Original Article

Significance of monitoring vascular endothelial growth factor, monocyte chemoattractant protein-1 and Interleukin-8 in diabetic macular edema towards early identification of nonresponders to ranibizumab therapy

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Purpose: Identification of nonresponders prior to anti-vascular endothelial growth factor (anti-VEGF) therapy would help in the judicious clinical management of diabetic macular edema (DME) patients. Thus, a systematic study was initiated to identify nonresponding DME patient population undergoing ranibizumab treatment to figure out additional inflammatory components that may contribute to their nonresponsiveness to anti-VEGF therapy. **Methods:** A total of 40 patients recruited to this investigator-initiated trial received intravitreal ranibizumab monthly for 3 months. The fourth- and fifth-month injections were according to PRN protocol and the sixth-month injection was mandatory. Best-corrected visual acuity (BCVA), central macular thickness (CMT), and VEGF in aqueous humor were measured for all the patients. Patients were grouped into responders/nonresponders on the formulated criteria and the levels of key pro-inflammatory cytokines were also measured between the two groups at baseline, 2 month and 5 months using cytometric bead array (CBA). **Results:** Eleven patients were categorized (29.72%) as responders and 10 patients (27.02%) as nonresponders. Nonresponders showed poorer BCVA (P = 0.024, 0.045, and 0.048 for 4, 5, and 6 months) and higher CMT (P = 0.021, 0.0008 and <0.0001 for baseline, 1, 2, 3, 4, 5, and 6 months) compared to responders. The cytokines IL-8, MCP-1 were significantly up regulated (P = 0.0048 and 0.029 for MCP-1 and IL-8) in nonresponders. **Conclusion:** Elevated MCP-1 and IL-8 levels found in the nonresponders could be used as a prognostic marker to identify these groups of patients and can help in developing alternative treatment options along with anti-VEGF therapy.

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Despite different anti-vacular endothelial growth factor (anti-VEGF) therapy for diabetic retinopathy (DR) with India projected to be the world capital of diabetes with 80 million patients by 2030,[1] identification of many million nonresponders is essential for alternate therapy development. Diabetic macular edema (DME) is one such complications of DR responsible for vision loss characterized by the accumulation of fluid in the macular region of the retina.[2,3] The global DME prevalence rate is 6.8% in diabetic patients.[4] Edema results from dysregulation of biochemical pathways, disintegration of blood–retinal barrier (BRB)[5,6] and accumulation of its by-products. With the advent of intravitreal anti-VEGF or corticosteroid injections for DME, the decades old laser photocoagulation therapy gradually disappeared.[7,8] The anti-VEGF/corticosteroid therapy suppress the inflammation either by inhibiting VEGF or by activating the genes involved in anti-inflammatory response.[9]

The anti-VEGF drug ranibizumab (Lucentis) is mainly used in treating DME because of its enhanced clinical outcome in terms of vision improvement and edema reduction.[10] RISE and RIDE studies showed that the macular thickness was reduced to 40% in 3 months following monthly ranibizumab injection.[11] However, there are patients refractory to anti-VEGF therapy with persistent edema[12] and that 30% of patients showed non responsiveness to anti-VEGF's[13,14] without much improvement in visual acuity and edema.

Therefore, it is essential to undertake a systematic, sequential monitoring of VEGF levels following each injection through the Investigator Initiated Trial using ranibizumab as the anti-VEGF agent to identify whether the persistent edema is due to nonsuppression of VEGF levels following treatment or not. Apart from identifying responders and nonresponders to ranibizumab therapy, inflammatory factors monocyte chemoattractant protein 1 (MCP-1) and Interleukin-8 (IL-8) involved in BRB integrity which may contribute to edema formation other than VEGF also need to be evaluated in responders and nonresponders. These inflammatory targets allow clinicians for a judicious approach in administering the

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anti-VEGF therapy as sustained VEGF neutralization might damage the retina and affect the vision. Moreover, development of alternate therapy against targets that is responsible for the elevation of MCP-1 and IL-8 may serve as supplementary to anti-VEGF therapy singly or in combined form.

