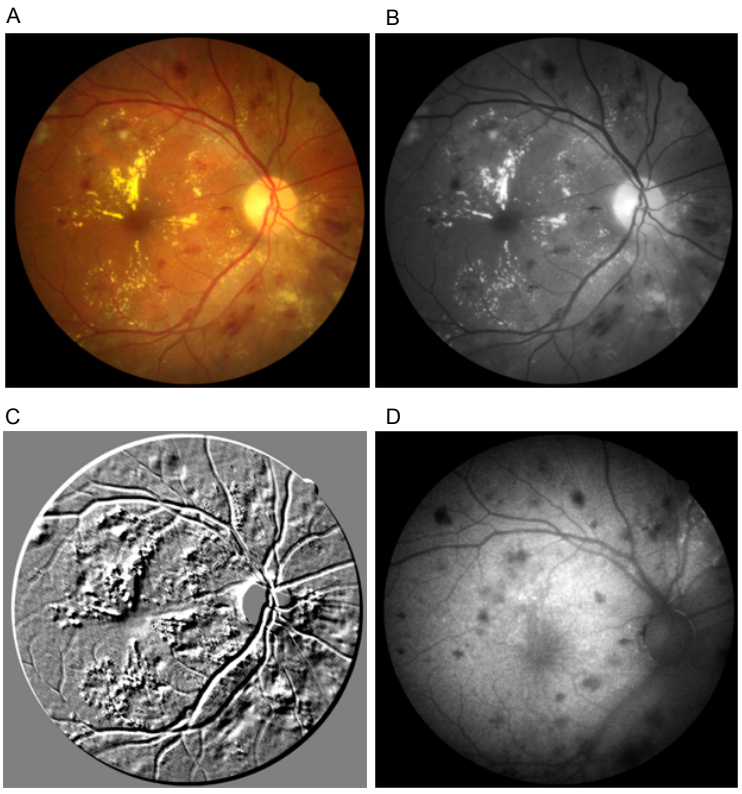

Figure 2: A. Color photo of patient with diabetic retinopathy and posterior pole exudates. **B.** Monochromatic view of red free extraction of color image. **C.** Topographical view of retina demonstrating 3D-like view of the retina with retinopathy and exudates. **D.** Fundus Auto-Fluorescence image demonstrating retinal hemorrhages and masking macular exudates.

Meeting of "Friends for Life". The children ranged from 5-30 years of age, with a mean age of 14 years. Seventy nine percent (79%) of the children were Caucasians, with twenty-one (21%) reported as "Other Race" and 56% were females. Duration and HbA_{1C} were self-reported, with a mean of 7.8 years (range 0.4 to 25 years) and 7.9%, respectively. Visual acuity was 20/20 for 57% of the right eye and 61% for the left eye. Table 1 provides patient demographics along with clinical characteristics of the screened population. Our success rate for artifact-free images was 92% for all screened children and a 5% pick-up rate was found for early signs of diabetic retinopathy in this population.

In subjects with positive findings, retinal pathology was noted on digital imaging and involved retinal hemorrhages and vascular changes consistent with microaneurysms. No subjects presented with proliferative diabetic retinopathy and one subject presented with a cataract.

DISCUSSION

The Friends for Life organization prides itself on several objectives; one of which is to connect families with one another, while educating them on the nature of type 1 diabetes and how to prevent its complications. The complications of type 1 diabetes are caused, but not limited to, duration or onset of diabetes, poor control of glucose levels (H_1AC) and a combination of poorly controlled HbA_{1C} and high blood pressure (Figures 3A and 3B).

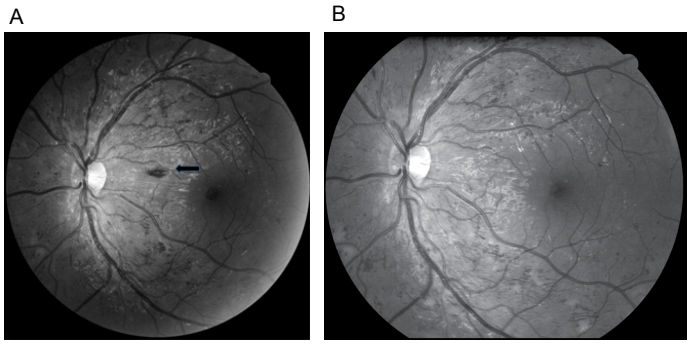

Figure 3. Retinal images of DM1 left eye. **A.** Complications of DM1 due to a combination of poorly controlled H_1AC and high blood pressure with associated flame hemorrhages. **B.** Result of improved control of H_1AC and high blood pressure three weeks post screening.

Routine, yearly retinal exams are performed to help manage and educate children and their families to maintain healthy retinas. Advanced imaging technologies permit us to utilize high resolution digital imaging to enable analysis of various layers of the retina and determine the health of the nerve fiber layer, retinal layers and the choroid. With diabetes, our primary interest lies in the retinal layer best visualized in the green (red free) layer. As the initial image resolution of the Canon CR-2 Plus AF is 18 Mp, when separating the layers into RGB, one is left with 3 images with resolutions of roughly 6 Mp each.

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Race		
	White	79%
	Other	21%
Age (y)		
	Mean	14
	Range	5 to 30
Gender		
	Male	44%
	Female	56%
Duration of DM1 (y)		
	Mean	7.8
	Range	0.4 to 25
Visual Acuity		
	Right	(20/20) 57%
	Left	(20/20) 61%

Table 1: Patient demographics and clinical characteristics.

This allows us to best observe changes which could be missed with lower resolution. In addition, when performing auto-fluorescence imaging (that does not require the injection of sodium fluorescein to the subject), we have been successful in identifying new areas of retinopathy not yet visible in baseline color retinal imaging as demonstrated in Figure 2D. The inability to determine an imaging technology as a gold standard presented as a limitation to our study. However in future studies, confirmation through office physical examination can be performed.

Although a gold standard for imaging technology has not been determined, these emerging technologies assist in an early diagnosis and are made readily available to patients and their families throughout the event.

CONCLUSION

Ocular complications associated with type 1 diabetes post +/- 12 years of onset of the disease may include: diabetic retinopathy, macular edema and cataracts if left unchecked. With the prevalence of diagnosis at a young age, type 1 diabetic patients can experience vision complications by the time the child is a teenager. Duration of the disease, poor glycemic control, elevated blood pressure and sedentary life style all pose as risk factors to retinopathy that if left unchecked, can lead to vision loss and even blindness. The purpose of yearly exams as recommended by the American Academy of Ophthalmology,[12] the American Academy of Optometry;[13] as well as, the American Diabetic Association[14] is to detect early ocular changes.

Retinal imaging used in mass screenings can help identify early retinal changes; as well as, aid in the management and care of retinas demonstrating changes associated with diabetes. Advances in digital imaging software can assist eye health care providers in the detection and isolation of ocular events. Remote reading centers can use advanced imaging tools to best isolate and detect subtle changes easily missed in early signs of retinopathy. In our experience, use of both remote telemedicine and high resolution imaging systems, with associated software management systems, can greatly aid in the intervention and maintenance of the patient's ocular care. Thus, early detection is pivotal in the management of and in maintaining vision and quality of life for children with type 1 diabetes.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest

CONSENT

Consent was obtained at the time of examination.

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Research

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Apelin Gene Therapy Increases Autophagy *via* Activation of Sirtuin 3 in Diabetic Heart

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ABSTRACT

Heart failure is the leading cause of death in diabetic patients. Recently we showed that apelin gene therapy attenuates heart failure following myocardial infarction. This study further explored the potential mechanisms by which apelin may reduce cardiac injury in Post-myocardial infarction (MI)) model of diabetes. Wild type and Sirt3 knockout (Sirt3 KO) mice were induced into diabetes by intra-peritoneal (i.p.) Streptozotocin (STZ). STZ mice were then subjected to MI followed by immediate intramyocardial injection with Adenovirus-apelin (Ad-apelin). Ad-apelin treatment resulted in over expression of apelin in the ischemic hearts of STZ mice. Apelin over expression led to a significant increase in Sirt3 expression. Apelin over expression significantly reduced $gp91^{phox}$ expression. This was accompanied by a significant reduction of reactive oxygen species formation. Ad-apelin treatment also dramatically reduced NF-κb-p65 expression in WT-STZ mice. Over expression of apelin further enhanced autophagy markers (LC3-II and beclin-1) expression in post-MI heart. Most intriguingly, knockout of Sirt3 in STZ mice abolished these beneficial effects of apelin treatment. *In vitro,* knockout of Sirt3 in EPCs significantly enhanced high glucose-induced ROS formation. Conversely, treatment of Sirt3 KO-EPCs with NADPH oxidase inhibitor led to two fold increase in LC3-II levels. Our studies demonstrate that apelin increases autophagy *via* up regulation of Sirt3 and suppression of ROS-NF-κb pathway in diabetic heart.

KEYWORDS: Sirtuin 3; Autophagy; ROS; Apoptosis; Myocardial infarction; Diabetes.

ABBREVIATIONS: HF: Heart Failure; DM: Diabetes Mellitus; BMCs: Bone Marrow Cells; STZ: Streptozotocin; LAD: Left anterior descendant artery; DHE: Dihydroethidium; Ad-GFP: Ad-green fluorescent protein; DAPI: Diamino-2-phenyl indole; MI: Myocardial Infarction.

INTRODUCTION

Long known to augment the risk for cardiovascular disease, Diabetes Mellitus (DM) increases mortality of patients with Heart Failure (HF) over that observed in HF patients without DM.[1] Cardiovascular disease is one of the major complications of DM.[2] Clinical studies show that Myocardial Infarction (MI) is the leading cause of morbidity and mortality in the patients with DM.[3,4] A population-based study also reveals that the incidence of MI in diabetic patients is significantly higher than non-diabetic patients.[5] Therefore, it is urgent to develop new agents for the treatment of post-MI heart failure in DM.

Apelin is a bio-activated peptide which binding to the apelin receptor (APJ).[6] Apelin has been shown to protect the heart against ischemia injury and reduce infarct size.[7] A recent study also showed that deficiency of apelin exacerbated ischemia-reperfusion injury.[8] We have reported that over expression of apelin promoted myocardial angiogenesis and improved cardiac function in post-MI diabetic STZ mice and these beneficial effects of apelin were mediated through activation of Sirt3.[9] Sirt3 is a member of a highly conserved family

of protein deacetylases, which is closely associated with the prolonged lifespan of human.[10] Sirt3 has been shown to regulate cardiomyoctye apoptosis, survival and cardiac hypertrophy.[11] Previously, we also observed that treatment with Bone Marrow Cells (BMCs) over-expressing apelin enhanced myocardial angiogenesis and functional recovery, accompanied by increased Sirt3 levels in the ischemic heart.[12] Our study further showed that knockout of Sirt3 blunted the protective effect of apelin in cultured EPCs.[12] These findings indicate a critical role of Sirt3 in apelin-mediated protective effect in post-MI heart.

The present study was designed to evaluate the functional role of Sirt3 in apelin-mediated beneficial effects against ischemic injury in diabetic mouse model. Wild type and Sirt3 knockout (Sirt3 KO) mice were treated with streptozotocin (STZ) to induced hyperglycemic DM model followed by myocardial infarction by ligation of Left Anterior Descendant (LAD) artery. Using this ischemic STZ mouse model, we have examined the effects of apelin gene therapy on the autophagy and ROS formation in ischemic hearts of diabetes. Moreover, we have explored the potential mechanisms by which apelin regulates myocardial autophagy in diabetes.

MATERIALS AND METHODS

All procedures conformed with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of University of Mississippi Medical Center (protocol identifier: 1280). The investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Experimental Animal Model Treatment

Wild type control and Sirt3 knockout (Sirt3 KO) mice (obtained from Jackson laboratory, Bar Harbor, Maine, USA) were bred by our lab. Experimental mice (male at 4-5 month age) were intra-peritoneal (i.p) injected with streptozotocin (STZ, 50 mg/kg, Sigma Co, MO, USA) for 5 days to induce diabetes. At 6 weeks, mice with blood glucose level >300 mg/dl were selected for the left anterior descending coronary (LAD) artery ligation to induce MI. Experimental STZ mice were anesthetized with ketamine (100 mg/kg) plus xylazine (15 mg/kg), intubated, and artificially ventilated with room air. A left thoracotomy was performed, and the LAD was exposed and ligated with 8-0 nylon suture. Ischemic areas were intramyocardial injected with adenovirus-apelin (Ad-apelin) and adenovirus-β-gal adenovirus (Ad-β-gal) at the dose of 1×10^9 PFU per heart at four sites. Experimental STZ mice were divided into 4 groups: (i) WT-STZ+Ad-β-gal (n=14 mice); (ii) WT-STZ+Ad-apelin (n=14 mice); (iii) SIRT3KO-STZ+Ad-β-gal (n=14 mice); and SIRT 3KO-STZ+Ad-apelin (n=14 mice). After 2 weeks of ad-apelin or Ad-β-gal gene therapy, mice were sacrificed by cervical dislocation under anesthesia with isoflurane.

Western Blot Analysis of PHD2, HIF-1α, NF-κb, Apelin, gp91^{phox}, Beclin-1 and LC3-I/II Expression

Hearts were harvested and homogenized in lysis buffer for Western analysis. Following immunoblotting, the membranes were blotted with HIF1-α, apelin, gp91^{phox} (1:1000, Cell Signaling, MA, USA), PHD2, NF-κb-p65, beclin-1 and LC3-I/II (1:1000, Santa Cruz, CA, USA) antibodies. The membranes were then washed and incubated with a secondary. Antibody coupled to horseradish peroxidase and densitometric analysis was carried out using image acquisition and analysis software (TINA 2.0).

ROS Formation Assays in Heart Tissue

To detect *in situ* generation of ROS in heart tissues, Dihydroethidium (DHE) staining was performed. DHE (1 nM, Molecular Probes, Oregon, USA) was applied to each heart tissue section and cover-slipped. Slides were incubated in a dark, humidified chamber at 37 °C for 30 min. The nuclei were counterstained with 4,6-diamino-2-phenyl indole (DAPI). The relative density of red (DHE) fluorescence was quantified by measuring 5 random fields per section using image-analysis software (Image J, NIH).

Endothelial Cell Progenitor Isolation, Culture and Identification

EPC was isolated and cultured from femur and tibia bone marrow of WT and Sirt3 KO mice as described previously.[13] Two EPC markers, IB4 (1:50 dilute) and CD34 (1:200 dilute), were used for EPC identification by immunohistochemistry.

EPC Treatment and Transfection

To mimic *in vivo* hyperglycemic conditions of DM model, EPC were exposed to high glucose (30 mmol/L) for 24 hours, and followed by transfection with Ad-apelin and Ad-β-gal (1×10^9 PFU) in serum-free medium. An Ad-green fluorescent protein (Ad-GFP) was applied to the cultured EPC as a marker to determine the transfection efficiency before transfected with Ad-apelin and Ad-β-gal.

To detect the intra-cellular ROS production in EPC, 1×10^4 cells were seeded in chamber wells and cultured for 24 hours to reach >80% confluence. Then CM-H2DCFDA (10 μmol/L, Molecular Probes, Oregon, USA) was added to chamber wells for 30 minutes. The nuclei were counterstained with 4,6-Diamino-2-phenyl indole (DAPI). The relative density of green (DCFDA) fluorescence was quantified by measuring 5 random fields per section using image-analysis software (Image J, NIH).

Statistical Analysis

Data are presented as the mean±standard deviation.

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Statistical analysis of data were performed with one-way ANOVA followed by the post hoc test and P values less than 0.05 were considered as significant.

RESULTS

Apelin Gene Therapy Increases Post-Mi Survival Rate of Mouse

WT-STZ mice receiving Ad-apelin or Ad-β-gal treatment were survived for 2 weeks of post-MI. There was no death in Sirt3 KO-STZ mice received Ad-apelin treatment after 2 weeks of post-MI, whereas, there was an approximately 42.9% death rate in Sirt3 KO-STZ mice received Ad-β-gal treatment ($P<0.001$).

Apelin Gene Therapy Upregulates Sirt3 Expression in the Hearts

As shown in Figure 1A, intramyocardial injection with Ad-apelin led to apelin overexpression in the hearts of WT-STZ mice. Sirt3 expression was significantly upregulated by apelin overexpression in the hearts of WT-STZ mice compared to WT-STZ mice treated with Ad-β-gal (Figure 1B).

Apelin Gene Therapy Attenuates Myocardial gp91^{phox} Expression and ROS Formation in Post-MI STZ Mice

Ad-apelin treatment significantly inhibited NADPH oxidase gp91^{phox} expression in post-MI STZ mice, but failed to reduce gp91^{phox} expression in post-MI Sirt3 KO mice (Figure 2A). Ad-apelin treatment significantly reduced ROS formation in the hearts of WT-STZ mice when compared with Ad-β-gal treatment. In Sirt3 KO-STZ mice, Ad-apelin treatment did not suppress ROS formation in comparison with Ad-β-gal treatment (Figures 2B and 2C).

Apelin Gene Therapy Elevates Autophagy Gene Beclin-1 and LC3-II Levels in Post-MI STZ Mice

Overexpression of apelin resulted in significant increases in beclin-1 and LC3-II expression in WT-STZ+MI mice when compared with Ad-β-gal treatment (Figures 3A and 3B). No significant alterations in beclin-1 or LC3-II expression were found in Sirt3 KO-STZ+MI mice received Ad-apelin treatment (Figures 3A and 3B). Using immunohistochemistry staining, we further confirmed that apelin overexpression dramatically increased number of LC3-II positive cells compared to Ad-β-gal treatment. In contrast, the Ad-apelin treatment did not change LC3-II levels in Sirt3 KO-STZ+MI mice (Figure 3C).

