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# Effect of intravitreal bevacizumab on macular thickness: exploring serum and vitreous proangiogenic biomarkers in patients with diabetic macular edema

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**Background/aim:** This study evaluates diabetic macular-edema (DME) patients for the effect of intravitreal bevacizumab (IVB) injection on macular thickness and proangiogenic biomarkers in serum and vitreous.

**Materials and methods:** Forty DME patients were analyzed for macular thickness (MT). Twelve proangiogenic biomarkers in serum and vitreous were analyzed before and after IVB.

**Results:** Significant decrease in MT with vitreal vascular endothelial growth factor-A (VEGF-A) was observed as expected after IVB, while serum VEGF-A did not follow a decreasing trend in contrast to VEGF-C, which decreased both in serum and vitreous. Other vitreal factors like bone morphogenetic protein-9 (BMP9) and fibroblast growth factor (FGF) were also significantly decreased, while endothelial growth factor (EGF) increased following IVB. Before IVB, significant negative correlations were vitreous BMP9 with serum FGF, vitreous human growth factor (HGF) and interleukin-8 (IL-8) with serum endothelin, and vitreous and serum FGF and serum placental growth factor (PLGF) with EGF. After IVB, negative correlations in serum vs. vitreous were found for both HGF and PLGF with BMP9, and angiopoietin with FGF. Cube average thickness was negatively correlated with serum FGF and positively correlated with vitreous PLGF and endothelin.

**Conclusion:** Vascular endothelial growth factors are not the only factors that cause macular edema in diabetic patients. The effect of IVB on different proangiogenic biomarkers indicated a complex interplay of other factors in DME.

**Key words:** Bevacizumab, central macular thickness, angiogenic factors, vascular endothelial growth factor, placental growth factor

## 1. Introduction

The National Institute of Health defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (1). Later, the World Health Organization in coordination with the International Labour Organization and International Program on Chemical Safety defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (2).

Much work is being done to identify proangiogenic biomarkers in diabetes and in diabetic retinopathy. Normal vascularization requires a balance between proangiogenic and antiangiogenic factors. Disturbance of this balance is seen in many retinal diseases including diabetic retinopathy. Anti-VEGF drugs are now widely used for diabetic retinopathy to combat this imbalance.

In the current study, the following angiogenic biomarkers were analyzed: angiopoietin 2, human growth factor (HGF), endothelial growth factor (EGF), fibroblast growth factor (FGF), placental growth factor (PLGF), vascular endothelial growth factor-A and -C (VEGF-A and VEGF-C), bone morphogenetic protein 9 (BMP9), follistatin, leptin, cytokines, interleukin-8 (IL-8), and angiogenic peptide (endothelin). Moreover, the effect of a single injection of intravitreal bevacizumab (IVB) on these factors was also studied in patients with diabetic macular edema (DME).

BMP9 is a special type of transforming growth factor (TGF)-β. It plays an important role in the development of normal vasculature and is seen to be strongly activated in blood vessels of diabetic patients (3,4). FGF is another proangiogenic factor, which is raised in serum of patients with diabetic retinopathy (5). It causes an increase in the vascular permeability, which results in DME (6). The VEGF

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family is composed of six isoforms: A, B, C, D, E, and PLGF. Human hepatic growth factor is also a proangiogenic biomarker and increases vascular permeability. Likewise, other proangiogenic factors have roles in macular edema.

With the advent of the era of intravitreal injections of anti-VEGF agents, it is observed that there are certain patients who do not respond to anti-VEGF therapies. It has led to the hypothesis that there are some other angiogenic factors that also play a role in causing macular edema. There must be a complex interplay of these factors with interrelated biochemical pathways, which makes treatment of DME a difficult challenge. This study was done to identify such proangiogenic factors.

## 2. Materials and methods

An interventional, time-series study was designed, in which 40 patients (M = 24, F = 16) with DME were selected from a tertiary care hospital of Lahore, Pakistan. The current study was conducted on the basis of Declaration of Helsinki principles and was approved by institutional review board of the hospital.

### 2.1. Study period and design

This was a prospective, interventional time-series study, conducted from January 2016 to December 2016.