Methods

This investigator initiated trial was held as a single centre study and was done in agreement with the Helsinki declaration and was in compliance with rules and regulations of institutional review board and ethics committee endorsement. Informed consent in writing was obtained from all participants.

Both male/female patients >18 years of age having Type II DM with center involving DME were recruited for the study. The inclusion criteria of the study were baseline BCVA of 20/30 to 20/200 (6/9 to 6/60 Snellen) and central macular thickness more than 300 microns on optical coherence tomography (OCT). Patients having media opacity which prevented good OCT and FFA, advanced proliferative diabetic retinopathy (PDR) who may need a surgical intervention soon, other progressive retinal diseases; any form of glaucoma including neovascular glaucoma which may reduce the media clarity; previous vitreoretinal surgeries; recent cataract surgery or pan-retinal laser photocoagulation in the study eye or focal/grid laser photocoagulation in the study eye or recent intravitreal injection in the last 6 months; untreated diabetes mellitus; untreated/uncontrolled hypertension, history of stroke or myocardial infarction or renal failure; identified hypersensitivity to ranibizumab or whichever components of formulation or fluorescein; women who are pregnant or lactating were excluded from the study.

The duration of the study for each patient was 6 months wherein first three and the last/sixth injections were mandatory and the in between treatments (fourth and fifth injections) were pro re nata (PRN) based (if central macular thickness was more than 300 microns). In all situations, 0.5 mg/0.05 mL ranibizumab (Novartis Pharma, Switzerland) was administered as a single intravitreal injection in accordance with the prescribing information. All patients underwent BCVA and CMT assessment during the study period. The Snellen BCVA values were subsequently converted into logMAR scale for statistical analysis and to letter score for representing improvement in visual acuity. The CMT was measured using optical coherence tomography (Zeiss Cirrus 4000°CT). About 100 µL of aqueous humor was taken prior to the intravitreal injection with a 1cc syringe with a 26 gauge needle under standard sterile condition through a paracentesis. Protease inhibitor cocktail was added to the samples and kept at –80°C until use.

As mentioned above, the first three injections and last sixth injection were mandatory in all patients and VEGF estimated during different time points were indicated as baseline, 1 month and 2 month, and so on. Aqueous humor samples were taken only during intravitreal injection. Baseline indicates the sample collected just before first injection and hence not undergone any treatment. The sample represented as 1 month is collected just before second injection and 2 month sample is collected just before third injection and so on. So the VEGF levels represented as 1 month is the effect of first injection and 2 month represents the effect of second injection etc. Fourth and fifth injections were PRN based and hence, VEGF levels could be estimated in only those patients who were injected at these time points.

The VEGF standards were prepared according to manufacturer's (human VEGF–A platinum ELISA kit, Invitrogen, USA) instructions. 40 µL of aqueous humor samples obtained at different time points in duplicates from DME patients were used to quantify the VEGF concentration. The absorbance was taken at 450 nm using a multi-plate reader (BioTeck, USA) and VEGF concentration were calculated in aqueous samples.

Based on the RISE and RIDE clinical trial results,[11,15] the criteria for responders was formulated when (1) macular thickness reduced more than 40% in the first 3 months (2) macular thickness became normal in 6 months (3) if only 4 injections (the mandatory injections) were needed in 6 months. In our study, "responders" label were given to patients who satisfy all the three criteria and "nonresponder" label did not meet all these three criteria.

The cytokines IL-6, IL-8, IL-12p70, IP-10, and MCP-1 were quantified simultaneously in aqueous humor samples (50 µL) of DME patients (responders and nonresponders) by using multiplexed human cytometric bead array (CBA) kit[16-18] (Becton Dickinson, San Diego, California). The samples and standards were prepared as per manufacturer's instructions. The data acquired was analyzed by FCAP Array software at our flow-cytometry facility. Individual cytokine concentrations were calculated based on their fluorescent intensities in comparison with the standard reference curve.

For the cytokine analysis, patient samples following ELISA VEGF quantification with a left over volume of at least 50 µl were selected. The cytokine measurement was done at 3 time points i.e., at baseline, 2 and 5 months for both responders and nonresponders.