Apelin Gene Therapy Attenuates NFκb Expression, but not Prolyl Hydroxylase-2 (PHD2) and HIF-1α Expression

To determine whether apelin regulates the transcriptional regulator gene expression, NFκb-p65 and PHD2/HIF-1α expression were examined. As shown in Figure 4A, the NFκb expression level was significantly higher in post-MI Sirt3 KO-STZ mice. Overexpression of apelin significantly reduced NFκb-p65 expression level in post-MI WT-STZ mice, but failed to inhibit its expression in post-MI Sirt3 KO-STZ mice. Moreover, apelin overexpression had little effects on HIF-1α and PHD2 expression both in WT and Sirt3 KO-STZ mice (Figures 4B and 4C).

Overexpression of apelin reduces ROS formation in EPC

To mimic STZ hyperglycemic condition *in vivo*, cultured EPCs were exposed to high glucose (30 mmol/L) for 24 hours before transfection with Ad-apelin. Overexpression of apelin significantly reduced high glucose induced ROS formation. Moreover, Knockout of Sirt3 abolished apelin-mediated suppression of ROS formation in EPC under high glucose conditions (Figures 5A and 5B). To determine the interactions of Sirt3, NADPH oxidase and ROS on autophagy gene expression, Sirt3KO-EPCs were exposed to NADPH oxidase inhibitor apocynin for 24 hours. As shown in Figure 5C, treatment of Sirt3KO-EPCs with Apo (200 and 400 microM) increased LC3-II expression.

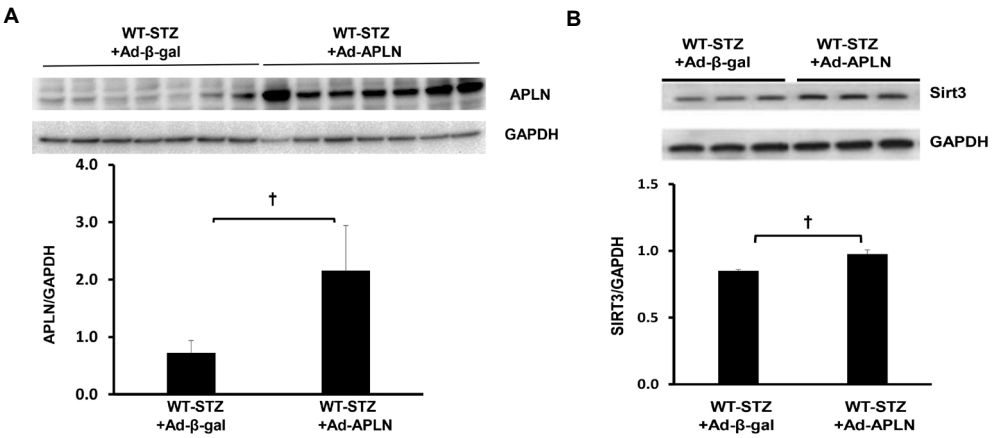

Figure 1: Apelin gene therapy on apelin and Sirt3 expression in post-MI STZ mice. (A) Ad-apelin treatment significantly increased apelin expression in the heart of WT-STZ (n=3, †P<0.05). (B) WT-STZ mice had a significant increase in Sirt3 expression in the heart after Ad-apelin gene therapy (n=3, †P<0.05)

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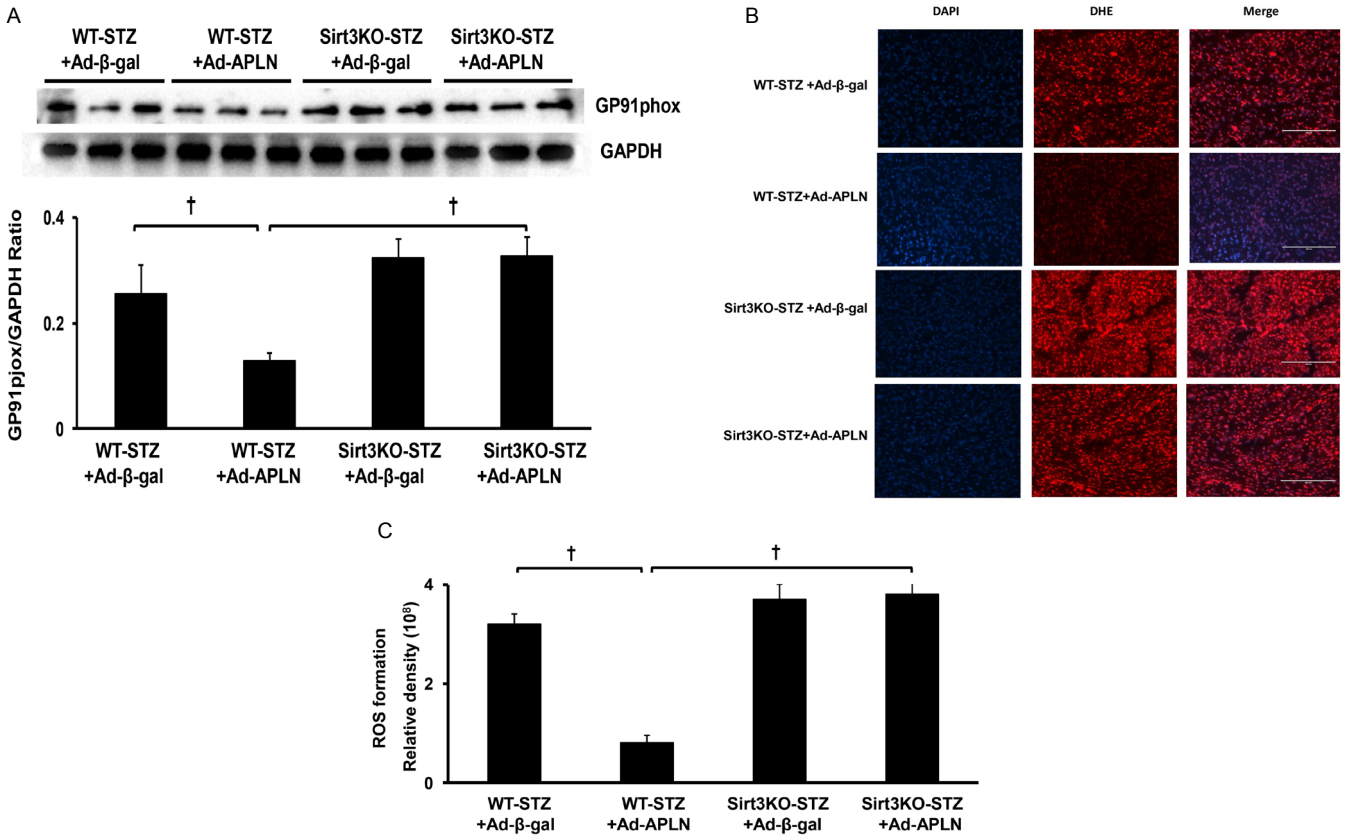

Figure 2: Apelin gene therapy on myocardial ROS formation. (A) apelin gene therapy significantly reduced gp91phox expression compared to WT-STZ mice treated with Ad-β-gal. Knockout of Sirt3 blunted apelin gene therapy-mediated gp91phox expression in Sirt3 KO-STZ mice (n=3 mice, †P<0.001). (B and C) DHE staining of ROS formation shows that Ad-apelin treatment dramatically inhibited ROS formation in the heart of WT-STZ mice (n=5, †P<0.001). Ad-apelin treatment had little effect on the ROS formation level in Sirt3 KO-STZ mice. WT-STZ mice treated with Ad-apelin had a lower ROS formation level than Sirt3KO-STZ, but did not reach significant difference. The ROS formation levels were similar between mice received Ad-apelin and Ad-β-gal treatment in Sirt3KO-STZ mice.

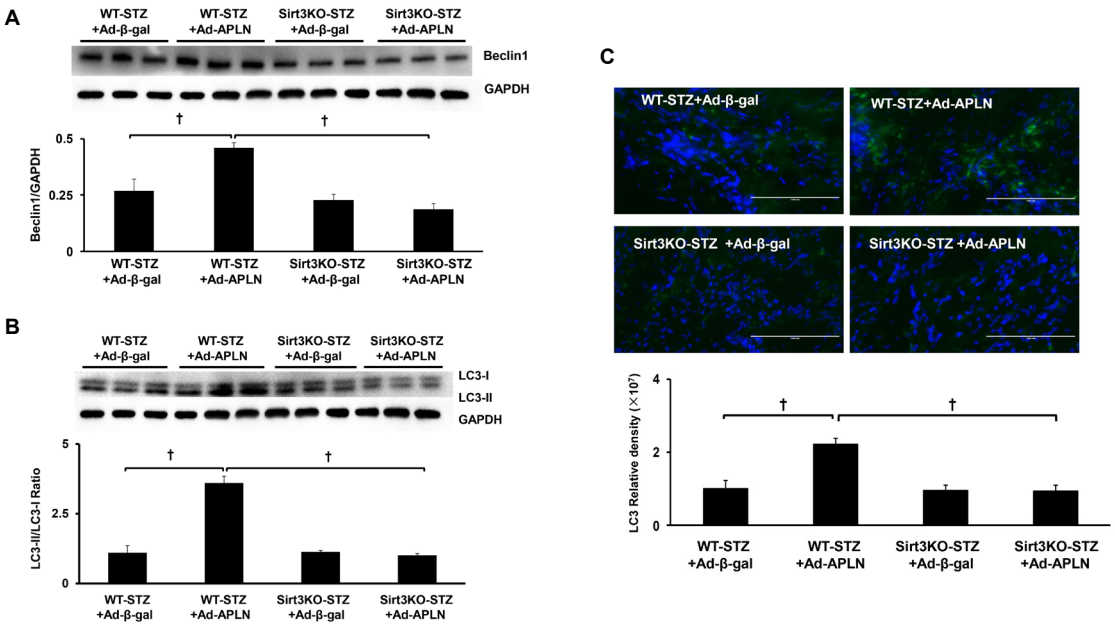

Figure 3: Apelin gene therapy on myocardial autophagy. (A) Apelin overexpression significantly upregulated beclin-1 expression compared to WT-STZ mice treated with Ad-β-gal. Knockout of Sirt3 blunted apelin gene therapy-mediated beclin-1 expression in Sirt3 KO-STZ mice (n=3 mice, †P<0.001). (B) Apelin gene therapy significantly enhanced LC3-II expression compared to WT-STZ mice treated with Ad-β-gal. Knockout of Sirt3 blunted apelin-mediated LC3-II expression in Sirt3 KO-STZ mice (n=3 mice, †P<0.001). (C) Immuno-staining of LC3-II levels in the hearts. Measurement of fluorescent density in the heart section showed that Ad-apelin treatment dramatically increased the LC3-II levels compared to Ad-β-gal injection In WT-STZ+MI mice. In contrast, the Ad-apelin and Ad-β-gal treatment did not change the LC3-II levels in Sirt3 KO-STZ+MI mice.

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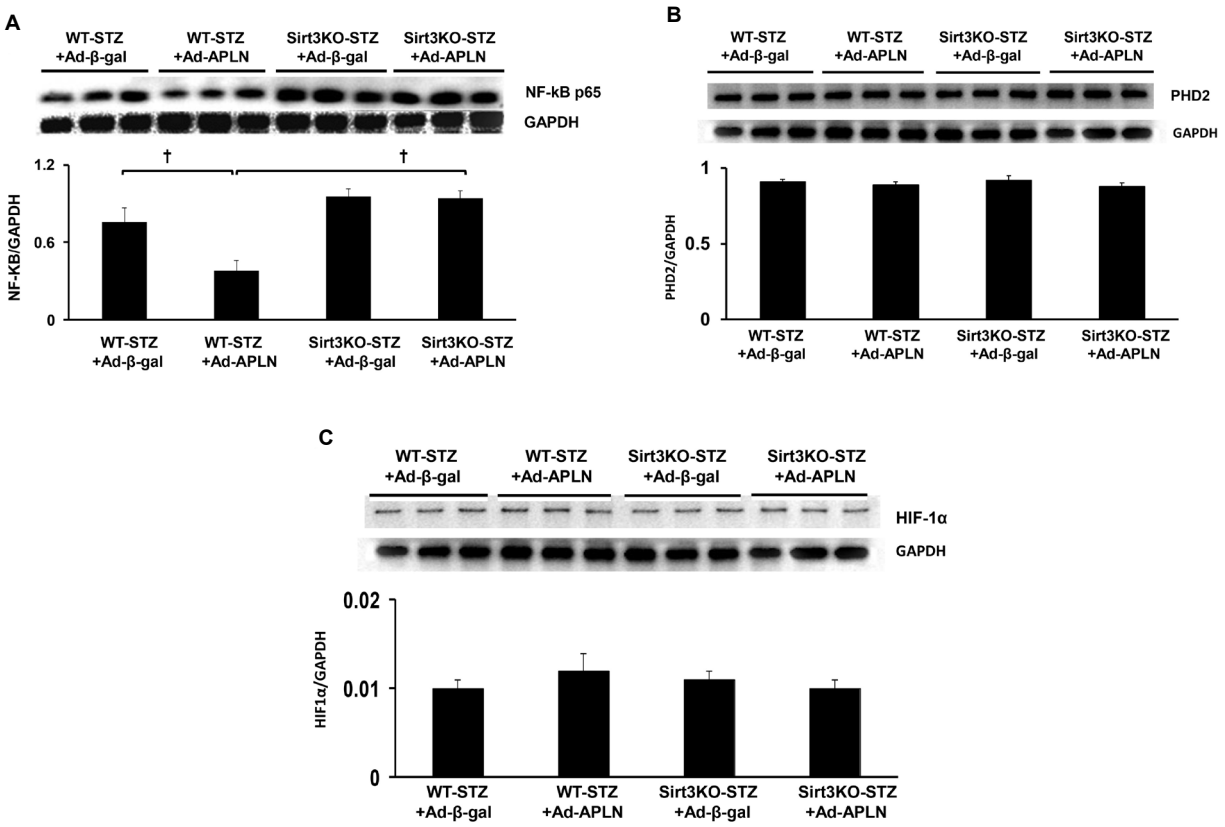

Figure 4: Apelin gene therapy on NF-kb and HIF-1α expression. (A) Ad-apelin treatment significantly reduced NFkb expression in WT-STZ post-MI mice in comparison with Ad-β-gal injection. Apelin gene therapy had little effects on the NFkb expression in Sirt3 KO-STZ mice. (B and C) Ad-apelin treatment did not alter HIF-1α and PHD2 levels in WT-STZ and Sirt3 KO-STZ post-MI mice in comparison with Ad-β-gal injection.

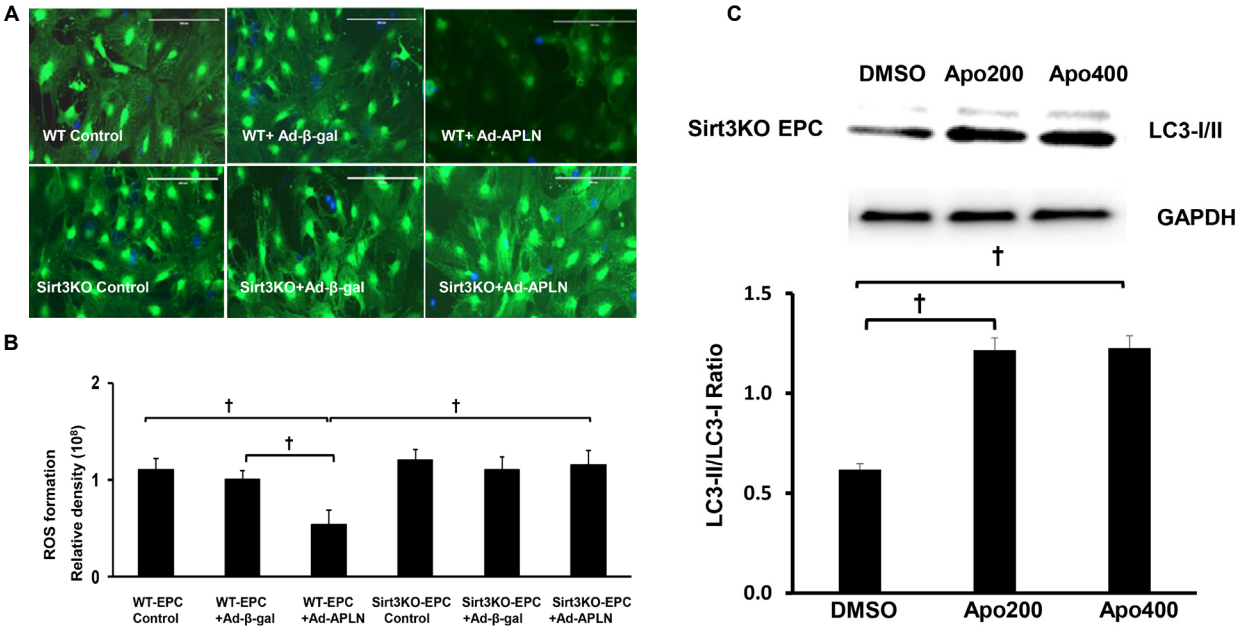

Figure 5: Knockout of Sirt3 abolishes apelin induced suppression of ROS formation in EPC. (A) Representative images of overexpression of apelin on ROS formation in EPC under High Glucose (HG) conditions *in vitro*. (B) Overexpression of Ad-apelin in EPC attenuated HG-induced ROS formation (n=5, †P<0.001). The inhibitory effect of apelin on HG-induced ROS formation was abolished in Sirt3 KO-EPC. (C) Treatment of Sirt3 KO-EPC with apo (200 and 400 μM) enhanced LC-3 II expression.

