APPENDIX 1

Diagnosis

Diagnosis of diabetic macular edema (DME) was confirmed according to WHO criteria.[A1] Diabetic macular edema and extent of severity of diabetic retinopathy (DR) were measured by two trained ophthalmologists using dilated funduscopic examination with slit-lamp-bio-microscopy by +90D and 3-mirror-lens, seven-field-digital-fundus-photography with fluorescence angiography and optical-conhorence-tonography (OCT). Grading and severity of retinopathy was assessed through modified Airlie House Classification of Early-Treatment-Diabetic-Retinopathy-Study (ETDRS) criteria and according to International Clinical Diabetic Retinopathy and Diabetic Macular Edema Severity Scales of the Global Diabetic Retinopathy Project Group.[A2,A3] Letter score were documented as per Snellen visual acuity chart.[A4,A5]

Criteria to Define Phenotype

In present study diabetic macular edema subjects were further cross classified into: focal diabetic macular edema with mild to moderate non-proliferative diabetic retinopathy (FDME+MNPDR, n=28), focal diabetic macular edema with severe NPDR (FDME+SNPDR, n=16), diffuse diabetic macular edema with SNPDR (DDME+SNPDR, n=15) and diffuse diabetic macular edema with proliferative diabetic retinopathy (DDME+PDR, n=27).

According to disease severity scales for diabetic retinopathy and diabetic macular edema, mild non-proliferative diabetic retinopathy is characterized by microaneurysms only. Moderate non-proliferative diabetic retinopathy is characterized by more than just microaneurysms with retinal haemorrhages, exudates and cotton wool spots but no intraretinal microvascular abnormalities or significant venous beading. Severe non-

proliferative diabetic retinopathy is characterized by one or more of the following, in the absence if proliferative diabetic retinopathy: more than 20 intraretinal haemorrhage in each of four quadrants, definite venous beading in two or more quadrants, prominent intraretinal microvascular abnormalities (IRMA) in one or more quadrants. Proliferative diabetic retinopathy is characterized by one or more of the following: extraretinal neovascularization (new vessels on the disc or elsewhere in the retina) with or without vitreous or preretinal haemorrhage. Severity of diabetic macular edema is classified into mild diabetic macular edema characterized some retinal thickening or hard exudates in posterior pole away from the centre of the macula. Moderate diabetic macular edema is characterized by retinal thickening or hard exudates near the centre of the macula but not involving the centre. Severe diabetic macular edema is characterized by retinal thickening or hard exudates involving the centre of the macula. Diabetic macular edema is angiographically divided into focal diabetic macular edema characterized by more than 67 % leakage originated from leaking microaneurysms in the whole edema area and diffuse diabetic macular edema characterized by less than 33 % leakage originated from leaking microaneurysms, and the rest from diffuse leaking capillaries in the whole edema area.[6]

Treatment Protocol

Eighty six diabetic subjects with vision impairing diabetic macular edema received 3 initial consecutive monthly injections of RBZ 0.5 mg followed by prompt grid / focal or panretinal laser photocoagulation (within 3-10 days after the last injection). Best Corrected Visual Acuity (BCVA) was measured using Snellen chart at base line (prior therapy) and at the interval of 4 weeks (±1 week) up to month 12. Central macular thickness (CMT) was determined through Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) at every follow-up visit. The main categories assessed were as follows: diabetic macular edema type

(focal / diffuse) in mild to moderate and severe non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy. The primary objective of the study to measure improvement in BCVA status and reduction in macular thickness at every visit till 6 months.

Laser Treatment (Focal and/or Grid and/or Scatter)

Modified ETDRS focal / grid laser photocoagulation technique was used in our treatment protocol. We directly treated all microaneurysms in areas of retinal thickening between 500 and 3000 μm from the centre of macula. A mild grey white burn of 50 μm spot size with burn duration of 0.1 second was applied beneath all microaneurysms. In grid treatment laser burn was applied to all areas of edema not associated with microaneurysms. Area considered for grid treatment was 500 – 3000 μm superiorly, nasally and temporally from the centre of macula. No burn was placed within 500 μm of the disc. Panretinal photocoagulation followed Diabetic Retinopathy Study photocoagulation technique as 800 – 1600 burns of 500 μm spot size of 0.1 second duration. Direct treatment of new vessels and microaneurysms or other lesions causing macular edema was followed. The primary efficacy end points included mean BCVA letter score change from baseline to month 12 and proportion of patients who gained ≥ 10 and ≥ 15 letters in BCVA. The secondary efficacy end point included mean reduction in central macular thickness. The safety assessments of the therapy was done by 12 month incidence of adverse events like endophthalmitis, intraocular haemorrhage, retinal detachment, raised intraocular pressure and changes in vital signs.

REFERENCES

A1. World Health Organization. Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation.2016. World Health

Organization. Available at: https://apps.who.int/iris/handle/10665/43588. Accessed July 7, 2019.