Data were tabulated with descriptive statistics such as mean, standard deviation (SD) and standard error (SE) for continuous variables as well as frequency and percentages for categorical variables. The statistical significance was analyzed by paired *t* test. SPSS software version 22.0 and Graphpad Prism software version 7.2 were used for the analysis.

Results

Forty patients were enrolled in this Investigator Initiated Trial and three of them were excluded. Two patients discontinued treatments due to adverse event and age-related issues. One patient had myocardial infarction and the second one had age-related lassitude and tiredness and hence did not come for the follow up treatment. The third patient had negative VEGF value at baseline and hence excluded from data analysis.

Mean patient age was 58 years (58.7 ± 8.8SD) with mean BCVA of 0.47 ± 0.244SD (logMAR) (6/15 ± 6/9 snellen) and CMT of 476.96 ± 144.04SD. Patient's demographic and clinical features are provided in Table 1. The color fundus images, fluorescein images and OCT images at baseline and following treatment with ranibizumab indicates the reduction in leakage and edema upon ranibizumab treatment compared to baseline [Fig. 1a-c].

In order to perform the trial, the baseline values for vision (BCVA), edema (CMT) were noted before the intravitreal ranibizumab injection [Table 1]. All the patients were treated

for 6 months and the change in these parameters from baseline to 6 month is represented in Table 2. Among the 37 patients, 12 (32.4%) received all the 6 injections, 14 (37.8%) received 5 injections and 11 (29.7%) received 4 injections in total.

The baseline mean VEGF value was 890.40 ± 92.47 pg/mL and at 6 month it was reduced to 527.95 ± 67.81 pg/mL with a statistical significance of $P < 0.0001$ [Table 2, Fig. 2a and b]. Since all the patients did not receive the stipulated 6 injections based on PRN protocol, up to three injections, better reduction in VEGF level was observed in comparison to the sixth injection time point [Fig. 2a]. For those who did not receive anti-VEGF injection at fourth or fifth or both time points, an increment in the VEGF levels was noticed in the average value of VEGF at the time of sixth injection as compared to the third month [Fig. 2a].

In concurrence, the reduction in edema level was found to be statistically significant at sixth month represented in the CMT values following anti-VEGF therapy compared to baseline [Table 2, Fig. 2c and d]. The CMT value plummeted from 476.96 ± 24.24 µm at baseline to 285.78 ± 18.91 mm at sixth month ($P < 0.0001$) [Table 2, Fig. 2c and d]. The edema reduction showed stability in value from mandatory injection time point (first 3 injections) to the sixth injection even in the absence of fourth or fifth or both injections as per PRN protocol.