DISCUSSION

Our previous studies demonstrated that SIR3 plays a central role in the apelin-mediated cardiac protection. We have shown that: (a) Sirt3 is essential for apelin-induced angiogenesis in response to ischemia in diabetes;[9] (b) loss of Sirt3 limits apelin-overexpression bone marrow cell-mediated angiogenesis and cardiac repair;[12] and (c) upregulation of Sirt3 by overexpression of apelin ameliorates diabetic cardiomyopathy in diabetic db/db mice.[14] In this study, we further demonstrated that apelin overexpression increases autophagy and reduces NADPH oxidase derived ROS formation in the ischemic hearts of diabetes. We also revealed that Sirt3 has a critical role in apelin-induced autophagy and suppression of ROS formation. Our present findings elucidate a novel mechanism by which apelin protects the ischemic heart of diabetes.

Autophagy is a highly conserved cellular process that maintains cell survival by releasing energy substrates *via* the lysosome-dependent pathway and by removing defective organelles.[15] Accumulating evidence also indicate that autophagy plays a key role for the maintenance of cardiomyocytes structure and function under ischemia and pressure-overload.[16-19] Enhancing autophagy protects against ischemia/reperfusion injury in cardiac myocytes.[20] Augmenting autophagy is emerging as a therapeutic strategy for acute myocardial ischemia.[21] In the present study, we showed that overexpression of apelin increased the expressions of two important autophagy markers, beclin-1 and LC-3II, in the ischemic heart. Intriguingly, knockout of Sirt3 abrogated this effect, suggesting that induction of autophagy through activation of Sirt3 may contribute to the protective action of apelin against ischemic injury.

NADPH oxidase subunit gp91^{phox}, known also as Nox2, is an enzyme solely dedicated to the generation of ROS.[22] Gp91^{phox} expression is upregulated in the ischemic heart and salvages cardiomyocytes from ROS-induced injury.[23] Knockdown of Nox2-NADPH oxidase using siRNA improves cardiac function following myocardial infarction.[24] Loss of NOX2 (gp91^{phox}) further reduces oxidative stress and prevents progression to advanced heart failure.[25] In this study, we showed that the apelin overexpression significantly inhibited gp91^{phox} expression in heart of STZ mice. This was accompanied by reduced ROS formation and increased autophagy gene expression. In contrast, knockout of Sirt3 in STZ mice completely abolished these beneficial effects of apelin gene therapy. As one of the major mitochondrial NAD+-dependent deacetylase, Sirt3 is involved in mitochondrial ROS formation.[26,27] Sirt3 regulates ROS production by directly binding and deacetylating mitochondrial complex I and II.[28] Sirt3 has been shown to attenuate hydrogen peroxide-induced oxidative stress through the preservation of mitochondrial function.[29] NADPH oxidase has been shown involved in autophagy activation in cardiomyocyte.[30] Previously, we have shown that loss of Sirt3 enhanced ROS formation and apoptosis in EPCs.[31] Consistent with these findings, we showed that knockout of SIRT3 abolished apelin-mediated suppression of ROS formation in heart tissue and cultured EPCs. Moreover, pharmacological inhibition of NADPH oxidase in Sirt3 KO-EPCs increased LC-3II expression. These data suggest a possible interaction among Sirt3, gp91^{phox} and ROS in the regulation of autophagy.

NF-κb and HIF-1α are two important transcriptional factors involved in the regulation of autophagy gene expression.[32,33] ROS-mediated NF-κb activation has been shown to lead to the autophagic degradation in fibroblasts.[34] Our data showed that apelin gene therapy significantly suppressed NF-κb expression, but has little effect on HIF-1α and its regulator PHD2 levels. Furthermore, knockout of Sirt3 abrogated apelin-induced suppression of NF-κb activation and failed to affect autophagy gene expression in ischemic heart of STZ mice. These data implicate that apelin may upregulate autophagy in infarcted heart of diabetic mice *via* suppression of NF-κb, rather that HIF-1α. We therefore hypothesized that deficiency (loss) of Sirt3 in diabetic heart may cause gp91^{phox} activation and increase ROS formation, which subsequently inhibits autophagy expression *via* activation of NF-κb. However, further study is warranted to clarify this signaling pathway.

In summary, the current study provides direct evidence that overexpression of apelin reduces ROS formation and enhances autophagy *via* upregulation of Sirt3. Our data suggest that modification of Sirt3 with apelin could be used as a novel therapy strategy for the treatment of diabetes-associated heart failure.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Combination Treatment with a Novel Polyherbal Formulation and Metformin: A Single Blind Placebo-Controlled Study in Patients with T2DM and Cognitive Impairments

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ABSTRACT

Background: Older people suffering from Type 2 Diabetes Mellitus (T2DM) are at major risk for age related cognitive dysfunction and dementia, mainly due to vascular complications. Studies have shown that T2DM is also associated with Alzheimer's Disease (AD) and is responsible for accelerating the pathology through insulin resistance. A polyherbal drug containing *Bacopa Monnieri, Hippophae rhamnoides* and *Dioscorea bulbifera* has shown a potent neuroprotective effect in management of cognitive deficits in elderly; and metformin a well-accepted antidiabetic agent responsible for lowering blood glucose in T2DM, can together provide an intriguing potential combination therapy for prevention and amelioration of cognitive impairments in T2DM patients.

Objective: The present study is aimed to evaluate the combined effect of a polyherbal drug and metformin on improving cognitive functions in patients suffering from T2DM.

Method: Elderly patients with an age range of 60-75 years diagnosed for T2DM were enrolled in the study and randomized into two groups; Group I=T2DM patients given metformin and placebo, Group II=T2DM patients given metformin and polyherbal drug. The subjects received the combination therapy of metformin (500 mg) and placebo or metformin (500 mg) and polyherbal drug (500 mg) twice daily for a period of 24 weeks. Estimation of Mini Mental State Examination (MMSE) score, blood glucose, HbA1c, insulin, lipid profile (total cholesterol, LDL-c, HDL-c, triglycerides), homocysteine, Interleukin-6 (IL-6), TNF-α and C-reactive protein (CRP) were measured at baseline and were repeated at three months and six months. The primary end point was a change from baseline to week 24 in MMSE score. Key secondary end points included change from baseline to week 24 in Digital Symbol Substitution (DSS); subtest of the Wechsler Adult Intelligence Scale-Revised), word recall (digital memory apparatus – Medicaid systems, Chandigarh, India), attention span (Attention Span Apparatus – Medicaid systems, Chandigarh, India), Functional Activity Questionnaire (FAQ) and Hamilton Depression Scale (HDS) score. Further inflammatory markers and level of oxidative stress were analysed using standard biochemical tests.

Result: The trial was performed in 120 elderly diabetic patients out of whom 112 patients

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completed the study for 24 weeks. Statistically significant differences were found between the two groups after intervention on cognitive performance indicated by MMSE, DSS, word recall, attention span, FAQ, HDS, and memory span scores; inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6), Interferon-α (INF-α) and Tumor necrosis factor-α (TNF-α); oxidative stress markers like Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione (GSH) and Thiobarbituric acid reactive substances (TBARS); and neurodegeneration marker Homocysteine (Hcy). However, no significant variations were indicated between groups in serum insulin and HbA_{1c} levels after intervention of 24 weeks.

Conclusion: The results of this study demonstrate the therapeutic potential of the combination therapy of a polyherbal drug and metformin. These results also support longer trials of this combination therapy for patients with mild cognitive impairment and diabetes.

KEYWORDS: Neurodegeneration; Type 2 diabetes; Polyherbal formulation; Metformin; Combination treatment.

ABBREVIATIONS: T2DM: Type 2 Diabetes Mellitus; AD: Alzheimer's Disease; HDS: Hamilton Depression Scale; FAQ: Functional Activity Questionnaire; TBARS: Thiobarbituric acid reactive substances; GSH: Glutathione; GPx: Glutathione peroxidase; Hcy: Homocysteine; CRP: C-reactive protein; IL-6: Interleukin-6; SOD: Superoxide dismutase; IGF: Insulin like growth factor; BMI: Body Mass Index; MMSE: Mini Mental State Examination; DSS: Digital Symbol Substitution; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HPLC: High-performance liquid chromatography.

INTRODUCTION

In developing countries, improvement in the health care has contributed to people living healthier and longer lives. This has resulted in an increase in the world ageing population and consequently an increase in age related disorders like dementia. Dementia is debilitating disease which involves variety of conditions that develop when nerve cells (neurons) in the brain die or do not function properly. Alzheimer's disease (AD) is accounted to be the most common form of dementia followed by vascular dementia and mixed dementia. The symptoms include memory loss, changes in behaviour, ability to think clearly, eventual impairment of one's ability to carry out basic bodily functions such as walking and swallowing, and ultimately lead to death. It has been estimated that 35.6 million people were suffering from dementia in 2010 and is projected to triple by 2050. Each year 7.7 million new cases are reported which implies that one new case in four seconds is diagnosed every day.[1] This disease constitutes a great burden not only to the patients, but is also devastating for the caregivers and is an enormous social and economic burden to the Society. Pharmaceutical interventions so far have been directed towards amelioration of the symptoms, no drug action stops the progression of the disease.

On the other hand, diabetes is common metabolic disease that is manifested in the form of hyperglycemia and glucose intolerance due to relative insulin deficiency, impaired effectiveness of insulin action, or both.[2] Worldwide estimations in 2011 indicate 366 million patients suffering from diabetes and these numbers are proposed to rise to 552 million by 203.[3] There are two types of diabetes mellitus; Type1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM), which differ in their etiology and clinical presentation. T1DM is an autoimmune disease in which the insulin producing cells of the pancreas are destroyed, and results in chronic hyperglycemia due to insulin deficiency. In contrast, T2DM is a result of insulin resistance and relative insulin deficiency that is caused due to inadequate response by target tissues like skeletal muscles, adipose tissue and liver, to circulating insulin and is often accompanied by raised insulin levels.

Many recent studies have implicated T2DM as a risk factor for cognitive dysfunction and dementia in the elderly. A positive correlation has been observed between the number of elderly individuals with T2DM and the number of people with diabetes and cognitive dysfunction. Rotterdam study is one of the earliest large scale epidemiological finding which showed that T2DM patients had an increased risk for developing dementia.[4] Another study in 2004 demonstrated a 65% increase risk of developing AD in T2DM patients.[5] Furthermore, a recent meta-analysis of population-based longitudinal studies in 2012 confirmed the relative risk of AD in subjects with T2DM was 1.46 times higher as compared with the subjects without T2DM.[6] Studies have revealed that AD is a metabolic disease, and its symptoms are associated with impairments in brain's responsiveness to insulin, glucose utilization and energy metabolism, which lead to increased inflammation, oxidative stress and more insulin resistance. These metabolic derangements contribute to the structural, functional, biochemical and molecular abnormalities that result in neuronal loss, synaptic disconnection, beta amyloid accumulation and tau hyperphosphorylation that are characteristic of AD pathology.[7] The concept of AD to be a metabolic disease stemmed from the observations that deficits in cerebral glucose utilization, were present either prior to or coincident with initial stages of cognitive dysfunction.[8-11] Even post-mortem studies of AD patient brains show molecular and biochemical evidence of insulin and Insulin like growth factor (IGF) resistance and impairments in signal transduction.[12,13] Either, the gene expression of insulin or IGF polypeptides in brain and cerebrospinal fluid is altered or there is reduced level of insulin binding to its receptor and decreased responsiveness to insulin stimulation.[12-14] Hence, AD related neurodegeneration has been hypothesized to be "Type 3 diabetes" or "brain insulin resistant state".

Current pharmacological interventions have made remarkable advances to treat and prevent classic microvascular and macrovascular complications in patients with T2DM,[15] however cognitive dysfunctions are not targeted. Elderly T2DM patients

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with cognitive impairment and dementia face major hindrance in self-care behaviour that is essential for the management of diabetes. Therefore, diabetes along with cognitive dysfunction in the elderly creates a large burden for the patients as well as their families and society. There is an urgent need to take brain protection into consideration while developing and implementing future treatments of diabetes for the elderly population. The underlying mechanisms of the association of T2DM with cognitive impairments need to be investigated and alternative disease modifying approaches are necessary for development of a treatment or method of prevention. Moreover, it may be beneficial to control the blood glucose levels to optimum and establish a combination therapy for enhancing cognitive preservation.

Traditional medicinal system has utilized the potential of medicinal plants since antiquity for the treatment of memory dysfunction. In the present study, the efficacy of a combination therapy of metformin and a US patented novel polyherbal formulation, containing *Bacopa monnieri*, *Hippophae rhamnoides*, and *Dioscorea bulbifera* in management of cognitive dysfunction in T2DM patients was investigated. The polyherbal formulation has demonstrated a potential role in treatment and prevention of neurodegenerative disorders in the elderly by significantly improving cognitive and neuropsychiatric measures, reducing oxidative stress and neuroinflammation.[16] Whereas, metformin is the first line pharmacologic treatment for T2DM, as it decreases hepatic glucose production and improves insulin sensitivity by augmenting glucose uptake in the peripheral tissues, mainly muscles.[17,18] Metformin's efficacy, security profile, beneficial cardiovascular and metabolic effects and its ability to be associated with other antidiabetic or neuroprotective agents provided the basis to use the combination therapy in the present study. With this background, this study was undertaken to explore the efficacy of the polyherbal drug in combination with metformin to ameliorate cognitive impairments in T2DM patients.

METHODS

Study Design, Participants and Treatment

The study was conducted as a randomized, single blinded, placebo controlled observational study. Elderly persons with diabetes >60 years of age were enrolled in the study for a period of 24 weeks.

Inclusion criteria were: Onset of T2DM for at least 10 years, fasting blood glucose levels <180 mg/dl, HbA1c level of 7 to 10%, Body Mass Index (BMI) <40 kg/m^2, no autoimmune disorder, no cardiac ischemic or renal disease, no chronic inflammatory disease or infection, no regular consumption of other herbal drugs, no consumption of any vitamin supplements <2 months before starting screening. Additional inclusion criteria were deterioration of memory along with at least three of the following five complaints: poor orientation, poor judgment and problem solving difficulties, trouble in the functioning of community affairs, inability to function independently in home and during hobbies and difficulties in personal care.

Exclusion criteria were: Non adherence to the study protocol (no consumption of more than 20% of the capsules), any sensitivity or unwanted effect to the test drug after the onset of the study, any variation in patients routine treatment i.e., variation in type and dose of the drugs to be consumed, and treatment with insulin, no consumption of any nutritional/ herbal supplements, consumption of alcohol or narcotic drugs.

Around 235 elderly were screened out of which 115 were excluded for not meeting the exclusion criteria and other reasons. Only 120 patients were randomized into two groups of 60 participants each; Group I: elderly subjects with diabetes who were given metformin and placebo, Group II: elderly patients with diabetes who were given metformin and test drug (polyherbal formulation).[16] A random list of numbers was determined by a computer-generated series with the proper sequence applied to container labels and supplied to participants in the order enrolled after being randomly assigned to the various treatment groups. This computer generated randomization scheme was developed and kept by the study sponsor. Metformin and test drug were given at a dose of 500 mg twice daily just after meals. The placebo tablets were prepared using dibasic calcium phosphate and microcrystalline cellulose and were identical in appearance to the test drug.[16] Follow up of the patients was performed by monitoring them weekly by phone to control them from non-consumption of capsules, prevent sample loss and record response to relevant questions. Compliance was determined by calculating the rate of tablet consumption before they received medication for next two weeks. The participants were also asked to contact the study centre if they experienced any medical problems during the study period and were advised to not make any changes in their usual diet, and make any self-reliant changes in the medication routine.

Measurements

Demographic characteristics including age, gender, height, weight, marital status, education, occupation, duration of onset of the disease, type and dose of medication used to control diabetes, cognitive impairment symptoms were assessed and recorded by interviewing the patients. Cognitive parameters and biochemical tests were recorded at baseline and at 12th and 24th week of intervention. Body Mass Index (BMI) was calculated by weight (kg) divided by height squared (m). Cognitive function was assessed following structured performance tests, which included mental status, verbal memory, complex psychomotor skills, and attention/executive functions. Mini mental state examination (MMSE) was used to assess mental status.[19] Memory scores were tested using the digital memory apparatus (Medicaid systems, Chandigarh, India) device for both immediate and delayed memory performance. Complex psychomotor

skill was examined using the Digital Symbol Substitution (DSS) test, which is a sub-test of the Wechsler Adult Intelligence Scale-Revised,[20] and has a score range of 0-93. Attention span scores were obtained using the electronic device-Attention Span Apparatus (Medicaid systems, Chandigarh, India). A well-trained psychologist/technical person administered all four tests in the same order to all the study patients. Depression was assessed by the Geriatric Depression Scale-15 (GDS-15),[21] which is a global test for depression with scores ranging from 0 to 15. Functional activity questionnaire (FAQ) scores were also obtained to test cognitive function of the participants.[22,23] After 12 h of fasting venous blood sample was taken from each patient by a laboratory technician at the beginning, follow-ups and end of intervention. The blood samples were collected in separate vacutainer tubes and were centrifuged at room temperature, at 3500 g for 10 min; the serum and plasma were stored at -80 °C until they were analysed. Blood glucose, HbA1c, serum insulin, and lipid profile which included total cholesterol, triglycerides, Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL) were analysed using standard tests using blood serum. The blood plasma level of homocysteine was determined by a High-performance liquid chromatography (HPLC) method. Levels of CRP, IL-6 and TNF-α were detected in the blood plasma by the ELISA method using test kits. SOD and GPx activities as well as GSH and TBARS levels were measured as markers for oxidative stress. SOD activity was estimated by the method of Misra and Fridovich, GPx activity was estimated by the method of Rotruck, et al. GSH levels as well as TBARS levels were detected in the blood by colorimetric assays.[24-29] Any adverse reactions were assessed by routinely monitoring liver function test (serum glutamic-oxaloacetic transaminase, glutamic pyruvic transaminase, bilirubin and alkaline phosphatase), total protein and serum albumin, urine test (urinary vanillomandelic acid, 17 ketosteroides and glucocorticoid) and kidney function tests (blood urea, serum creatinine, and uric acid) using standard laboratory procedures.