A2. Grading Diabetic Retinopathy from Stereoscopic Color Fundus Photographs-an Extension of the Modified Airlie House Classification. ETDRS Report Number 10. Early Treatment Diabetic Retinopathy Study Research Group. Ophthalmology 1991;98:786–806.

A3. Wilkinson CP, Ferris FL, III, Klein RE, et al. Proposed International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales. Ophthalmology 2003;110:1677-82.

A4. Frank, R. N. On the Pathogenesis of Diabetic Retinopathy: A 1990 Update. Ophthalmology 1991;98:586-93.

A5. Snellen H. [Sample Letters to Determine Visual Acuity]. Berlin: Peters; 1868.

A6. Mitchell P, Bandello F, Schmidt-Erfurth U, et al. The RESTORE Study: Ranibizumab Monotherapy or Combined with Laser versus Laser Monotherapy for Diabetic Macular Edema. Ophthalmology 2011,118:615-25.

APPENDIX 2

Detailed Methodology for Enzyme-linked Immunosorbant Assay (ELISA)

VEGF165A were quantified by enzyme-linked immunosorbant assay (ELISA) kit using from My Biosourse, Cataloge No.MBS017242 from undiluted plasma of the study subjects. This ELISA kit recognizes both native and recombinant Human VEGF 165A and VEGF165b the isoforms of VEGF. The immunogen used in this kit is full length recombinant human VEGF-165 protein. As per manufacturer supporting document the sensitivity of this kit is 5.0pg/ml and no significant cross-reactivity or interference between this analyte and analogues is recorded. Detailed of methodologies and in-house (My Biosourse) datasets were documented in published manual. We evaluated the concentration of VEGF 165A using the following methods:

VEGF165A = VEGF165 (A+B) – VEGF165 B

VEGF165B were determined in plasma by Enzyme linked immunosorbant assay (ELISA) kit using from My Biosourse, Cat. MBS720132/MBS109074. As per manufacturer in-house validation VEGF165B kits does not possess any cross reactivity with VEGF165A and other analogues. The detection antibody is rabbit polyclonal and the immunogen is a recombinant full-length protein corresponding to Human VEGF 165B. The standard is a synthetic Human VEGF 165B peptide. The lab has tested this kit with human serum/plasma samples and the sensitivity of the kit was 1 pg/ml. Both Intra-assay CV (%) and Inter-assay CV (%) is less than 15%. Detailed of methodologies and in-house (My Biosourse) datasets were documented in published manual.

VEGFR1 were quantified from plasma via ELSA based methods using commercially available kit viz. Ray Bioteh (Cat. ELH-VEGFR1). The kit is an in-vitro enzyme-linked

immunosorbent assay for the quantitative measurement of human VEGFR1 in serum (human VEGFR1 concentration is low in normal serum/plasma, and may not be detectable in this assay), plasma and cell culture supernatants. This assay employs an antibody specific for human VEGFR1 coated on a 96-well plate. Standards and samples are pipetted into the wells and VEGFR1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human VEGFR1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of VEGFR1 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. The reproducibility of the kit was Intra-Assay CV%: <10% and Inter-Assay CV%: <12%, respectively with sensitivity 1.5ng/ml. The kit shows no cross-reactivity with the following cytokines viz. human Activin A, AgRP, Angiogenin, ANG-1, Angiostatin, Cathepsin S, CD40, Cripto-1, DAN, DKK-1, E-Cadherin, EpCAM, Fas L, Follistatin, Galectin-7, ICAM-2, IL-13 R1, IL-13R2, IL- 17B, IL-2R alpha, IL-2 R beta, IL-23, LAP, NrCAM, PAI-1, PDGF-AB, Resistin, SDF-1 beta, sgp130, Shh N, Siglec-5, ST2, TGF-beta 2, Tie-2, TPO, TRAIL R4, TREM-1, VEGF-C.

VEGFR2 and R3 were quantified through the ELSA kit manufactured by RayBiotech with matched principle from undiluted plasma samples through kit Cat. ELH-VEGFR2 and ELH-VEGFR3. Manufacturer's in house lab reports established that the kit viz. ELH-VEGFR2 devoid of the cross reactivity with human Angiopoietin-1, Angiostatin , BMP-7, CD14, CD30, CD40, CD40 Ligand, CTLA-4, CXCL16, Dkk-4 , DR6, Endostatin, ESelectin, Follistatin, HB-EGF, HVEM, ICAM-2, IGF-II, IL-10 Ra, IL- 10 Rb, IL-18, IL-9 , IL-2 Ra, IL-2 Rb, IL-5 Ra, LAP, L-Selectin, M-CSF R, MMP-1, 2, 3, 7, 8, 9, 10 and 12, PDGF-AB,

SDF-1b, Tie-1, Tie-2, TIMP-3. VEGFR3 viz. ELH-VEGFR3 kit shows no cross-reactivity with human Angiogenin, BDNF, BLC, ENA-78, FGF- 4, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL- 7, IL-9, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, I-309, IP-10, G-CSF, GM-CSF, IFN-gamma, Leptin (OB), MCP-1, MCP-2, MCP-3, MDC, MIP-1 alpha, MIP-1 beta, MIP-1 delta, PARC, PDGF, RANTES, SCF, TARC, TGF-beta, TIMP-1, TIMP-2, TNF-alpha, TNF-beta, TPO, VEGF with a sensitivity 15pg/ml.