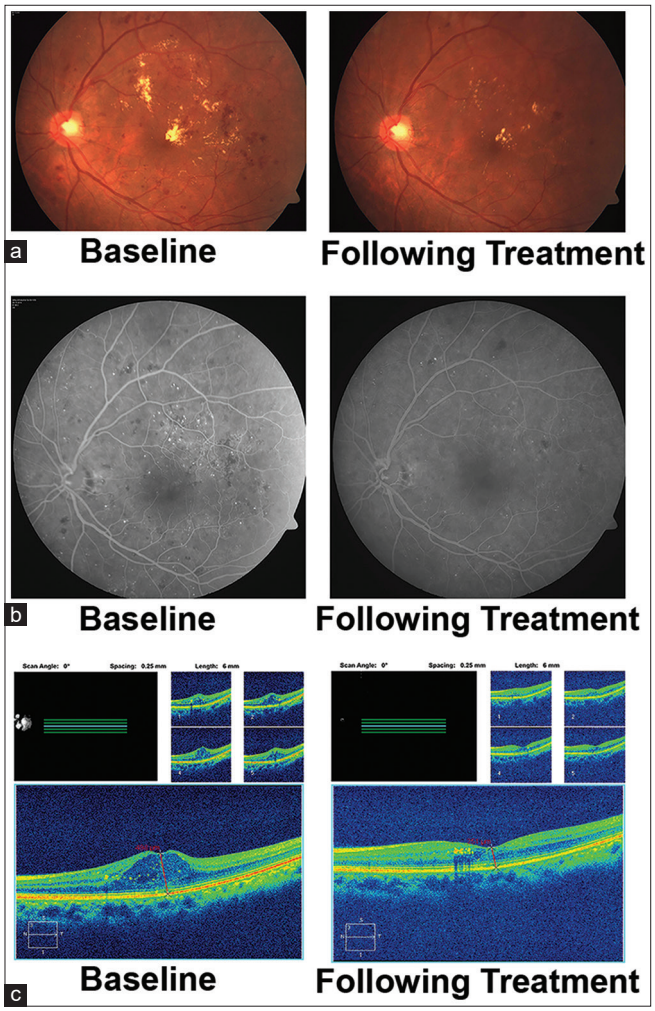

Figure 1: Images showing baseline and following treatment clinical characteristics. (a) Representative color fundus images at baseline and following treatment. (b) Representative fluorescein images at baseline and following treatment. (c) Representative OCT images at baseline and following treatment. OCT-optical coherence tomography

To further confirm the efficacy to anti-VEGF therapy, we evaluated changes in BCVA at baseline and after therapy. Significant improvement in the vision from baseline to sixth month following ranibizumab therapy was shown by logMAR value reduction [Fig. 2e, f]. Even in the absence of fourth or fifth or both injections based on PRN protocol, the visual acuity improvement remained stable to that of visual acuity following 2nd injection [Fig. 2e]. The vision was improved from a logMAR value of 0.47 ± 0.043 (6/15 snellen) at baseline to 0.28 ± 0.038 (6/9 snellen) (10 letter improvement) at 6 month with a $P < 0.0001$ [Table 2, Fig. 2f].

The overall analysis showed significant reduction in VEGF and CMT with improvement in BCVA following ranibizumab treatment from baseline to sixth injection [Table 2 and Fig. 2]. Further, we categorized responders and nonresponders to anti-VEGF treatment based on criteria elaborated in methodology. Eleven patients (29.72%) satisfied the three criteria for responders. Meanwhile, 10 patients (27.02%) satisfied the criteria for nonresponders.

Subsequently, the change in pattern of BCVA and CMT were analyzed for these two groups [Fig. 3a and b]. After third injection, the responder group did not receive next two injections according to PRN protocol because their CMT fell below 300 microns. Even in the absence of two injections, the BCVA did not show any further deterioration. The BCVA was changed from 0.36 to 0.14 (6/12 to 6/7.5) (10 letter improvement) for responder group ($P = 0.0006$) and 0.43 to 0.32 (6/15 to 6/12 snellen) (5 letter improvement) for nonresponder group ($P = 0.039$)

Table 1: Clinical demographics and baseline parameters of patients

	Mean±SD
Age, yrs	58.7±8.8
HbA1c, mmol/mol	8.6±1.7
Visual acuity, LogMAR	0.47±0.244
CMT/CRT, mm	476.96±144.04
VEGF, pg/ml	890.40±562.52
Category	**No. (%)**
Gender	
Male	22 (55)
Female	18 (45)
Eye	
Right	18 (45)
Left	22 (55)

Table 2: VEGF, CMT and BCVA values at baseline and at 6th month in Ranibizumab treated patients. The data represented with±SEM

Clinical parameter	Baseline±SE	6th Month±SE	*P*
BCVA (LogMAR)	0.47±0.043	0.28±0.038	<0.0001
CMT (mm)	476.96±24.24	285.78±18.91	<0.0001
VEGF (pg/ml)	890.40±92.47	527.95±67.81	<0.0001