Ethical Considerations

The study aims and methods were explained to the patients and the informed written consent was received from them if they were interested in participation. The study protocol was approved by the institution's ethical committee at Institute of medical science, Banaras Hindu University and SRM University. The clinical trial titled ''Prevention and management of age related neurodegenerative disorders-an Ayurvedic intervention'', was registered (No. K.11022/10/2009-DCC) with Dept. of AYUSH (Ministry of Health and Family Welfare, Govt. of India). The study was approved by Institutional ethical committee, and was undertaken as an additional pilot trial under the CTRI/2014/12/005312 trial, to specifically study subjects with diabetes and cognitive impairment.

Data Analysis

All data are expressed as mean±SD. The unpaired student t test was performed to compare the results obtained from the different groups. All statistical analysis was done using the Graphpad prism ver. 2.0. Per-protocol analysis was performed and statistical significance was regarded at $P<0.05$.

RESULT

Demographic Data of Subjects

A total of 120 elderly subjects with diabetes participated in the study, out of which the 112 participants completed the study. The following cases were excluded from the intervention: 4 patients had no tendency to continue, 3 patients were irregular for weekly follow-ups due to travel, 1 patient could not be contacted; the remaining 112 participants continued till the end of the trial were all investigated (Figure 1). The compliance of consuming capsules in both the groups turned out to be more than 90%, thus demonstrating a well adherence to the study protocol by the patients. All the patients who received the combination therapy, 66(58.9 %) were male and 46(38.3%) were female. The mean age of the patients in group I and II were 65.92±6.87 and 67.48±8.35 respectively. The baseline characteristic of the patients before the study is presented in Table 1. There were no statistically significant differences between the variable in the two groups (Table 1). The adverse events reported in the study were mild in severity and included nausea, drowsiness and constipation. The study parameters after 24 weeks of treatment, including neuropsychological parameters, memory span, biochemical, inflammatory and oxidative stress parameters were compared between group I and II and are shown in Tables 2, 3, 4 and 5 respectively.

Effect on Neuropsychological Parameters

To assess the therapeutic potential of metformin in combination with test drug for neuroprotective effects in patients with diabetes, several neuropsychological parameters such as memory, mental status, complex psychomotor skills, and attention/executive functions were analysed and are shown in Table 2. After 24 weeks of intervention the neuropsychological parameters were compared in elderly patients with diabetes who received a combination therapy of metformin and placebo or metformin and test drug. Significant improvement in the MMSE score ($P<0.0001$), DSS score ($P<0.0001$), word recall ($P<0.0001$), attention span ($P<0.0001$), FAQ ($P=0.0320$), and HDS score ($P<0.0001$) was observed. Similar statistical analysis on the short term memory ($P<0.0001$), and long term memory scores ($P<0.0001$) confirmed significant improvements in group II that received a combination therapy of the test drug with metformin.

Effect on Biochemical and Inflammatory Markers

The levels of inflammatory markers and other biochemical parameters have been reported in Table 3. No signifi-

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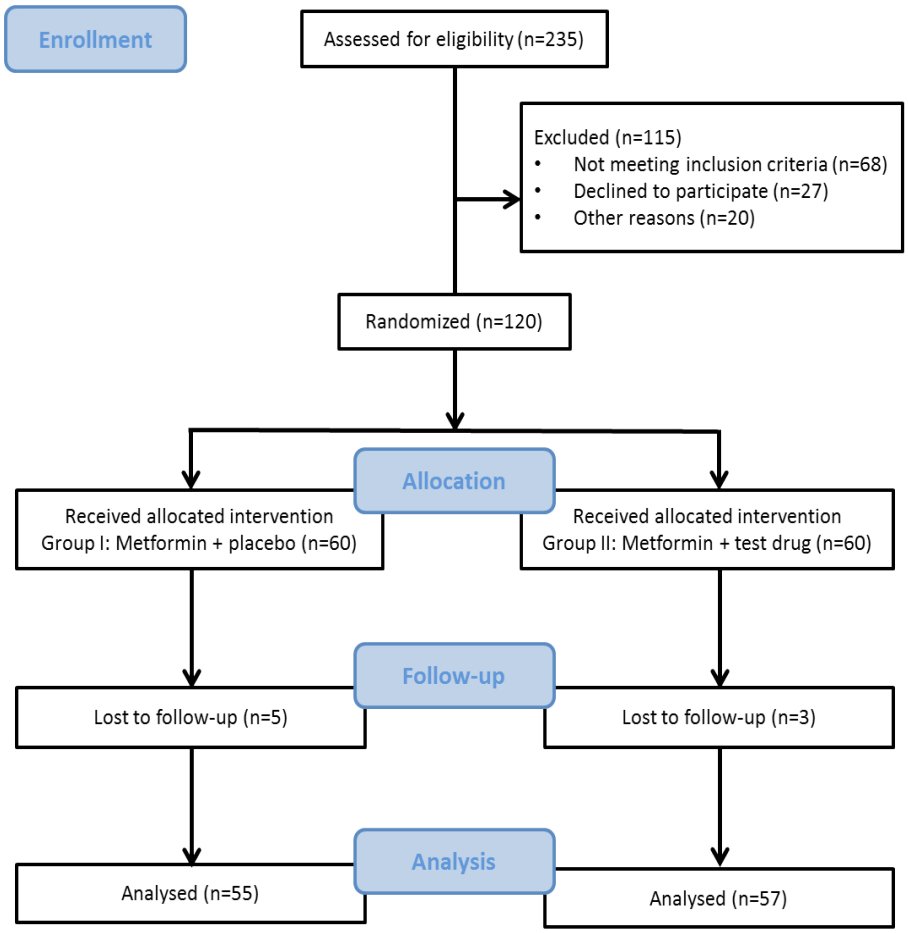

Figure 1: Flow chart of the study.

Variables	Group I (N=55) Mean±SD	Group II (N=57) Mean±SD	*P*- value
Male (N(%))	31(56.36)	35(61.4)	
Female (N(%))	24(43.63)	22(38.59)	
Age (years)	65.92±6.87	67.48±8.35	0.28
Body mass Index (kg/m^2)	25.04±6.85	26.39±5.27	0.24
Blood glucose (mg/dL)	156.17±43.58	164.27±54.91	0.39
HbA1c (%)	10.09±2.31	9.63±3.24	0.39
Serum Insulin (mU/ml)	8.39±3.48	7.85±2.72	0.36
Serum Cholesterol (mg/dL)	219.58 ± 41.56	228.41±33.75	0.22
Serum Triglycerides (mg/dL)	225.88±62.04	234.71±48.90	0.40
Serum LDL (mg/dL)	122.42±11.73	118.42±14.82	0.12
Serum HDL (mg/dL)	46.45±3.72	48.73±5.01	0.16
Homocysteine (Hcy) (nmol/L)	21.852±5.921	22.52±3.91	0.48
C-reactive Protein (CRP) (mg/dL)	4.853±1.574	5.063±1.74	0.50
Interleukin-6 (IL-6) (pg/dL)	1.392±0.041	1.401±0.026	0.17
TNF-α (pg/ml)	987.26±91.23	1010.53±105.21	0.21
MMSE score	15.24±2.90	15.93±2.16	0.56

Table 1: Comparison of the qualitative and quantitative variables between the two groups before the intervention.

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Parameter	Group I (N=55)			Group II (N=57)			*P* value (comparison between Group I and II after 24 weeks)
	Initial	After 12 weeks	After 24 weeks	Initial	After 12 weeks	After 24 weeks	
MMSE score	15.24±2.90	15.12±3.31	14.89±2.01	15.93±2.16	16.28±3.20	17.95±3.11	<0.0001
DSS score	41.98±12.49	40.80±11.97	41.72±12.05	39.72±10.91	43.85±3.85	47.90±13.06	<0.0001
Word recall score	4.87±1.22	5.06±1.30	4.69±2.21	5.02±1.83	6.42±1.69	6.85±2.04	<0.0001
Attention Span score	6.68±2.13	6.35±1.87	6.49±1.32	6.98±1.63	7.55±2.04	8.09±2.12	<0.0001
FAQ score	19.842±4.316	18.975±3.922	18.895±3.116	20.101±5.824	18.702±6.102	17.186±4.993	0.0320
HDS score	14.867±3.085	13.352±4.113	12.886±3.591	15.854±4.753	16.104±4.902	17.372±5.112	<0.0001

Values are expressed as mean±SD
Group I (N=55) elderly diabetics treated with metformin and placebo, group II (N=57) elderly diabetics treated with metformin and test drug, Statistical analysis have been done to compare scores after 24 weeks treatment between group I *vs.* II
MMSE: Mini Mental State Examination; DSS: Digital Symbol Substitution; FAQ: Functional Activity Questionnaire; HDS: Hamilton Depression Scale

Table 2: Effect of combination therapy on neuropsychological parameter scores.

Memory span (Score)							*P* value (comparison between Group I and II after 24 weeks)
	Group I (N=55)			Group II (N=57)			
	Initial	After 12 weeks	After 24 weeks	Initial	After 12 weeks	After 24 weeks	
STM score	7.84±1.63	7.39±1.82	7.1±1.58	6.99±1.81	7.83±1.20	8.25±2.04	<0.0001
LTM score	5.98±1.02	5.82±0.97	5.66±1.22	5.61±1.13	5.89±1.35	6.75±1.38	<0.0001

Group I (N=55) elderly diabetics treated with metformin and placebo, group II (N=57) elderly diabetics treated with metformin and test drug, Statistical analysis have been done to compare scores after 24 weeks treatment between group I *vs.* II.
STM: Short Term Memory; LTM: Long Term Memory

Table 3: Effect of combination therapy on memory span.

Parameter	Group I (N=55)			Group II (N=57)			*P* value (comparison between Group I and II after 24 weeks)
	Initial	After 12 weeks	After 24 weeks	Initial	After 12 weeks	After 24 weeks	
Hcy (nmol/L)	21.852±5.921	20.956±6.321	23.721 ±5.456	22.52±3.91	20.832±5.391	18.193±3.885	<0.0001
CRP (mg/dL)	4.953±1.574	4.392±2.014	5.012±2.101	5.063±1.74	4.223±1.735	3.851±1.080	0.0003
IL-6 (pg/dl)	1.392±0.041	1.52±0.058	1.428±0.085	1.401±0.026	1.274±0.093	1.12±0.092	<0.0001
TNF-α (pg/ml)	987.26±91.23	1012.41±89.23	992.06±92.2	1010.53±105.21	889.32±86.35	760.25±93.26	<0.0001
Serum Insulin (mU/ml)	8.39±3.48	6.94±2.85	7.08±3.10	7.85±2.72	6.34 ±2.11	6.12±1.98	0.0525
HbA1c (%)	10.09±2.31	10.35±1.94	9.01±1.58	9.63 ±3.24	9.54 ±1.75	8.89±1.18	0.6489

Values are expressed as mean±SD
Group I (N=55) elderly diabetics treated with metformin and placebo, group II (N=57) elderly diabetics treated with metformin and test drug, Statistical analysis have been done to compare scores after 24 weeks treatment between group I *vs.* II.
Hcy: Homocysteine; CRP: C-reactive Protein; IL-6: Interleukin 6; TNF- α: Tumor necrosis factor alpha

Table 4: Effect of combination therapy on inflammatory markers and biochemical parameters.

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Parameter	Group I (N=55)			Group II (N=57)			*P* value (comparison between Group I and II after 24 weeks)
	Initial	After 12 weeks	After 24 weeks	Initial	After 12 weeks	After 24 weeks	
SOD (U/g Hb)	1592.11±106.21	1422.31±115.32	1342.41±102.98	1612.23±196.52	1423.32±211.32	1226.32±150.26	<0.0001
GPx (U/g Hb)	69.31±10.21	68.21±11.25	71.58±14.65	67.24±9.65	61.38±12.985	58.96±9.35	<0.0001
GSH (U/g Hb)	1.721±0.115	1.498±0.098	1.352±0.142	1.689±0.102	2.208±0.206	2.65±1.25	<0.0001
TBARS (nmol/g Hb)	152.68±40. 17	163.68 ±64.05	173.45±56.81	158.78±65.421	139.81±34.72	128.17±29.68	<0.0001

Values are expressed as mean±SD
Group I (N=55) elderly diabetics treated with metformin and placebo, group II (N=57) elderly diabetics treated with metformin and test drug, Statistical analysis have been done to compare scores after 24 weeks treatment between group I *vs.* II.
SOD: Superoxide dismutase; GPx: Glutathione peroxidase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances

Table 5: Effect of combination therapy on oxidative stress markers.

cant statistical differences were observed at the beginning of the intervention. However, there was gradual increase in the Hcy levels in group I, suggesting a progressive neurodegeneration due to glucose intolerance. Comparison of Hcy levels between groups I and II at the end of 24 weeks showed significant decrease in Hcy levels (<0.0001). Similar trend of decrease in CRP level (P=0.0003) was observed in the group that received the test formulation in addition to metformin. Additionally, the levels of inflammatory markers IL-6(P<0.000) and TNF-α (P<0.0001) were significantly lowered after 24 weeks of intervention. According to the results, no statistical differences were observed at the beginning and end of intervention in terms of serum insulin (P=0.0525) and HbA1c (P=0.6489) levels.

Effect on Oxidative Stress

The effect of the test drug was also assessed on antioxidants like SOD, GPx, GSH and TBARS. A slight increase in oxidative stress markers, SOD, GPx and TBARS was observed in group I that received metformin and placebo. Whereas, the levels of SOD (P<0.0001), GPx (P<0.0001), GSH (P<0.0001), and TBARS (P<0.0001) showed significant decrease at the end of the intervention between group I and II. This demonstrates the ability of test drug to lower oxidative stress in elderly patients with diabetes.

DISCUSSION

The present study indicated that combination treatment of (polyherbal formulation) in addition to metformin in elderly patients with diabetes for 24 weeks causes improvement in cognitive functions. One of its definitive outcomes is a significant change in MMSE scores as well as in DSS, attention span, word recall, FAQ and HDS scores, thereby showing the effect of the polyherbal formulation in controlling neurodegeneration in T2DM patients. On the other hand, a significant decrease was found in oxidative stress and inflammatory markers in the group that received metformin and test drug compared to the group that received metformin and placebo. Therefore, considering the results of these parameters especially MMSE, inflammation and oxidative stress, it may be concluded that the polyherbal formulation is effective in preventing progressive neurodegeneration associated with diabetes.

Aging is a major risk factor for AD and evidences from recent studies suggest that brain insulin resistance is one of the major factor that contribute to mild cognitive impairment or dementia and AD.[12,30-34] The molecular and biochemical consequences of insulin resistance in the brain are caused due to impairments in the insulin signalling pathway that compromises neuronal survival, energy production, gene expression, plasticity and white matter integrity.[33,35,36] The brain undergoes a starvation state due to deficit in glucose uptake and utilization, thus causing oxidative stress, impairments in homeostasis and increased cell death. Impairments in insulin signalling results in neurodegeneration due to increased activity of kinases that aberrantly phosphorylates tau, generation of reactive oxygen species that damages proteins, nucleic acids and lipids, leads to accumulation of amyloid beta monomers and plaques, causes mitochondrial dysfunction and increase signalling through pro-inflammatory and pro-apoptosis cascades.[35-38] Also insulin resistance is associated with down-regulation of genes needed for cholinergic function, thereby further compromising neuronal plasticity, memory and cognition.[36,38]

The basis of the neuroprotective effects of the test formulation is corroborated by several studies in animals and humans. The polyherbal formulation is a combination of *Bacopa monnieri*, *Hippophae rhamnoides* and *Dioscorea bulbifera*. Several studies have demonstrated the nootropic effect of *Bacopa monnieri* is imparted via the action of triterpeniod saponin called bacosides that show acetylcholinesterase inhibition, acetyltransferase activation, b-amyloid reduction, and increased cerebral blood flow.[46] *Hippophae rhamnoides* on the other hand contains high concentration of flavonoids, fat soluble vitamins, folic acid, various fatty acids, phytosterols, essential amino acids and quercetin as the active phytomolecule. The fruit pulp extract *Hippophae rhamnoides* have a potent antioxidant property that imparts

a neuroprotective role by preventing oxidative damages. In addition, high concentration of folic acid in the fruits helps in regulating homocysteine metabolism by lowering the elevated levels associated with neurodegeneration.[39,40] Moreover, *Dioscorea bulbifera* extracts contain diosgenin and studies have shown its potent anti-inflammatory, anti- hyperglycemic and anti-obesity properties.[41-44] Its role in preventing neurodegeneration can be postulated to be *via* management of neuroinflammation caused due to hyperglycaemia, hyperlipoproteinaemia and obesity. After assessing the pharmacological activities of the plant extracts it was concluded that whole plant of *Bacopa monnieri*, fruit pulp of *Hippophae rhamnoides* and rhizome of *Dioscorea bulbifera* possessed AChE inhibitory activity, anti neuroinflammatory and antioxidant properties.[45] Several preclinical analysis and clinical trials have proven the efficacy of these plants in management of cognitive deficits in aged population.[16,43,46-50]

Subsequently, metformin in addition to its antidiabtic properties has been also recently recognized as a potential treatment for neurodegenerative disorders such as AD. Studies have shown that metformin prevents apoptotic cascade in endothelial cell type by inhibiting Permeability Transition Pore (PTP) and blocking the release of cytochrome-c that will lead to cell death.[51-53] Another study demonstrated that insulin in addition to metformin activates insulin signalling pathway and potentiates insulin's effects on amyloid reduction, improves neuronal insulin resistance and glucose uptake.[53] Moreover metformin has a role in promoting neurogenesis in rodent and human cultures by activating protein kinase C-CREB binding protein (PKC-CBP) pathway, thereby recruiting neural stem cells that regenerates brain by endogenously repairing the injured areas.[54]

Since the aging population is increasing at an alarming rate, hence people suffering from this T2DM associated cognitive dysfunction will become increasingly larger problem. The need of the hour is to understand the underlying mechanisms and pathophysiology of this specific condition that may lead to development of better therapeutics. It is essential to control optimal level of blood glucose and explore the best combination of medication for establishing and enhancing cognitive preservation. Also, cognitive dysfunction in T2DM may start at a relatively early stage, thus starting early management may be important to prevent not only dementia but also other complications.[55]

The present study demonstrates a high percentage of patient compliance which attests the clinical findings of improvement in cognitive functions and can be regarded as strong point of the study. The main drawback of the study is small number of patients and a short follow-up. However, few studies have been done to clinically test combination drugs to manage T2DM and cognitive dysfunction and this study proves the requirement of combination treatment in this specific condition. Therefore, it is suggested that forthcoming similar investigations should be done for longer duration with larger sample size. Moreover, studies are warranted to understand the mechanism of action of the test drug in improving cognitive functions in T2DM patients.