The specificity and cross reactivity for all the biomolecules is hard to achieve due to limited skills and knowledge hence chance of minute cross reactivity may exist with some rare analogues. Sequence based or mass spectrometer based quantification methods may be more precise but it seems less cost effective in terms of biomarker at least for the developing and underdeveloped world. Understanding the limitation of the specific methods viz. ELISA, we believe the observation.

Detailed Statistical Methodology

The statistical methodologies mentioning the statistical program and software we used. In present study we have used ‘R’ programming language and used Graphpad prism for the Figure 1 and 2. The R function aov() were used to perform the one way ANOVA for understand the difference of the cytokines like VEGF and its receptors protein expression for the phenotype of our study group like non responder, 5 letter gain, 10 letter gain and 15 letter gain that are categorized as an outcome of the therapy. The function summary. aov() is used to summarize the analysis of variance model. As the ANOVA test is significant for the cytokine sVEGFR2, hence we computed Tukey HSD (Tukey Honest Significant Differences using R function: TukeyHSD()) for performing multiple pairwise-comparison between the means of groups.

Again if we calculate the power of the study it relies in different outcome on different variables. For Table 1 the variable like central macular thickness reduction among responder and non responder group consider the outcome as continuous variable and the difference of mean about 166um with the sample size of 86, the power of the observation here is 90% considering the type-1 error (α) 0.001 and type-2 error (β) is 0.1 further for the variable sVEGFR2 considering the observed mean of the study, using type-1 error (α) 0.02 and type-2 error (β) is 0.1, the power may achieved 90% with the inclusion of 52 samples but in present study we included 86 samples. In case of Table 2 for the clinical phenotype diffuse diabetic macular edema with proliferative diabetic retinopathy as a non-responder phenotype in with the dichotomous end point where the response of therapy for the phenotype is about 20% and non-response for the phenotype is 50%, The probability of a type-1 error; finding a difference when an alpha cut-off of 4% (0.04) -- indicating a 4% chance that a significant difference is actually due to chance and is not a true difference, then the power of the observation is 80% with the type II error 0.2 by including 82 subjects but here the data presented for 86 samples. With the aid of these, calculation the power varied from 90% to 80% upon the observed significant variable. As the least power observed here is 80% hence overall we can say the power of the study is 80%.

In table 2, in this analysis, we used model in terms of case and control for non-responder and responder, respectively. In this model we assume non responder as ‘case’ as it reveals adverse or ‘bad’ outcome measures or outcome-A and responder as ‘control’ as it reveals ‘good’ outcome measures or outcome-B for the therapy. Like simple case control association study for 2X2 contingency table, here we make 2X6 contingency table to calculate odds ratio in the form of 2X2 calculation for each phenotype. As for example for genetic association study in case control design we used the 2X2 contingency format for the genotype or categorical

variable AA(may coded as ‘0’), Aa(may coded as ‘1’) and aa (may coded as ‘2’) on the basis of present and absent of each genotype in case and control group, Instead of ‘0’, Aa (‘1’) and aa (‘2’), here we used the ‘disease phenotype’ as categorical variable like focal diabetic macular edema with MNPDR (coded as ‘1’), focal diabetic macular edema with SNPDR (coded as ‘2’), diffuse diabetic macular edema with SNPDR (coded as ‘3’) and diffuse diabetic macular edema with PDR (coded as ‘4’). Odds ratio calculated for each phenotype in flowing methods using ‘R’[R version 3.6.1 (2019-07-05)] under the package ‘statistic’.

In Table 4, the linear regression was performed by ‘R’ with the function ‘ln’ to find the association of the predictive marker with the secondary outcome like central macular thickness reduction. Central macular thickness reduction used as dependent variable(y) in this model where diabetic macular edema phenotype used as independent variable and numerical coding increases with the disease severity like FDME+MNPDR coded as ‘1’, FDME+SNPDR coded as ‘2’, DDME+SNPDR coded as ‘3’ and DDME+PDR coded as ‘4’ to predict the impact of independent variables on treatment outcome. The data reveals that the estimates for the regression model is 125.4 (P: .0005) in negative direction that reflects magnitude of central macular thickness reduction is inversely proportionate to disease severity less diseases severity focal diabetic macular edema with MNPDR that coded as ’1’ acquire highest central macular thickness reduction and most severe phenotype DDME+PDR that coded as ‘4’ acquire lowest central macular thickness reduction to get benefit of specific treatment module like 3 loading dose of RBZ+Laser. The study revealed that with central macular thickness reduction corroborated the significant association with diabetic macular edema phenotype/ disease severity and its response to RBZ+Laser; P= .0005 (Table 3).