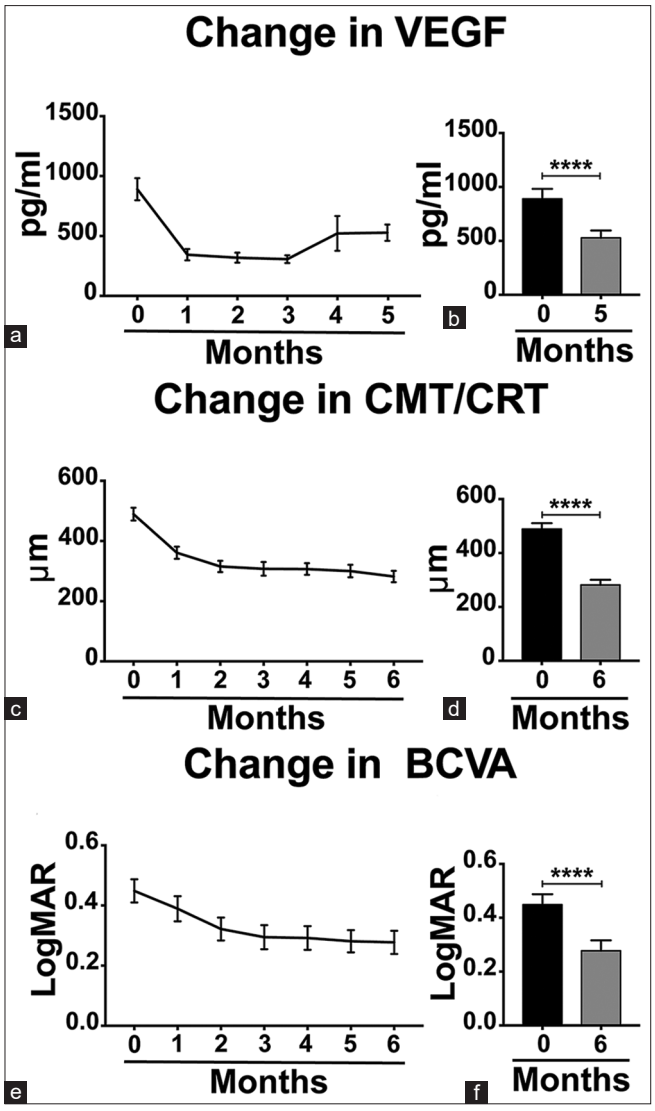

Figure 2: Change in levels of VEGF, CMT/CRT and BCVA during ranibizumab treatment. (a) VEGF levels from baseline after ranibizumab injections at different time points. (b) VEGF levels at baseline vs. 6 month. (c) CMT/CRT changes at different time points from baseline after ranibizumab injections and (d) at baseline vs. 6 month. (e) BCVA changes at different time points from baseline after ranibizumab injections and (f) at baseline vs. 6 month. Data represented with mean ± SEM. *** $P < 0.0001$

indicating a better visual improvement in responders than nonresponders [Fig. 3a]. In responder group, the CMT level was reduced to normal value during the mandatory three injections and remained as such till the sixth injection despite not receiving the fourth and fifth injections [Fig. 3b]. In contrast, the nonresponder group received all the injections and failed to show any significant reduction in CMT [Fig. 3b] despite receiving all the injections with significant reduction in VEGF levels throughout the study following 1st injection [Fig. 4]. In the responder group, the VEGF reduced to a significant level after the 1st injection itself ($P = 0.002$) and then maintained in the same till the three mandatory injections [Fig. 4] and were not given the next two injections due to their normal CMT values [Fig. 3b]. As a result, their VEGF levels increased to the baseline value at 5 month [Fig. 4].

To further appraise the involvement of other inflammatory mediators, we evaluated the change in pattern of key pro-inflammatory mediators mostly reported to be present in DME condition and have role in maintaining the integrity of BRB as BRB breaching is a major event responsible for edema formation. The cytokines IL-6, IL-8, MCP-1, IP-10, and IL-12p70 were measured in responders and nonresponders at baseline, 2 and 5 months.

Among the 11 responders, only 6 patients had sufficient volume of aqueous humor for CBA analysis. Similarly, only 4 patients among the nonresponders had required volume of aqueous sample for analysis. The change in levels of cytokines at different time points in these two categories of patients was represented in Fig. 5.

Notably, the levels of pro-inflammatory cytokines MCP-1, IL-8 and IL-6 were found up regulated in the nonresponders [Fig. 5]. IL-8 and MCP-1 levels showed significant difference between responders (14.02 ± 7.5 for IL-8 and 339.95 ± 114.4 for MCP-1) and nonresponders (46.96 ± 29.9 for IL-8 and 799.27 ± 262.03 for MCP-1) at 5 month with $P = 0.0296$ and 0.0048, respectively [Fig. 5b and e]. Significant difference in MCP-1 level between the two groups at 2 month (387.85 ± 130.1 for responders and 747.38 ± 268.8 for nonresponders) could be seen [Fig. 5e, $P = 0.0209$]. Though IL-6 was elevated in nonresponders, but wasn't significant compared to responders. Likewise, IP-10 and IL12p70 did not show significant difference between the two groups.