CONCLUSION

The observations from the present study indicate that a combination therapy of a polyherbal formulation in addition to metformin for 24 weeks has a potential to be a new therapeutic for patients suffering from cognitive impairments associated with T2DM. Results show a significant improvement in neuropsychological parameters including learning and memory functions in the group that received polyherbal formulation. The study also suggests these changes occur due to neuroprotective effects of the polyherbal formulation by reducing oxidative stress and inflammation. Further, analysis is needed to confirm routine use of this combination therapy.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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AUTHOR CONTRIBUTIONS

G.P.D is the study co-ordinator who conceived and designed the study protocol, he had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. A.S drafted the manuscript, and both A.S and P.K.S contributed to the interpretation of findings, and preparation of the final manuscript. P.U and S.S performed the literature search and statistical analysis, contributed to the data

collection with supervision from G.P.D., A.A, K.I and V.N.M, who together developed and designed the study and oversaw the data acquisition and analysis. No potential conflicts of interest relevant to this article are present.

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Research

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Association of Total and Differential White Blood Cell Counts to Development of Type 2 Diabetes in Mexican Americans in Cameron County Hispanic Cohort

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ABSTRACT

Objective: To evaluate the relationship between total and differential White Blood Cells (WBC) counts with time to transition to type 2 diabetes in Mexican Americans using prospective data from the Cameron County Hispanic Cohort (CCHC).
Results: Multivariable Cox proportional hazards regression models revealed that obese Mexican-American cohort participants whose total WBC count or granulocyte count increased over time had 1.39 times higher risk and 1.35 times higher risk respectively of transition to type 2 diabetes when compared to overweight participants. The granulocyte or total WBC count in participants with BMI>=35 were significant risk factors for transition to type 2 diabetes.
Conclusions: Increased total WBC and WBC differential counts, particularly lymphocytes and granulocytes, are associated with risk of transition to type 2 diabetes in obese Mexican Americans, after adjusting for other potential confounders. Screening and monitoring the WBC counts, including lymphocytes and granulocytes can help with monitoring potential transition to type 2 diabetes.

KEYWORDS: Type 2 diabetes; BMI; WBC; Cox proportional hazards regression; Effect modification; Statistical interaction.

ABBREVIATIONS: WBC: White Blood Cells; CCHC: Cameron County Hispanic Cohort; Il-6: Interleukin 6; IL-8: Interleukin 8; CRP: C-reactive protein; BMI: Body Mass Index; CRU: Clinical Research Unit; ADA: American type 2 diabetes association; IFG: Impaired Fasting Glucose; VIF: Variance Inflation Factors; HR: Hazard Ratio.

INTRODUCTION

The prevalences of obesity and type 2 diabetes in the United States (US) are high. More than one-third of US adults are obese, but age-adjusted obesity in Hispanics is higher at 42.5%. In 2014, 9.3% of the US population was reported to have type 2 diabetes but

the age-adjusted rate of diagnosed type 2 diabetes in Mexican Americans nationally was also higher at 13.9%.[1] CCameron County, Texas is a USA-Mexico border community with 80.5% Mexican Americans[2] where we have a community-based cohort, the Cameron county Hispanic cohort (CCHC). Data from this cohort shows that the Cameron county population is characterized as having extremely high rates of obesity, type 2 diabetes, cardiovascular disease along with metabolic complications and other diseases.[3-8] Obesity is a strong risk factor for type 2 diabetes[9] and also plays a significant role in development of low-grade inflammation.[10] It has been shown that many low-grade inflammatory markers, including elevated levels of Interleukin 6 (Il-6), Interleukin 8 (IL-8), C-reactive protein (CRP) and Tumor Necrosis Factor alpha (TNF-α), are associated with insulin resistance and type 2 diabetes.[11] Total white blood cell (WBC) count is another inflammatory marker which has been shown to be associated with obesity, impaired glucose tolerance, cardiovascular diseases and type 2 diabetes.[12,13] However, there are a few studies that demonstrate association of inflammatory markers with development of type 2 diabetes.[14-16] In particular, in some studies total WBC count and/or WBC differential counts, lymphocytes and granulocytes, were found to be independent risk factors for development of type 2 diabetes.[17-22] In addition, the studies of total WBC counts and WBC differential counts and development of type 2 diabetes were conducted in different populations with respect to gender, ethnicity, and glycemic status; however there is no information of how total and differential WBC counts are associated with transition to type 2 diabetes in Mexican-American people of mixed European and Amerindian ancestry.

The aim of our study was to assess potential additive associations of total and differential WBC counts (lymphocytes, monocytes, and granulocytes), CRP, and Body Mass Index (BMI) with time to transition to type 2 diabetes in Mexican Americans using prospective data from the Cameron County Hispanic Cohort. We further evaluated effect modification of BMI and BMI levels on (a) total WBC counts, (b) lymphocytes, (c) monocytes, and (d) granulocytes, and (e) CRP in association with time to transition to type 2 diabetes.

METHODS

Study Population

The Cameron County Hispanic Cohort (CCHC), as previously described,[4] was initiated in Cameron County, Texas in 2004 and now numbers more than 3000 participants of age 18 years or older. The initial purpose of the Cohort was to address the burden of chronic diseases such as obesity and type 2 diabetes and their related conditions in Mexican-Americans, one of the fastest growing ethnic groups in the United States. The participants in this nested study were drawn from the CCHC and included on the basis of completion of two or more visits, as follows: (1) participants in a nested study, the type 2 Diabetes Risk Study (DRS), consisting of cohort participants with pre diabetes, but not type 2 diabetes, at their initial visit (fasting blood glucose 100 to 125 gm/dl) and who then visited quarterly over 5 years, or (2) participants visiting the CRU for their regularly scheduled 5- or 10-year follow up visits. Six hundred and thirty six participants fulfilled the inclusion criteria and their data were therefore admitted to this study.

Data Collection

At their first visit all CCHC participants who agreed to participate in the cohort provided written informed consent including permission to be contacted for further studies and to visit again in 5 year intervals. After agreeing to join the DRS nested study a further consent form was signed by participants. All participants visited the Clinical Research Unit (CRU) where they received detailed physical evaluations and responded to questionnaires in either Spanish or English by choice. Socio-demographic and clinical characteristics, biomarkers, family medical history and medications were assessed by trained staff members[4] and serial measurements of the variables were routinely entered into the database. Biological specimens and questionnaires were obtained at all visits according to standard CCHC protocols.[4]

The institutional review boards (IRBs) at The University of Texas Health Science Center and the University of Texas at Brownsville reviewed and approved the protocol and the informed consent forms with permissions to collect and store de-identified data and specimens for this and other studies.

Outcome Variable

The outcome variable of interest was time to first transition from no diabetes and pre-diabetes to type 2 diabetes. The time variable, measured in months, was created as the interval between the date of the follow-up visits and baseline visit. All participants without any evidence of type 2 diabetes after the baseline visit, death or lost-to-follow up were censored at their last observation dates. Type 2 diabetes was defined by the 2010 definitions of the American type 2 diabetes association (ADA)[23] and includes a glycated Hemoglobin (HbA1c) of 6.5% or greater and or a fasting blood glucose >126 mg/dl. Participants were categorized to have type 2 diabetes if they answered that they had been told by a health care provider that they have type 2 diabetes, or if they were taking hypoglycemic medications or their laboratory findings met the 2010 ADA criteria for type 2 diabetes.

Independent Variables

The independent variables of interest were BMI, total and differential WBC counts (lymphocytes, monocytes, and granulocytes), and C-reactive protein (CRP) measured at the time of the event or censoring. Other covariates, such as age at the time of the event or censoring, gender, family history of type 2 diabetes, pre-diabetes status, smoking, and triglycerides were included in the regression analysis. Body mass index (BMI) was

calculated as weight in kilograms divided by height squared in meters (kg/m^2). Height was measured to the nearest 10th cm using a stadiometer. Weight (to the nearest tenths of a kilogram) was measured on a calibrated beam balance. BMI groups were created as BMI<25 (normal), 25≤BMI<30 (overweight), and BMI≥30 (obese). Family type 2 diabetes history (yes/no) was defined based on patients' reported type 2 diabetes status or high blood sugar status ever diagnosed for biological father, biological mother, or siblings. Pre-diabetes was defined as participant having Impaired Fasting Glucose (IFG) between 100 and 126 mg/dl or HbA1c between 5.7% and 6.5%.

STATISTICAL ANALYSIS

Descriptive statistics of baseline demographics and clinical characteristics were conducted. Categorical variables were described using frequency and percentages. Continuous variables were described with mean and standard deviation. SI units are used throughout.

Cox proportional hazard regression model was conducted to estimate hazard ratios and 95% confidence intervals using person-period data. Since the time to type 2 diabetes was right-censored for the participants who did not transition to type 2 diabetes, Breslow's method was used to handle ties in the data. We fit several univariable Cox proportional hazard regression models for the time to transition to type 2 diabetes including for BMI, total WBC counts, WBC differential counts and CRP. Next, we fit several multivariable Cox proportional hazard models for the time to transition to type 2 diabetes by each significant variable total WBC counts, WBC differential counts and CRP, while adjusting for (1) BMI, and (2) BMI, age, gender, pre-diabetes status, type 2 diabetes family history, smoking and triglycerides.[24-27] In addition, the interactions between BMI and total WBC counts, and BMI and WBC differential counts were tested by including the product of the two variables in the models. The statistical significance of the interaction term in the models was assessed by the overall chi-square test (for interaction with categorical BMI) and the local chi-square test statistic of the coefficient estimates, and by using a likelihood ratio test for the nested models with and without an interaction term. Assessment for multicollinearity between the variables included in the models was performed using Spearman correlation coefficient and Variance Inflation Factors (VIF). The function form of the continuous covariates included in univariable and multivariable Cox proportional hazard regression models were evaluated and as a result, variable CRP was log-transformed and serum triglyceride level was included in the models along with its quadratic form. The proportionality assumption of the hazards in the final models was tested graphically using Schoenfeld and scaled Schoenfeld residuals. Since family type 2 diabetes history and pre-diabetes status did not satisfy the proportional hazard assumption, all models that included these two variables involved stratified analysis using strata statement in SAS Proc PHReg. In the stratified models, the regression coefficients are assumed to be the same in each stratum although the baseline hazard functions may be different.[28] The key assumption of the stratified proportional hazards models that the covariates are acting similarly on the baseline hazard function in each stratum was tested by using likelihood ratio test.[28] Cox-Snell residuals were used to assess the overall fit of the final models.

All statistical analysis was performed using SAS 9.4.[29] All statistical tests were two-sided and were performed using significance (alpha) level of 0.05.

RESULTS

From the original cohort of 3002 subjects, 636 participants who were diabetes free at a baseline visit and had more than one follow-up were included in this study. These 636 subjects did not differ from the rest of the cohort participants in terms of gender, age, number of years lived in Brownsville, and household income. The maximum observation period was nine years and the mean follow-up was 4.4 visits. The participant's main demographic, anthropometric and clinical baseline characteristics are presented in Table 1. The mean (SD) age of participants was 44.6(14.04) years. The majority of the participants were females (68.5%), born in Mexico (68.9%) and the average number of years lived in Brownsville, TX was of 20.7±15.45 years. More than half of the participants (52%) had no high school education and were full-time or part-time employed (53%). Only 26.6% of the participants had any form of health insurance, and 55% of these had private insurance.

On average, the participants at a baseline visit were overweight and obese with mean BMI 30.6±6.17 (48.8% obese and 36.1% overweight). More than half (53.2%) of the participants had pre-diabetes. Baseline mean WBC count, mean lymphocyte, monocyte and granulocyte counts were within the normal range.

We observed 107 participants (16.8%) who transitioned to type 2 diabetes over the entire follow-up period. Individuals who transitioned to type 2 diabetes compared to those who did not had higher baseline measurements of most clinical characteristics measured, with the exception of triglyceride and cholesterol levels. They were older with higher BMI and waist circumference measurements (Table 1).

There is a weak (r<0.30) pairwise correlation between total WBC count, lymphocytes, granulocytes, monocytes and BMI, age, gender, smoking status, family history of type 2 diabetes, pre-diabetes, and triglycerides. However, the correlation analysis showed that there was a high dependency between total WBC counts and granulocytes (r=0.91, p-value<0.0001), total WBC counts and lymphocytes (r=0.60, p-value<0.0001), and between total WBC counts and monocytes (r=0.51, p-value<0.0001). A weak positive relationship was found between lymphocytes and granulocytes (r=0.31, p-value<0.0001).

Univariable Cox proportional hazard regression

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Socio-Demographic Characteristic and Clinical Characteristic	Total n=636	Converted to Type 2 diabetes n=107	Not Converted n=529
Categorical variables	n(%)	n(%)	n(%)
Male	200(31.5)	34(31.8)	166(31.4)
Female	436(68.5)	73(68.2)	363(68.6)
Born in Mexico	438(68.9)	74(69.2)	364(68.8)
USA	198(31.1)	33(30.8)	165(31.2)
With no high school education	328(51.6)	62(57.9)	266(50.3)
With high school education	308(48.4)	45(42.1)	263(49.7)
Employed	334(52.5)	52(48.6)	282(53.3)
Unemployed	302(47.5)	55(51.4)	247(46.7)
Married	429(68.6)	65(60.8)	364(68.9)
Not married	206(32.4)	42(39.2)	164(31.1)
Insured	169(26.6)	33(30.8)	136(25.8)
Uninsured	466(73.4)	74(69.2)	392(74.2)
Smoke	180(28.3)	30(28.0)	150(28.4)
Do not smoke	456(71.7)	77(72.0)	379(71.6)
Normal weight(BMI<25)	96(15.1)	6(5.7)	90(17.0)
Overweight(25≤BMI<30)	229(36.1)	28(26.4)	201(38.1)
Obese(BMI≥30)	309(48.8)	72(67.9)	237(44.9)
Pre-diabetes	337(53.2)	89(83.2)	248(47.1)
No type 2 diabetes	296(46.8)	18(16.8)	278(52.9)
Continuous variables	**Mean(SD)**	**Mean(SD)**	**Mean(SD)**
Age	44.6(14.0)	47.9(13.2)	44(14.1)
Years of education	11.2(5.3)	10(4.9)	11.5(5.4)
Years lived in Brownsville	20.7(15.5)	23.5(17.1)	20.1(15.1)
Annual Household Income	$20, 404($22, 944.3)	$16, 195.4($17, 424.0)	$21, 221.4($23, 801.6)
Waist circumference(cm)	100.1(13.5)	106.8(15.0)	98.8(12.8)
Hip circumference(cm)	110.1(11.8)	114.2(13.2)	109.2(11.4)
Waist-to-hip ratio	0.9(0.1)	0.9(0.1)	0.9(0.1)
BMI	30.6(6.2)	33.5(7.4)	30(5.7)
Number of White Blood Cells x 10^9 /L (n=328)	6.4(1.5)	6.9(1.7)	6.3(1.4)
Number of Lymphocytes x 10^9 /L (n=328)	2.2(0.6)	2.3(0.6)	2.2(0.6)
Number of Monocytes x 10^9 /L (n=328)	0.4(0.2)	0.4(0.2)	0.4(0.2)
Number of Granulocytes x 10^9 /L (n=328)	3.8(1.2)	4.2(1.3)	3.8(1.1)
Triglyceride mmol/L	1.8(1.5)	2.0(1.6)	1.8(1.4)
C reactive protein mmol/L	51.4(61.0)	66.7(79.0)	48.6(56.2)
Glycated hemoglobin(HbA1c)% mmol/mol IFCC /%	5.4(0.6) 36(6.6)	5.6(0.5) 38(5.5)	5.3(0.6) 34(6.6)
Mean FBG mmol/L	5.3(0.6)	5.8(0.5)	5.2(0.5)
Low density lipoprotein mmol/L	3.0(0.9)	2.9(0.8)	3.0(1.0)
High density Lipoprotein mmol/L	1.3(0.3)	1.2(0.3)	1.3(0.3)
Total cholesterol mmol/L	5.1(1.1)	4.9(0.7)	5.1(1.2)
HOMA_IR: MMOL_GLUC*ins/22.5	22.2(16.2)	28.8(18.1)	21.0(16.2)
Insulin pmol/l	93.6(65.5)	112.2(66.8)	89.4(64.7)

Table 1: Baseline demographic and clinical characteristics (SI units) for type 2 diabetes free participants at a baseline visit using Cameron county Hispanic cohort data, 2003-2014.

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analysis showed that the total WBC count [HR=1.17, 95% CI (1.06-1.28), p-value=0.0011], lymphocytes [HR=1.79, 95% CI (1.32-2.42), p-value=0.0002], granulocytes [HR=1.14, 95% CI (1.02-1.27), p-value=0.0229], and BMI [HR=1.07, 95% CI (1.04-1.10), p-value<0.0001] were significantly associated with transition to type 2 diabetes (Table 2). When BMI was modeled as a categorical variable with three levels (normal BMI <25, overweight 25≤BMI<30 and obese BMI≥30) the chi-square test resulted in significant Hazard Ratio (HR) in obese compared to normal BMI group [HR=3.95, 95% CI (1.60-9.77), p-value=0.003] and in obese compared to overweight group [HR=1.77, 95% CI (1.14-2.73), p-value=0.0105]. Using family history and pre-diabetes stratified multivariable Cox proportional hazard regression models, after controlling for age, gender, smoking status, and triglycerides, the total WBC count, lymphocytes, granulocytes, and BMI, remained statistically significantly associated with the development of type 2 diabetes (Table 2).

In the multivariable regression models (Table 3), controlled for other potential confounders, there was a significant interaction between total WBC counts and continuous BMI [HR=1.02, 95% CI (1.00-1.03), p-value=0.0203] which was confirmed with log likelihood ratio test for the nested models, with and without the interaction term (p-value=0.0439) (Figure 1). In addition, there was a significant interaction effect between granulocytes and BMI [HR=1.02, 95% CI (1.00-1.04), p-value=0.0147, log likelihood ratio test p-value=0.0331] (Table 3). Lymphocytes were significantly associated with development of diabetes (Table 3) with HR=1.68 [95% CI (1.22-2.32), p-value=0.0015].

In the multivariable Cox regressions conducted with BMI as a categorical variable (Table 4) a significant interaction effect was found between BMI and total WBC count (log likelihood ratio test p-value=0.0192). The hazard of transition

Variables	Crude Hazard Ratios for developing type 2 diabetes		Adjusted* Hazard Ratios for developing type 2 diabetes	
	HR(95% CI)	P value	HR(95% CI)	P value
Total WBC x 10^9/L	1.17(1.06-1.28)	0.0011	1.18(1.07-1.31)	0.0008
Lymphocytes x 10^9/L	1.79(1.32-2.42)	0.0002	1.72(1.26-2.35)	0.0006
Monocytes x 10^9/L	2.58(0.78-8.56)	0.1226	2.27(0.71-7.31)	0.1692
Granulocytes x 10^9/L	1.14(1.02-1.27)	0.0229	1.16(1.04-1.30)	0.0108
log(C reactive protein)	1.12(0.91-1.37)	0.2993	1.11(0.97-1.27)	0.1235
BMI	1.07(1.04-1.10)	<0.0001	1.04(1.003-1.09)	0.0365
BMI groups				
25≤BMI<30 *vs.* BMI <25	2.23(0.86-5.79)	0.098	1.96(0.75-5.13)	0.1697
BMI≥30 *vs.* BMI <25	3.95(1.60-9.77)	0.003	3.15(1.25-7.94)	0.0148
BMI≥30 *vs.* 25≤BMI<30	1.77(1.14-2.73)	0.0105	1.61(0.99-2.60)	0.0529

**Hazard ratios adjusted for age, gender, smoking, and triglycerides, and stratified by family history for type 2 diabetes and pre-diabetes status using strata statement in SAS Proc PHReg.*

Table 2: Univariable and multivariable Cox proportional hazard regression analyses of time to first type 2 diabetes on follow-up measures of total WBC count, WBC count differentials, CRP and BMI using Cameron county Hispanic cohort data, 2003-2014.

Models	Models with X=WBC		Models with X=Lymphocytes		Models with X=Monocytes		Models with X=Granulocytes		Models with X=ln(CRP)	
Variables	HR(95% CI)	P value	HR(95% CI)	P value	HR(95% CI)	P value	HR(95% CI)	P value	HR(95% CI)	P value
X	0.67(0.41-1.08)	0.1018	1.68(1.22-2.32)	0.0015	1.85(0.53-6.38)	0.3332	0.58(0.33-1.01)	0.0553	1.04(0.90-1.20)	0.6475
BMI	0.92(0.82-1.03)	0.148	1.05(1.02-1.08)	0.001	1.05(1.02-1.08)	0.0007	0.94(0.85-1.03)	0.1868	1.05(1.02-1.08)	0.0012
X*BMI	1.02(1.00-1.03)	0.0209					1.02(1.00-1.04)	0.0147		
Hazard ratio for X by the level of the interacting variable BMI										
X and BMI=20	0.95(0.77-1.16)						0.89(0.70-1.13)			
X and BMI=25	1.03(0.89-1.20)						0.99(0.83-1.17)			
X and BMI=30	1.13(1.01-1.26)						1.10(0.96-1.26)			
X and BMI=35	1.23(1.09-1.38)						1.23(1.06-1.41)			
X and BMI=40	1.34(1.14-1.57)						1.36(1.13-1.65)			

**All models were stratified by family history for type 2 diabetes and pre-diabetes status using strata statement in SAS Proc PHReg and adjusted for age, gender, smoking, and triglycerides.*

Table 3: Multivariable Cox proportional hazard regression models*for time to first type 2 diabetes on follow-up measures of total WBC count, WBC count differentials, CRP and BMI as continuous using Cameron County Hispanic Cohort data, 2003-2014.

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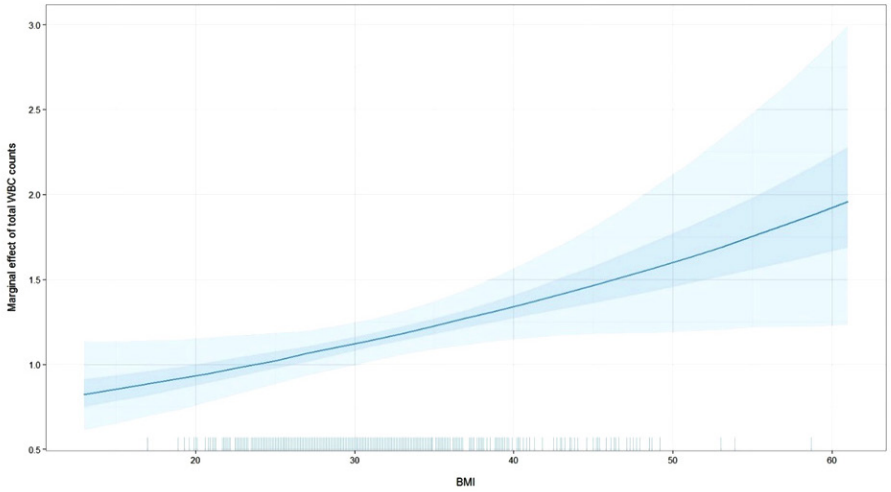

Figure 1: Simulated adjusted hazard ratio with the 95% probability interval of simulations of total WBC counts by unit increase in BMI.

to type 2 diabetes in thousands increase per microliter in total WBC count is 1.39[95% CI (1.07-1.81), p-value=0.0127] times higher in obese individuals compared to overweight individuals. This was also shown graphically in Figure 2 with estimated hazard ratios for total WBC counts by BMI levels. The line for BMI ≥35 lies above the lines for BMI<25 and 25≤ BMI<35, respectively, and it was crossed with the line for 25≤ BMI<35 but paralleled with the line for BMI<25. The local chi-square test in the adjusted models showed a significant HR 1.35[95% CI (1.01-1.81), p-value=0.0432] for increased levels in thousands granulocytes blood cells count per microliter comparing obese to overweight individuals (Table 4, Figure 3).

To further determine if granulocytes and lymphocytes were associated independently of each other with transition to type 2 diabetes, additional Cox proportional hazards models were fitted for (1) granulocytes and lymphocytes together and (2) for granulocytes and lymphocytes together controlled for BMI, age, gender, smoking status, family history of type 2 diabetes, pre-diabetes, and triglycerides. Table 5 shows that lymphocytes were independently associated with transition to type 2 diabetes after controlling for granulocytes and the other potential confounders. In addition, there was a significant interaction between granulocytes and BMI[HR=1.02, 95% CI (1.00-1.04), p-value=0.0177, log-likelihood ratio test p-value=0.0189] indicating that the effect of granulocytes on transition to type 2 diabetes is different for different values of BMI, after controlling for the effect of lymphocytes and the other potential confounders. More specifically, the HR for granulocytes was significant when BMI≥35 [HR=1.18, 95% CI (1.01, 1.37)] and the hazard ratio increased as the values of BMI increased.

DISCUSSION

Prospective data from our Mexican American population-based cohort revealed that total white blood cell counts, particularly lymphocytes and granulocytes were associated with risk of transition to type 2 diabetes. This remains true in both crude and controlled analyses using BMI and other potential confounders such as age, gender, smoking status, family history of type 2[18] diabetes, pre-diabetes, and serum triglyceride levels. Other studies conducted on different populations in respect to gender, geographic location, ethnicity, and glycemic status, have reported significant relationship between incidence of type 2 diabetes and the WBC count and WBC count differentials.[17-22] In a multicenter, multi-ethnic study using a range of glucose tolerance levels in USA states population without type 2 diabetes, Lozeno, et al. reported a significant association between higher lymphocyte counts and incidence of type 2 diabetes, after controlling for smoking, family history of type 2 diabetes, fasting blood glucose and BMI but did not find significant association between total WBC count and incidence of type 2 diabetes after controlling for the other covariates.[18] In a prospective cohort study of young, normoglycemic men with normal WBC counts, conducted in Israel, Twig, et al. reported that total WBC count was an independent risk factor for development of type 2 diabetes after controlling for age, smoking, family history of type 2 diabetes, fasting blood glucose, triglycerides and BMI.[20] In a prospective cohort study of Pima Indians with baseline normal glucose tolerance, Volzorova, et al. also found total WBC counts to be an independent risk factor for development of type 2 diabetes, after controlling for age, gender and percent of body fat.[17] Similarly, Jiang, et al. reported that elevated total WBC count was independently associated with deterioration in glucose metabolism in middle-aged and elderly Chinese.[19] In addition, neutrophils, which comprise the majority of granulocytes, were found significantly associated with incident type 2 diabetes.[30]

Although it is hard to compare WBC differentials between people living in different environments our findings confirmed these previous findings that increasing total WBC counts, lymphocytes and granulocytes to be independent predictors of transition to type 2 diabetes in Mexican Americans when controlling for other potential confounders. Moreover, we further examined the presence of statistical interaction effects in the Cox proportional hazards regression models, towards better ex-

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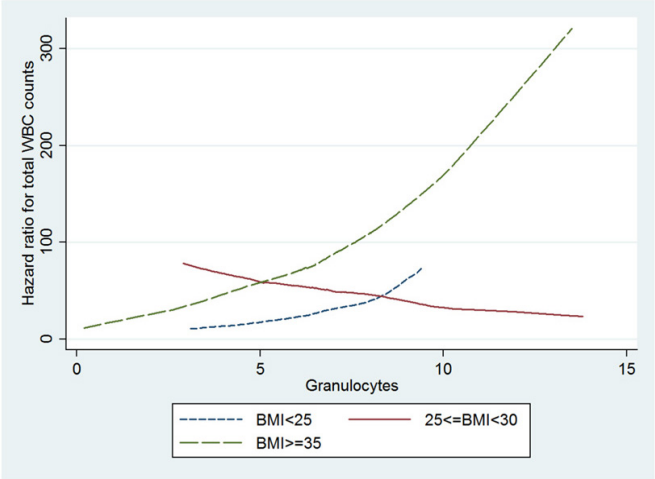

Figure 2: Estimated adjusted hazard ratios for total WBC counts by BMI levels.

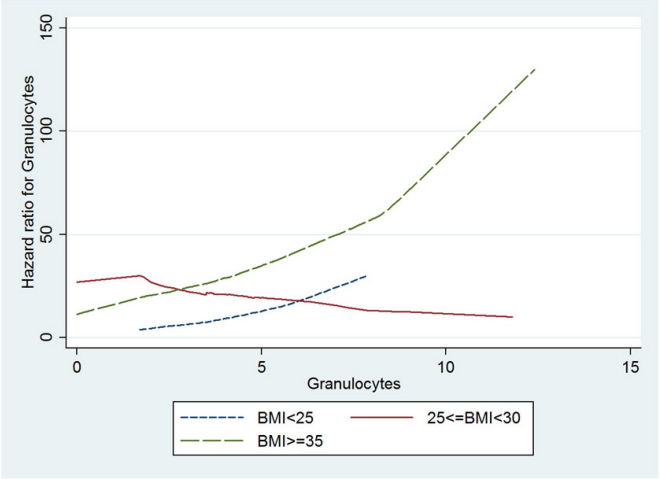

Figure 3: Estimated adjusted hazard ratios for granulocytes by BMI levels.

Models	Models with X=WBC		Models with X=Lymphocytes		Models with X=Monocytes		Models with X=Granulocytes		Models with X=log(CRP)	
Variables	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR(95% CI)	P value
X	1.45 (0.89-2.35)	0.1384	1.08 (0.25-4.72)	0.9229	2.18 (0.66-7.19)	0.1996	1.38 (0.86-2.23)	0.1869	1.06 (0.92-1.22)	0.4055
25≤BMI<30 *vs.* BMI<25	33.89 (0.81-1419.9)	0.0645	2.39 (0.16-35.38)	0.5265	1.92 (0.73-5.04)	0.186	11.25 (0.73-172.51)	0.0822	1.81 (0.69-4.77)	0.2308
BMI≥30 *vs.* BMI <25	6.34 (0.18-224.11)	0.3097	0.96 (0.08-11.77)	0.9757	3.12 (1.24-7.87)	0.0158	4.97 (0.38-65.93)	0.2239	3.02 (1.19-7.68)	0.02
X*25≤BMI<30 vs. BMI<25	0.63 (0.37-1.09)	0.0967	0.87 (0.16-4.56)	0.8651			0.67 (0.39-1.15)	0.1457		
X*BMI≥30 *vs.* BMI <25	0.88 (0.53-1.46)	0.6227	1.9 (0.42-8.71)	0.4078			0.9 (0.55-1.48)	0.682		
X* BMI≥30 *vs.* 25≤BMI<30	1.39 (1.07-1.81)	0.0127	2.2 (0.96-5.04)	0.0635			1.35 (1.01-1.81)	0.0432		
Hazard ratio for X by the level of the interacting variable BMI										
X and BMI<25	1.45 (0.89-2.35)		1.08 (0.25-4.72)				1.38 (0.86-2.23)			
X and 25≤BMI<30	0.92 (0.73-1.15)		0.93 (0.44-1.99)				0.92 (0.71-1.19)			
X and BMI≥30	1.27 (1.13-1.44)		2.05 (1.42-2.94)				1.25 (1.09-1.43)			

˙All models were stratified by family history for type 2 diabetes and pre-diabetes status using strata statement in SAS Proc PHReg and adjusted for age, gender, smoking, and triglycerides.

Table 4: Multivariable Cox proportional hazard regression models* for time to first type 2 diabetes on follow-up measures of total WBC count, WBC count differentials, CRP and BMI categories using Cameron county Hispanic cohort data, 2003-2014.

planation of the development of the type 2 diabetes. Our study indicated that total WBC count and granulocytes and BMI were jointly associated with development of type 2 diabetes. Higher levels of total WBC and granulocyte count were stronger predictors of incident type 2 diabetes in obese in compare to overweight individuals. Similar findings were reported only for total WBC counts in the cohort study of young normal glycemic Israeli men conducted by Twig G, et al.[20] Our results suggest that obese Mexican-American participants with higher levels of total WBC count had 1.39 times higher risk of transition to type 2 diabetes when compared to overweight participants. Likewise, obese Mexican-American with higher granulocytes counts had 1.35 times higher risk of conversion to type 2 diabetes when compared to overweight participants. Additionally we showed that lymphocyte and granulocyte count are independent risk factors for transition to type 2 diabetes, in particular, granulocyte count is a risk factor for categories II and III obese people (BMI>=35).

Though granulocytes (principally neutrophils) are considered the arm of the innate immune response and lymphocytes that of the acquired immune responses, it is now clear that there is considerable and complex cross-talk and collaboration between cells of both these systems both in acute and chronic disease.[31] Both these systems are implicated in the chronic inflammatory syndrome of type 2 diabetes.[32] Infiltration of visceral

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Models	Model 1		Model 2		Model 3*		Model 4*	
Variables	HR(95% CI)	P value	HR(95% CI)	P value	HR(95% CI)	P value	HR(95% CI)	P value
Granulocytes x 10^3 /uL	1.1(0.97-1.24)	0.1279	1.05(0.92-1.20)	0.4413	1.1(0.97-1.26)	0.1448	0.55(0.31-1.00)	0.0489
Lymphocytes x 10^3 /uL	1.7(1.25-2.32)	0.0008	1.57(1.13-2.17)	0.0068	1.6(1.15-2.22)	0.0054	1.58(1.14-2.21)	0.0066
BMI			1.06(1.03-1.09)	<0.0001	1.05(1.02-1.08)	0.0022	0.93(0.84-1.03)	0.1768
Granulocytes x 10^3 /uL* BMI							1.02(1.00-1.04)	0.0177
Hazard ratio for X by the level of the interacting variable BMI								
Granulocytes and BMI=20							0.85(0.66-1.10)	
Granulocytes and BMI=25							0.95(0.78-1.15)	
Granulocytes and BMI=30							1.06(0.91-1.23)	
Granulocytes and BMI=35							1.18(1.01-1.37)	
Granulocytes and BMI=40							1.31(1.07-1.61)	

**Models adjusted for age at the time of the event or censoring, gender, smoking, and triglycerides and stratified for family history of type 2 diabetes and pre-diabetes status using strata statement in SAS Proc PHReg;*

Table 5: Multivariable Cox proportional hazard regression analyses of time to first type 2 diabetes on follow-up measures of lymphocytes and granulocytes using Cameron county Hispanic cohort data, 2003-2014.

adipose tissues with neutrophils, macrophages and lymphocytes is now recognized to be associated with both obesity and type 2 diabetes.[32-34] Whether this process is associated with increased numbers of circulating cells of these lineages is unknown, but in Table 2 it is interesting to note that increases in the lymphocyte lineages are noticeably associated with development of type 2 diabetes.[32] Overall these observations suggest that it is reasonable to hypothesize that the increase in total and differential WBC counts that we observe may be markers of increase in the inflammatory syndrome and increasing insulin resistance prior to development of overt type 2 diabetes.[32]

STRENGTH AND LIMITATIONS

CCHC data is representative by design of this single ethnicity border population characterized with high prevalence of type 2 diabetes and other conditions and biomarkers for chronic diseases.[3-7] Although many of the participants do not have the same follow-up time, the response rate of the CCHC participants is high. To our knowledge, this study was the first conducted in a Mexican-American population, to assess risk factors leading to the development of type 2 diabetes. In addition our study has some novel findings that in non-diabetic and pre-diabetic population lymphocytes is an independent of granulocytes factor of incident type 2 diabetes regardless of the levels of BMI, while granulocytes is an independent risk factor when BMI>=35.