Discussion

In this investigator initiated trial, apart from the conventional overall changes in CMT and BCVA assessments,[19,20] we here sequentially assessed following each anti-VEGF Injection, the VEGF levels along with CMT and BCVA to identify responders from nonresponders. These groups were further analyzed to determine the levels of cytokines leading to identification of elevated levels of IL-8 and MCP-1 which are BRB integrity modulators along with few other cytokines following ranibizumab therapy.

Altogether, anti-VEGF therapy led to VEGF reduction accompanied by significant reduction in CMT and improvement in vision [Fig. 2a-f] is in agreement with the previous anti-VEGF therapy trials.[11,21,22] However, direct sequential VEGF evaluation along with CMT and visual acuity measurements following each anti-VEGF injection were not available in other trials or studies. Our sequential systematic monitoring of VEGF levels following each injection revealed a steeper reduction from baseline value of 890 pg/mL to a level of 343 pg/mL following the first injection. This level was maintained throughout till the end of the sixth injection [Fig. 2a]. Notably, patients who were not given the fourth or fifth or both injections as per PRN protocol, the levels of VEGF showed an upward trend [Fig. 2a] in responders where the BCVA and CMT were normal indicating that sudden return or fluctuations in VEGF levels close to baseline of 890 pg/mL is not altering the BCVA and CMT indicating that VEGF levels are not the only mediators of retinal pathology with respect to CMT and BCVA.

Though the anti-VEGF therapy is effective in reducing the edema as evidenced by reduction in CMT and improving visual acuity [Fig. 2a-f], 30% patients did not respond to therapy based on the formulated criteria demonstrated by no significant

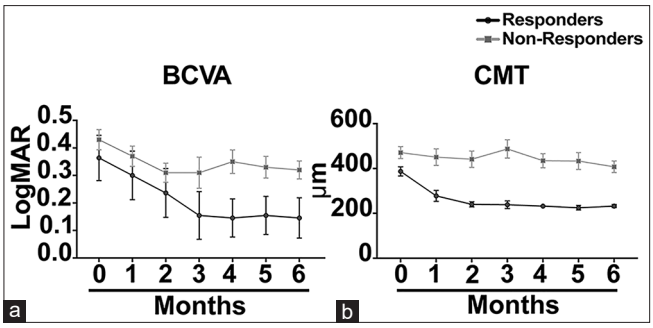

Figure 3: Comparison of change in BCVA and CMT/CRT in responder and nonresponder group. (a) Change in BCVA in responders and nonresponders. (b) Change in CMT/CRT in responders and nonresponders. Data represented with Mean ± SEM. The injections and time points were discussed in detail in methodology section

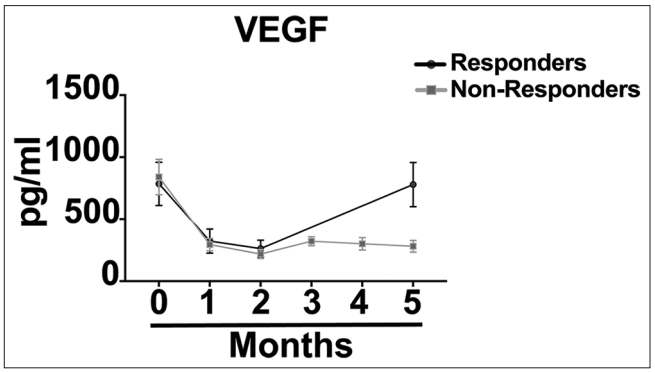

Figure 4: Comparison of change in VEGF in responder and nonresponder group. Data represented with mean ± SEM. The injections and time points were discussed in detail in methodology section

improvement in the CMT or BCVA [Fig. 3a and b]. Importantly in nonresponders, despite VEGF levels showing reduction like seen in responders, their CMT showed no significant change from the baseline value even during the first three consecutive injections and during the following injections [Fig. 3b]. Thus, existence of a group of patients without significant improvement in CMT and visual acuity despite down regulation of VEGF levels was established. [Fig. 3a & b, Fig. 4]. These observations clearly indicate that in the nonresponder group, VEGF is not the major determinant for edema formation and hence sustained intraocular reduction of VEGF by anti-VEGF without addressing the alternate cause may not improve the clinical situation of such patients.

Thus, we explored inflammatory mediators that have been shown to have an established role in the aggravating retinal pathology.[14] The role of inflammatory molecules such as IL-1β, IL-6, IL-8, IP-10, MCP-1, IL-10, IL-12, PlGF and VEGF in the pathogenesis pertaining to DR, DME and PDR have been shown in different studies.[23,24] However, clear demarcation into responders vs. nonresponders with respect to sequential VEGF monitoring following anti-VEGF therapy was absent in these studies. In our report, we systematically evaluated key cytokines MCP-1 and IL-8 along with others that are found to alter the BRB integrity. Importantly, we found that nonresponders showed statistically significant high levels of key cytokines MCP-1 and IL-8 [Fig. 5b and e].

MCP-1 regulating the migration of monocytes/macrophages in response to inflammation[25] and increased levels of MCP-1 in DME is exemplified in different studies.[26] This may be due to its ability to change the vascular permeability by altering the tight junction proteins.[27] Thus, increased MCP-1 levels found in nonresponders could attribute to the increased edematous and inflammatory condition manifested by high CMT and BCVA in these patients.

Similarly, in the context of MCP-1, significant IL-8 up regulation in nonresponders despite low VEGF levels is noteworthy. Different studies demonstrated elevated levels of IL-8 in DME patients aqueous in comparison with nonDME[26] and also the ineffectiveness of anti-VEGF treatment towards reduction in their levels.[28] IL-8 has been shown to induce loss of integrity of BRB and increased edema and CMT levels.[29] Collectively, it is clear that despite low VEGF levels, the high CMT seen in nonresponders could be attributed to the high IL-8 levels along with MCP-1 promoting the inflammation and edema.

Elevated IL-6 we observed may positively correlate with macular thickness[26,30] as IL-6 has multiple roles with respect to neuroprotection[31] as well as VEGF induction.[32] IL-6 induces production of matrix metallo-proteinases which in turn aggravate the retinal pathology by altering the BRB permeability.[33] Thus, elevated levels of IL-6 may have an implication in neuroprotection and pathology of the retina. Notably, following anti-VEGF bevacizumab injection, IL-6 levels were not found decreased in DME.[34]

Even though sample size is limited in our study, significant IL-8 and MCP-1 with increased CMT and lower BCVA values even after fifth injection with low VEGF levels is an indication of nonresponsiveness to anti-VEGF therapy for DME. Thus, elevated IL-8 and MCP-1 levels could serve as an early prognosis aqueous biomarker to identify a nonresponder. However, further analysis with more patient samples are required to consolidate our observation.

Despite anti-VEGF treatment showing vision improvement and reduction in edema following anti-VEGF therapy, 27% of patients were nonresponsive to therapy with poor visual acuity and edema compared to baseline. Interestingly, these patients showed significantMCP-1 and IL-8 up regulation with elevated IL-6 levels compared to responders. The study implies involvement of factors other than VEGF in edema formation in nonresponders. MCP-1 and IL-8 could serve as prognosis marker for nonresponsiveness to therapy and warrant alternative therapy for nonresponders for better clinical outcome.

Conclusion

Elevated MCP-1 and IL-8 levels found in patients with DME, who are nonresponders to ranibizumab could be used as a prognostic marker to identify this specific subgroup, and consider alternative therapeutic options in such patients.