Our study has some limitations. Based on the results from the analysis we did not find any association between CRP and transition to type 2 diabetes as shown in several studies.[35-38] We did not find any interaction between continuous BMI and lymphocytes. The reason is that the study was performed based on available data of 636 participants and detecting statistically significant smaller interaction effects in the Cox proportional hazard regression models may be underpowered.[39] Moreover, it is known, that larger sample sizes are needed to study interactions involving continuous variables.[39]

CONCLUSIONS

Increased total WBC and WBC differential counts, particularly lymphocytes and granulocytes, are associated with risk of transition to type 2 diabetes, particularly among the obese Mexican-American, after adjusting for other potential confounders. Our study emphasizes the predictive importance of total WBC counts, lymphocytes and granulocytes in obese participants, in particular the role of lymphocytes and granulocytes. Our observations emphasize the central role of chronic inflammation in the pathology of type 2 diabetes, and that this process is underway well before diagnosis. Since inflammation plays a role in the development of type 2 diabetes understanding its role is important in development of prevention strategies for type 2 diabetes. Since our study suggests that screening and monitoring the WBC counts, lymphocytes and granulocytes can help with predicting potential transition to type 2 diabetes administering anti-inflammatory medications in addition to life-style changes may bolster efforts in preventing this disease.

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Author Contributions: Research idea and study design: KPV, MHR, JBM; Data acquisition: JBM, SPF; statistical analysis/interpretation: KPV; Wrote the manuscript: KPV; Reviewed/edited manuscript: JBM, SPH, MHR, ML, RLO. Each author: provided intellectual content; contributed significantly to the preparation and/or revision of the manuscript; and approved the final version of the manuscript. KPV takes responsibility for the integrity of the data and the accuracy of the data analysis.

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DISCLOSURE

None of the authors have any conflicts of interest to declare.

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Case Report

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Type 1 Diabetic and Hypertensive Retinopathy: Case Presentation and Review of Literature

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ABSTRACT

Background: Type 1 Diabetes (T1D) Mellitus is a complex, chronic illness that affects half a million children under the age of 15 years. Complications associated with diabetic retinopathy can be prevented with continued self-management of Blood Glucose (BG) and Blood Pressure (BP) into adulthood. In this case, we present a 20-year-old man with a 15 year history of T1D who loses control of his BG and BP for 2 years.
Methods: Blood pressure, visual acuity and intraocular pressures were measured at the time of visit. Non-mydriatic retinal imaging was performed using a Canon CR-2 Plus AF with a resolution of 18 megapixel. A Spectral Domain (SD)-OCT provided a 5 micron resolution of the posterior pole including the macula/fovea. Optical Coherence Tomography Angiography (OCTA) (Optovue, Inc., Fremont, CA, USA) captured 6*6 mm angiograms centered on macula. TeamViewer™ was used to perform remote tele-presence tele-ophthalmology.
Results: Color Fundus Photo (CFP) of the subject in 2013 showed few hemorrhages with virtually no signs of retinopathy although his BP, last Glycated hemoglobin (HbA1c) and BG were uncontrolled (130/91 mm Hg, 13+, 421 mg/dL, respectively). Two years later, after 15 years of diabetes, his BP, last HbA1c, and BG are still uncontrolled (142/62 mm Hg, 13.5%, and 319 mg/dL, respectively). CFP and tele-consultation confirms severe Non-proliferative diabetic retinopathy (NPDR), after 131 days since last annual eye examination, with 259 retinal hemorrhages and 12 Intraretinal microvascular abnormalities (IRMAs) in his left eye. OCT was normal, but OCTA identified areas of retinal telangiectasia and micro-aneurysm formation. 21 days following NPDR diagnosis, he reduced BP to 122/78 mm Hg, HbA1c to 10%, and BG to 115 mg/dL. CFP showed 80 fewer hemorrhages and 10 IRMAs. 57 days following NPDR diagnosis, subject had BP of 107/72 mm Hg and BG of 124 mg/dL. CFP showed 180 fewer hemorrhages and 13 IRMAs.
Conclusions: As BG and BP were decreased and maintained within normal levels, the subject benefited from reduction in retinopathy findings. This case identifies the role non-mydriatic retinal imaging, OCT, and OCTA may play in the assessment and follow-up of patients with long duration type 1 diabetes. Tele-ophthalmology can be an important tool in the follow-up and second opinion of screened patients. An emphasis on BP monitoring can play an important role in the better management of patients with type I diabetes. Close monitoring and maintenance of BP below 130/80, fasting BG under 120 mg/dL, and HbA1c<10% can help reduce NPDR microvascular complications and save vision.

KEYWORDS: Type 1 diabetes mellitus; Diabetic retinopathy; Hypertensive retinopathy; Non-

proliferative diabetic retinopathy; Digital imaging; OCT; OCTA Angiography; Flame hemorrhage; Dot hemorrhage; IRMA; Telemedicine; Blood Pressure; Blood Glucose; Hypertension.

ABBREVIATIONS: T1D: Type 1 Diabetes; BG: Blood Glucose; BP: Blood Pressure; OCTA: Optical Coherence Tomography Angiography; CFP: Color Fundus Photo; NPDR: Non-proliferative diabetic retinopathy; IRMAs: Intraretinal microvascular abnormalities; IDF: International Diabetes Federation; HbA1c: Glycated hemoglobin; VA: Visual Acuity; IOP: Intraocular pressures; FAF: Fundus autofluorescence; FFL: Friends for Life; ETDRS: Early Treatment Diabetic Retinopathy Study; DICOM: Digital Imaging and Communications in Medicine; HIPPA: Health Insurance Portability and Accountability Act; UH: University Hospital; CSC: Clinical Sciences Centre; DNA: Deoxyribonucleic acid; HTN: Hypertension; UKPDS: UK Prospective Diabetes Study; HOT: Hypertension Optimal Treatment; CMOS: Complementary Metal Oxide Semiconductor.

INTRODUCTION

Type 1 diabetes (T1D) mellitus is an autoimmune process that selectively destroys the insulin-producing beta cells in the islets of Langerhans, resulting in an absolute deficiency of insulin.[1] The International Diabetes Federation (IDF) recently reported that the worldwide incidence of T1D affects nearly 500,000 children under the age of 15 years.[2] The largest reported numbers can be found in Western Europe and North America.[3] However, the highest mortality rate due to T1D in children is highest in sub-Saharan African countries such as Sudan, which shows 42.6 deaths per 100,000 children less than 15 years of age while in the United States, the incidence is 0.63 per 100,000 children.[4] It is clear that T1D is a serious health priority throughout the world.

Most recently, progress has been made in determining cellular malfunctions in patients with T1D so that effective treatments can be developed. Scientists from the Clinical Sciences Centre (CSC) in West London have shown that microRNA 375 is released into the bloodstream in large quantities as soon as the pancreas cells that produce insulin begin to die.[5] In addition, scientists from Joslin Diabetes Center at Harvard Medical School have discovered higher levels of miR-200 protein and consequently impaired Deoxyribonucleic acid (DNA) repair in stem cells from T1D patients with severe complications compared to T1D with absent or mild complications.[6] Their next step will be to determine whether these molecules can be detected in the bloodstream and used reliably as effective bio-markers for T1D diagnosis and complications.

The rising prevalence of T1D and its vascular complications are a major cause of concern, especially regarding the development of serious diseases that affect the kidneys, nerves, cardiovascular system, and the eyes. Diabetic retinopathy, one of the most common complications of T1D, is classified as microangiopathy affecting the retinal vasculature, including microvascular leakage from collapsing inner blood-retinal barrier (retinal oedema) and microvascular occlusion.[7] Reports suggest that individuals with T1D should undergo a retinal exam after 5 years of onset or by 15 years of age to screen for and prevent retinopathy.[8] Retinal hemorrhages are often associated with long history of diabetes[9] and high blood glucose as well as long duration of elevated blood pressures.[10] Other than glycemic and systemic BP control, no other biochemical factors have conclusively been demonstrated to protect against diabetic complications.[11]

The target fasting BG and HbA1c are 80-130 mg/dL and <7%, respectively.[8] The target BP is <130/80.[12] Loss of control of these important health parameters in a child with T1D results in progressive retinopathy.[13] Research has established the importance of BG control to prevent development and progression of diabetic retinopathy. Prolonged exposure to hyperglycemia induces a large number of cellular level alterations in vascular tissue, including non-enzymatic glycosylation of proteins and lipids and increased oxidative stress[14] in addition to substantially increased retinal oxygen consumption.[15] BP control is being advocated for the same purpose, as patients with T1D and uncontrolled hypertension are twice as likely to have reduced vision when compared to patients with diabetes and controlled BP.[16]

MATERIALS AND METHODS

Protocol for Screening

Visual Acuity (VA) and Intraocular pressures (IOP) were performed using an automated VA instrument from Canon RK-F2 Full Auto Ref-Keratometer (Tokyo, Japan) and Canon TX-20 Full Auto Tonometer (Tokyo, Japan) to capture pertinent data. BP was measured manually using Korotkoff technique while the patient sat comfortably.

The use of a Canon CR2 Plus AF (Tokyo, Japan) non-mydriatic retinal imaging system with a 18 Mp Complementary Metal Oxide Semiconductor (CMOS) sensor provided color and Fundus autofluorescence (FAF) fundus images. Image management and post processing of digital imagery was done using Canon's proprietary software, image SPECTRUM™ V 3.01 (Irvine, California, USA), to manage all electronic data including VA, tonometry as well as importing OCT and OCTA images for use in tele-reading.[17]

A Spectral Domain (SD)-OCT provided high-resolution structural imaging of sequelae of posterior segment manifestations in the subject's previously healthy eye. The following year, the subject was re-imaged using an optical coherence tomography angiography (OCTA) (Optovue, Fremont, California, USA) in addition to the previous imaging equipment. A certified diabetic reader quantified the extent of retinopathy and its progression over time by performing retinal hemorrhage count.

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TeamViewer™ (Tampa, Florida, USA) was used to perform remote tele-presence tele-ophthalmology.

Case Presentation Results

A 20-year-old Hispanic male with 15-year history of T1D has been followed remotely by our University based Telemedicine (Rutgers New Jersey Medical School in Newark, NJ, USA) during the annual meetings of Friends for Life (FFL): International Children with Diabetes (Orlando, Florida, USA: http://www.childrenwithdiabetes.com) for the past 5 years, beginning in 2010. FFL provides education and support for children with diabetes and their family members in order to maintain a healthy, typical lifestyle with diabetes.

The subject was diagnosed with T1D DM in 1999 after experiencing flu-like symptoms, and treated with a Medtronic insulin pump (Minneapolis, Minnesota, USA). In 2010, he attended friends for life (FFL) and participated in a comprehensive ophthalmic screening. During his initial visit, we noted a BG of 184 mg/dL and BP of 128/78. (Figures 1 and 2) A family history revealed the presence of type 2 diabetes (Father) and hypertension (Mother and Father). His retinal images were free of retinopathy with no hemorrhages and showed no signs of hyperglycemic or hypertension damage. (Table 1, Figure 3)

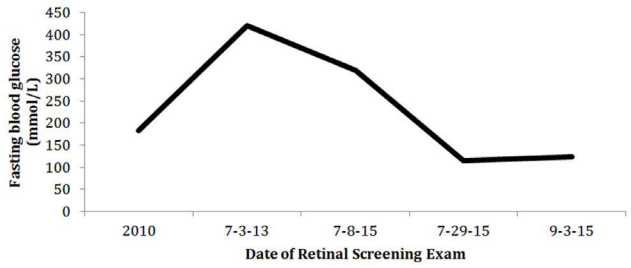

Figure 1: The subject's fasting blood glucose levels are recorded in real time evaluation.

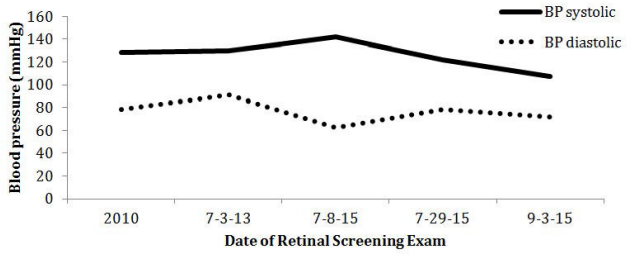

Figure 2: The subject's blood pressure values are recorded in real time evaluation.

The subject returned three years later in 2013 to the annual FFL meeting when he was 18-years-old and underwent a follow-up comprehensive ophthalmic screening. At the time he was taking fast-acting Novolog with pump but would occasionally use long-acting Lantus when not on the pump to control his T1D. His Body Mass Index (BMI) was 19.8, and he was within normal weight range. His self-reported BG was 421 mg/dL and last HbA1c was 13%. (Table 1, Figures 1,3 and 5) His blood pressure was 130/91 with a pulse of 106 bpm. (Table 1, Figure 2) Fundus images of the left eye showed 2 small dot hemorrhages, no flame hemorrhages, and a single IRMA, (Figures 3 and 4, Tables 1 and 2) as interpreted by a certified diabetic reader.

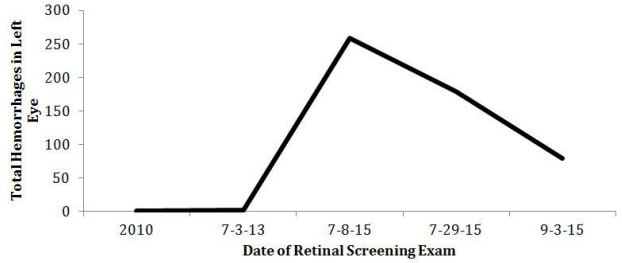

Figure 3: The subject's total flame and dot hemorrhages in the left eye are recorded in real time evaluation.

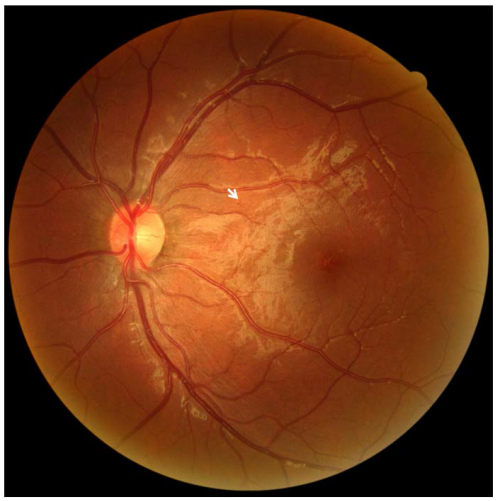

Figure 4: The subject's CFP was imaged in 2013. A 45-degree view of the subject's left eye posterior pole identified a few dot-blot hemorrhages (example as shown by arrow) and a single IRMA. No diabetic retinopathy was detected.

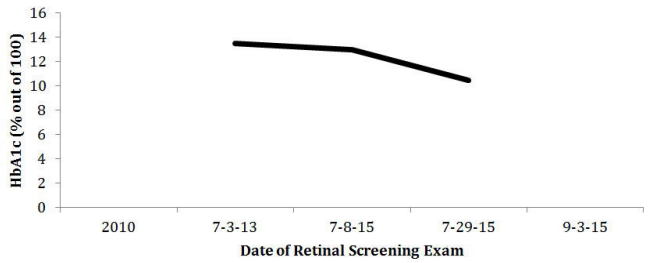

Figure 5: The subject's HbA1c levels are recorded in real time evaluation.

In January 2015, his pediatrician placed him on Enalapril (Kenilworth, NJ, USA) BP medication control. However, after only taking the medication once, the subject decided not to continue the treatment as he felt the medicine resulted in numbness and pain in his legs. During that time, he also halted use of

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Left eye	Jul 2010	Jul 11, 2013	Jul 8, 2015	Jul 29, 2015	Sept 3, 2015
Dot hemes	0	2	221	168	77
Flame hemes	0	0	38	11	2
IRMAs	0	1	12	10	13
T1 DM history (years)	10	13	15	15	15
BP (mmHg)	128/78	130/91	142/62	122/78	107/72
HbA1c (%)	n/a	13+	13.5	10.5	n/a
BG (mmol/L)	184	421	319	115	124
VA (left eye)	20/80 uncorrected	20/30 corrected	20/30 corrected	20/30 corrected	n/a

Table 1: The subject's left eye retinal fundus images were evaluated for counted hemorrhages and IRMAs. His BP, fasting BG, last HbA1c, VA of his left eye, and duration of T1 DM history were also noted.