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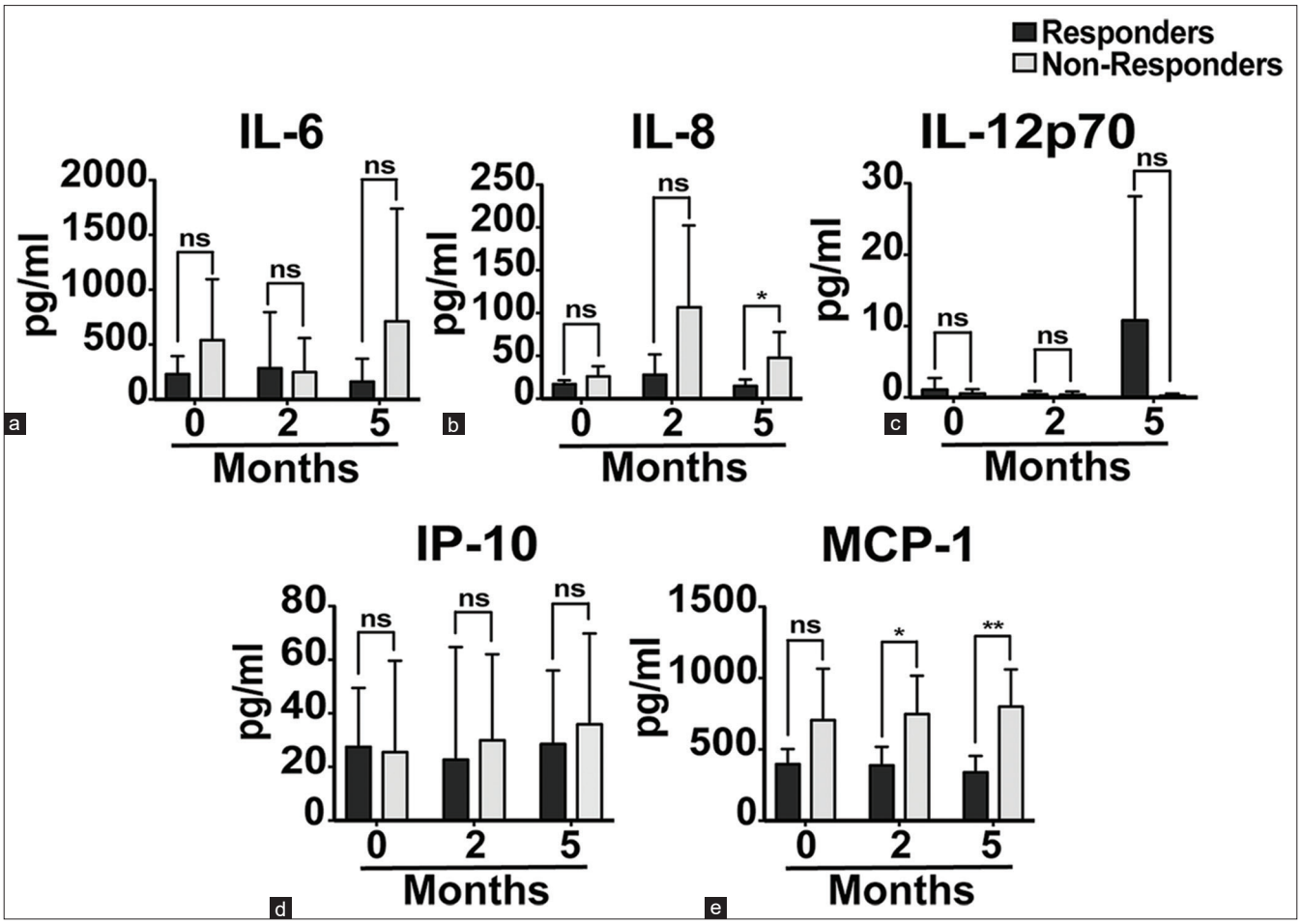

Figure 5: Cytokine levels analysis in responders and nonresponders. Comparison of cytokine levels at different time points following VEGF treatment between responders and nonresponders group (a) IL-6, (b) IL-8, (c) IL-12p70, (d) IP-10, and (e) MCP-1. Data represented with mean ± SD. Statistical analysis was done by using unpaired *t* test and */** represents that the *P* value is significant. The injections and time points were discussed in detail in methodology section

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Conflicts of interest

There are no conflicts of interest.

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