Location	Dot hemorrhage	Flame hemorrhage	IRMA	Total micro-vascular changes
July 11, 2013				
45° posterior pole	1	0	0	3
Outer inferior	0	0	0	
Outer superior	1	0	1	
Total	2	0	1	
July 8, 2015-131 days following last diabetic retinal exam; initial NPDR diagnosis				
45° posterior pole	108	25	3	271
Outer inferior	40	4	3	
Outer superior	73	9	6	
Total	221	38	12	
July 29, 2015- 21 days post NPDR diagnosis				
45° posterior pole	98	8	3	189
Outer inferior	34	1	3	
Outer superior	36	2	4	
Total	168	11	10	
September 3, 2015- 57 days post NPDR diagnosis				
45° posterior pole	48	2	3	92
Outer inferior	6	0	4	
Outer superior	23	0	6	
Total	77	2	13	

Table 2: The subject's left eye retinal fundus images were evaluated prior to NPDR diagnosis, on the day of medical NPDR diagnosis, 21 days following NPDR diagnosis, and 57 days following NPDR diagnosis. A board certified diabetic reader counted the number of flame and dot hemorrhages as well as IRMAs in the various regions.

his automated Medtronic insulin pump as he felt he was experiencing discomfort and dermal allergic reaction to the plastic tubing. He reverted to self sub-cutaneous injections 3 times per day. During that period he reported an uncontrolled diet consisting of mostly of fast food due to his busy life style.

On February 27, 2015, the subject underwent a full diabetic eye examination in a private optometric clinic in Paramus, NJ using a biomicroscope slit lamp and both 90-diopter and 20-diopter lens. No retinopathy was noted. The medical plan was for the subject to revisit in 6 months for retinal reevaluation and to see his ophthalmologist should he notice any sudden vision changes.

One hundred and thirty-one days following his most recent diabetic eye examination with now a history of 15 years T1D DM, the subject participated in FFL ocular screening. We noted his BP was 142/62 mm Hg and his fasting BG was 319 mg/dL while his May 2015 HbA1c was 13.5% (Table 1, Figures 1, 2 and 5). We noted a sudden change in his retinal findings of

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the posterior pole (45 degrees) consistent with diabetic retinopathy. A total of 221 dot hemorrhages, 38 new flame hemorrhages, and 12 IRMAs were counted in the left eye by a certified diabetic reader. (Figures 3 and 6, Tables 1 and 2) A wide field retinal image of his undilated eye was ordered for his left eye with four Early Treatment Diabetic Retinopathy Study (ETDRS) fields of 45 degrees each. An automated montage was created using image SPECTRUM™ seen in Figure 7. This allowed for more comprehensive remote tele-consultation, evaluating the extent of hemorrhages as well as stress on the retina caused by the uncontrolled elevated BP. Retinal montage shows the majority of retinal hemorrhages and vascular changes appeared in the posterior pole and were mostly absent in the periphery of the wide field image. Flame hemorrhages are associated with high BP and could be seen mostly within the arcades along with retinal stress.

Tele-consultation was conducted in accordance with Digital Imaging and Communications in Medicine (DICOM) and Health Insurance Portability and Accountability Act (HIPPA) protocols from Orlando, Florida, USA with our primary location at University Hospital with board certified ophthalmologists in Rutgers, New Jersey, USA. TeamViewer™ software was configured to share and review the subject's retinal images including color, red free, FAF, OCT, and OCTA. We used a cell phone connection to discuss the details of VA, IOP, BP, glucose levels as well as the subject's history. His identifiers were never used during communication or through use of TeamViewer™. The retinal findings were classified as severe NPDR as defined by the presence of at least one of the following: venous beading, IRMAs, or hemorrhages/microaneurysms present in a standard 45 degree field of view of the posterior pole photograph in at least two quadrants. (Figure 6, Table 2)[18]

A board certified retinal specialist remotely interpreted the OCT and OCTA images. He reported that the OCTA showed areas of retinal telangiectasia and occasional micro aneurysm formation. (Figure 8) Review of the digital CFP revealed hemorrhages associated with diabetic and hypertensive retinopathy. (Figure 6) However, the microvascular changes seen in the OCTA (Figure 8) were not recognized in the CFP (Figure 6) and offered further insight into the extent of retinopathy than the CFP suggested.

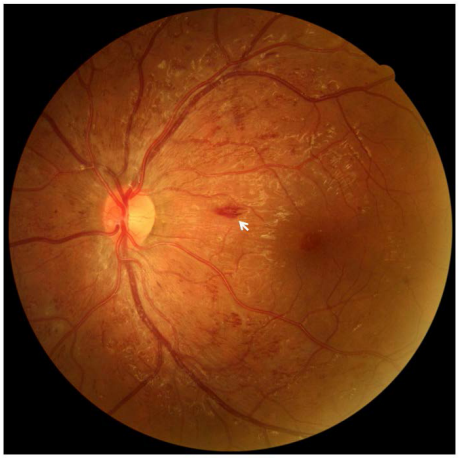

Figure 6: The subject's CFP was imaged on July 8, 2015 (131 days after his last diabetic eye examination, which had no reported retinopathy). A 45-degree view of the left eye posterior pole identified a significant number of dot-blot hemorrhages, flame hemorrhages (example depicted by arrow), and IRMAs. A board certified ophthalmologist diagnosed the patient with NPDR.

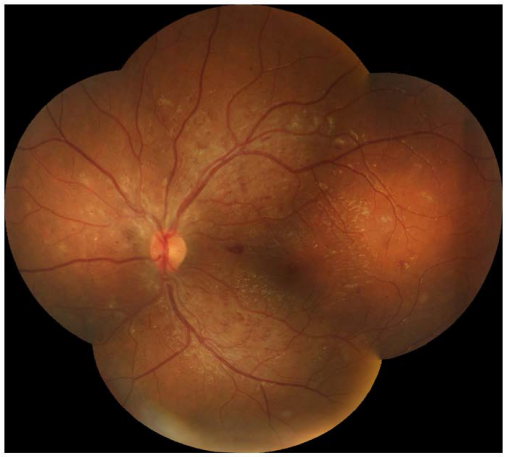

Figure 7: The subject's left eye CFP automated montage was created on July 8, 2015. ETDRS fields of 45-degree view from each quadrant were used to create a wide field retinal image. The hemorrhages and vascular changes were centrally located and noticeably absent in the periphery of the wide field image.

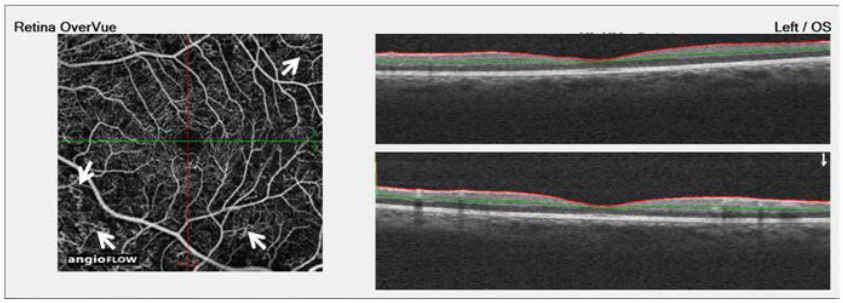

Figure 8: The subject's left eye (OS) OCT-A image was taken 131 days after his last diabetic eye examination, which had reported no signs of retinopathy. The left image depicts microvascular changes (examples highlighted by arrows) consisting of retinal telangiectasia and occasional micro aneurysm formation. This shows that the hemorrhages and microvascular changes noted in the CFP could be due to hypertension and not diabetic retinopathy. The right image shows that there is no macular edema but there were some vascular changes. These details were not seen in CFP.

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After confirming the potential urgency for vision loss and end organ damage, the subject was scheduled to be seen by his primary physician and ophthalmologist in NJ 21 days after the Orlando retinal screening because the subject resides in New Jersey. He was directed to resume taking daily Enalapril BP medicine immediately and to strive to greatly reduce his Hb1AC (from >13%).

On his first visit to University Hospital (UH) in Newark (21 days after FFL 2015), the subject maintained his VA at 20/30 in both eyes and had reduced his BP from 142/62 to 122/78 after resuming Enalapril medication. According to his neurologist, the tingling leg sensation that had caused the subject to cease medication was attributed to peripheral neuropathy onset. His July 2015 Hb1AC was reported to be 10%, and his BG was 115 mg/dL after he had made significant changes to his daily diet. (Table 1, Figures 1 and 5) His left eye seen in Figure 9 showed that the central flame hemorrhage had resolved and his retinal hemorrhages had reduced from 259 dot hemorrhages to 179 dot hemorrhages. (Figure 3, Tables 1 and 2)

On his second visit to UH (57 days after FFL 2015), the subject had a BP of 107/72 while his fasting BG was reported to be 124 mg/dL (Table 1, Figures 1 and 2). His left eye was imaged (Figure 10) and showed his retinal hemorrhages had reduced to 79 hemorrhages. (Figure 3, Tables 1 and 2) His newly imaged OCT showed absence of macular edema and minimum foveal swelling. (Figure 11)

DISCUSSION

Types of Retinal Hemorrhages and Microvascular Changes

There are two main types of posterior pole hemorrhages and a microvascular change that can affect patients with long duration or poorly controlled BG and hypertension.

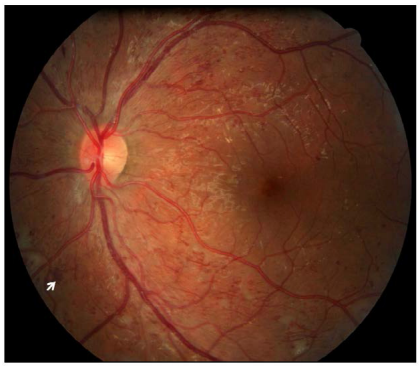

Figure 9: The subject's CFP was imaged 21 days following NPDR detection. A 45-degree view of the posterior pole in the subject's left eye showed that the central flame hemorrhage had resolved and there were 30% fewer total retinal hemorrhages since NPDR diagnosis. An example of an IRMA is demonstrated by the arrow.

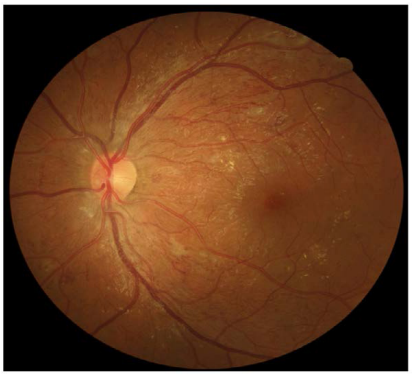

Figure 10: The subject's CFP was imaged 57 days following NPDR detection. A 45-degree view of posterior pole in the subject's left eye showed approximately 70% fewer counted retinal hemorrhages since diagnosis of NPDR.

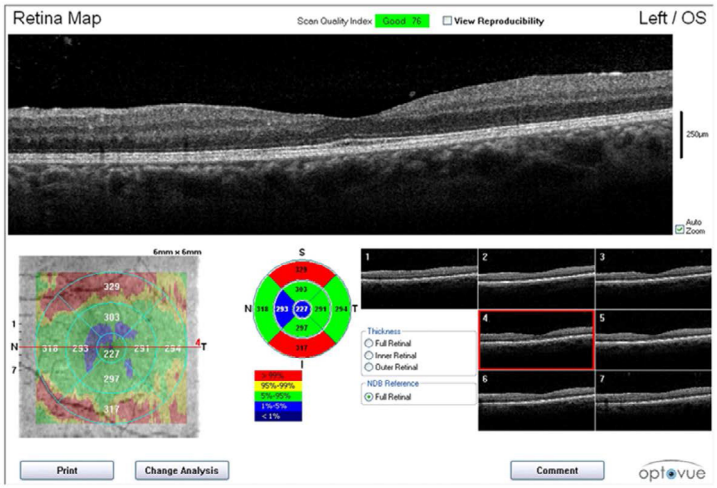

Figure 11: The subject's left eye OCT was imaged 57 days following NPDR detection. The OCT wellness reportdemonstrated in monochromatic scans that there is an absence of macular edema. The bottom-left 6 mm x 6mm scan illustrates thickening of the macular tissues represented in red. The top and bottom right images show that the fovea has minimum swelling.Close observation needs to be carried out on a 30-day basis.

Dot hemorrhages (Figure 4) consist of small, bright red dots seen within the inner nuclear, the outer plexiform, and the outer nuclear layers. After a 15-year medical history of T1D, many develop these dot hemorrhages when the walls of thin retinal vessels are weakened and rupture.[19] Due to their deeper location, dot hemorrhages take a longer time to resolve and affect pre-venular capillaries, thus signaling the presence of venous congestive disease.[20]

Flame hemorrhages (Figure 6) occur in the inner layer of the nerve fiber layer, and are mostly associated with severe hypertension that remains chronically elevated.[21,22] These hemorrhages exhibit a characteristic flame shape resulting from blood traveling through axons of the ganglion cells.[23] They are most frequently seen within the posterior pole, the area between the optic disc and the macula, and affect the superficial and peripapillary capillary beds within the retina.[24] Due to their superficial nature, they tend of resolve within six weeks when blood pressure returns to normal.[25]

In the more advanced retinopathy, intraretinal microvascular anomalies (IRMAs) (Figure 9) can be observed as dilated, tortuous capillary loops.[26] They are found in areas of non-perfusion and typically connect retinal arterioles with retinal venules.[27] These changes are indicative of severe non-proliferative diabetic retinopathy that may progress to proliferative diabetic retinopathy.[28]

With the progression of microvascular diabetic complications, tight BG control is important in the management of retinopathy. It is recommended that close to normal glucose levels should be maintained in patients in the earlier stages of diabetes.[28] However, overall management of T1D by controlling high blood pressure may be as important as high BG levels,[29-32] as hypertension causes more severe retinal hemorrhages that can be remediated with antihypertensive therapy after a few months.[33,34]

Hypertension and Diabetes

Hypertension (HTN) is a common comorbidity in diabetes, and is present in 20-60% of the diabetic population, which is 1.5-3 times higher than the age-matched non-diabetic population.[35] HTN damage is associated with potentially devastating outcomes, including both macrovascular and microvascular complications.[36] Several studies have sought to encode the ideal blood pressure goal for the diabetic population.

The UK Prospective Diabetes Study (UKPDS) demonstrated the significant reduction in microvascular events, primary retinopathy, seen in patients with high blood pressure control (goal<150/85 mm Hg) as compared to a more lenient regimen (goal<180/105 mm Hg).[37] The Hypertension Optimal Treatment (HOT) study suggested that patients with diabetes benefit from a blood pressure goal of diastolic≤80 mm Hg compared to diastolic BP≤90 mm Hg; individuals who maintained the diastolic BP≤80 mm Hg experienced a 51% reduction in major cardiovascular events.[38] The African Centre for the Constructive Resolution of Disputes (ACCORD) trial declared the lack of improvement in clinical outcomes with intensive blood pressure treatment (systolic<120 mm Hg) when compared to standard treatment goals (systolic<140 mm Hg).[39] As a result of these studies, the current established blood pressure goal for patients with diabetes is systolic <130 mm Hg and diastolic <80 mm Hg.[8] This is the goal used for the management of all diabetics, as there is limited evidence supporting the value of different blood pressure goals amongst T1D and T2 diabetics.

Management of BG and BP in patients with diabetes can quickly get out of control following puberty. For T1D adolescents and young adults, there is frequent failure to achieve good glycemic control, and it has been reported that mean HbA1c is highest during the ages of 11-19.[40] In addition, hypertension is established as an independent risk factor for diabetic retinopathy, rather than by association with nephropathy.[41] It has been reported that patients with diabetes who have uncontrolled hypertension double their risk for reduced vision as compared to patients with diabetes and controlled BP.[42]

The duration of diabetes is one of the strongest predictors for progression of retinopathy. It has been reported that the prevalence of retinopathy is 80% in those who have had a 15-year history of T1D.[43] If the macula is not involved, retinopathy can go unnoticed during this critical 15-year time period. However, the co-existence of uncontrolled hypertensive and long-term diabetic retinopathy further increases the possibility for rapid vision loss.[44] Once the macula is involved, central vision may deteriorate rapidly and visual prognosis must be evaluated on an individual basis.

RESULTS

Of the 320 patients that were screened during FFL 2015, 43 patients (13%) had a history of T1D for equal to or greater than 15 years. Of the 43, eight patients (19%) also had hypertension (BP>130/80). Out of 8, 7 patients (88%) had HbA1c<12%.

Our subject was exceptional in that although he had been seriously counseled for at least 2 years since FFL 2013 regarding his hyperglycemia (HbA1c=13.5%) and hypertension (BP=142/62), he failed to follow a more judicious lifestyle as proposed by the FFL educational program. In 2013, with a 13-year history of T1D, he did not have retinopathy. Two years later in 2015, with a 15-year history of T1D and continued uncontrolled BG and BP for >2 years, it took 131 days for severe NPDR to result. After regaining BP and BG control for >8 weeks (57 days) following NPDR diagnosis, the patient demonstrated signs of microvascular remediation through fewer counted dot and flame hemorrhages.

CONCLUSION

The subject had maintained good control of type 1 diabetes through most of his life, including the difficult phases of puberty. His decisions to cease tight control of BG and not follow the direction of his pediatric endocrinologist to take medication to control his high BP contributed to severe NPDR, as well as the peripheral neuropathy that he currently experiences in his feet.

T1D is a lifelong concern that can affect the psychological well being of affected children, as well as all members of a family providing lifelong self-care. However, our data suggests that education, tight control of all aspects affecting diabetes, support from family members, as well as groups such as FFL help maintain a healthier and normal lifestyle with diabetes.

Although we have seen promising return to better control of the subject's diabetes and clearing of nearly 70% of his retinal hemorrhages from his severe NPDR diagnosis, some damage may be end organ reversible over time, while other damage may become permanent. Further studies in T1D, specifically looking at the role of controlled hypertension combined with hyperglycemia in patients who have retinopathy or long history of T1D are needed. Medical management of patients with T1D should include blood pressure monitoring at each ophthalmic clinical visit in order to help detect inappropriately fluctuating and high blood pressure patterns. Early detection of microvascular and macrovascular changes using digital retinal imaging along with OCT and OCTA can help detect various types of hemorrhages and vascular changes before symptoms of permanent vision loss manifest.

CONFLICTS OF INTEREST

No authors have conflicts of interest.

CONSENT

Consent was obtained at the time of examination.

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