### 2.2. Exclusion and inclusion criteria

Patients with type 2 diabetes having DME were included in the study. The exclusion criteria were: patients with type 1 diabetes, patients with any other ocular or systemic disease, elevated blood pressure, evidence of vitreoretinal interface abnormality on spectral domain-optical coherence tomography (SD-OCT), and intravitreal corticosteroids or anti-VEGF A agents, laser photocoagulation, or intraocular surgery during the previous 6 months.

### 2.3. Outcome measures

The primary outcome measure was to find out the values of serum and vitreous proangiogenic markers and central macular thickness before and after IVB. The secondary outcome was to determine correlations among these biomarkers in vitreous and serum of patients with DME.

### 2.4. Methods

Clinical history, including ocular as well as systemic history, was taken. A consent form and the patient's proforma were duly filled out prior to examination. Ocular examination included distance and near visual acuity, pupillary reactions to light and accommodation, slit-lamp biomicroscopy, and tonometry. DME was diagnosed with the help of +78D lens indirect ophthalmoscopy and macular thickness was measured using SD-OCT (Carl Zeiss, USA, model 4000). In macular thickness parameters, central subfield thickness and cube average thickness (CAT) were taken into account. On the next day of OCT, 5 mL of blood and 0.1–0.2 mL of vitreous samples were drawn by using needle aspiration under strict aseptic conditions and bevacizumab (1.25 mg/0.05 mL) was injected into the vitreous cavity. All these procedures were done on the same day. Vitreous sampling and intravitreal injections were done using separate needles. Patients were given topical antibiotics four times a day. OCT was repeated after 4 weeks and blood and vitreous samples were drawn again. IVB injection was repeated in the patients with residual macular thickening and the patients who did not require another injection were excluded from the study. Serum was separated from all blood samples and all the samples were stored at –20 °C in properly labeled vials after adding 1X protease inhibitor to reduce the protein degradation. The patient proforma and consent forms were duly filled out prior to sampling.

Twelve analytes, angiopoietin 2, leptin, BMP9, IL-8, HGF, EGF, FGF, PLGF, endothelin, follistatin, VEGF-A, and VEGF-C, were studied. The xMAP flow cytometry technique was used to analyze these analytes. The Bio-Plex multiplex immunoassay system was adopted, which utilizes xMAP technology licensed from Luminex to permit the multiplexing of up to 100 different assays within a single sample. A human angiogenesis/growth factor magnetic bead panel kit (catalog # HAGP1MAG-12K) was used for the current study. Flow cytometry is the simultaneous measurement of multiple physical characteristics of a single cell as the cell flows in suspension through a measuring device. It measures the optical and fluorescence characteristics of a single cell.

### 2.5. Statistical analysis

The statistical analysis of all the analytes was conducted by Mann–Whitney U test. Correlation analyses were done to estimate predicted associations among different factors, using Spearman's rank correlation. All statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). The significance level was set at $P < 0.05$.

## 3. Results

The average age of the patients was 55 years (39–75 years). There were 60% males and 40% females having 58% right and 42% left therapeutic eyes. All the study eyes were phakic. Effects of IVB on macular parameters are shown in Table 1. Serum endothelin ($P = 0.003$) and VEGF-C ($P = 0.006$) were significantly decreased after IVB (Figures 1A and 1B), while serum PLGF ($P = 0.034$) and serum FGF ($P = 0.000$) were significantly raised after IVB (Figures 1A and 1B). Vitreous BMP9, FGF, VEGF-A, and VEGF-C were significantly decreased ($P = 0.01$, 0.04, 0.00, and 0.00, respectively) and vitreous EGF levels were increased ($P = 0.00$) after IVB (Figures 2A–2C).

Spearman's correlation before IVB therapy showed a negative correlation of vitreous BMP9 with serum FGF ($r = –0.515$, $P = 0.029$), serum PLGF ($r = –0.499$, $P = 0.035$)

**Table 1.** Macular thickness parameters before and after IVB in the therapeutic eyes.

| OCT parameters | OCT therapeutic eye | | |
|---|---|---|---|
| | Before therapy | After therapy | P-value |
| Central macular subfield thickness (μm) | 410.13 ± 24.25 | 299.55 ± 15.99 | <0.01 |
| Macular cube volume ($mm^3$) | 12.81 ± .36 | 11.39 ± 0.26 | <0.01 |
| Macular cube average thickness (μm) | 355.69 ± 10.35 | 304.69 ± 9.03 | <0.01 |

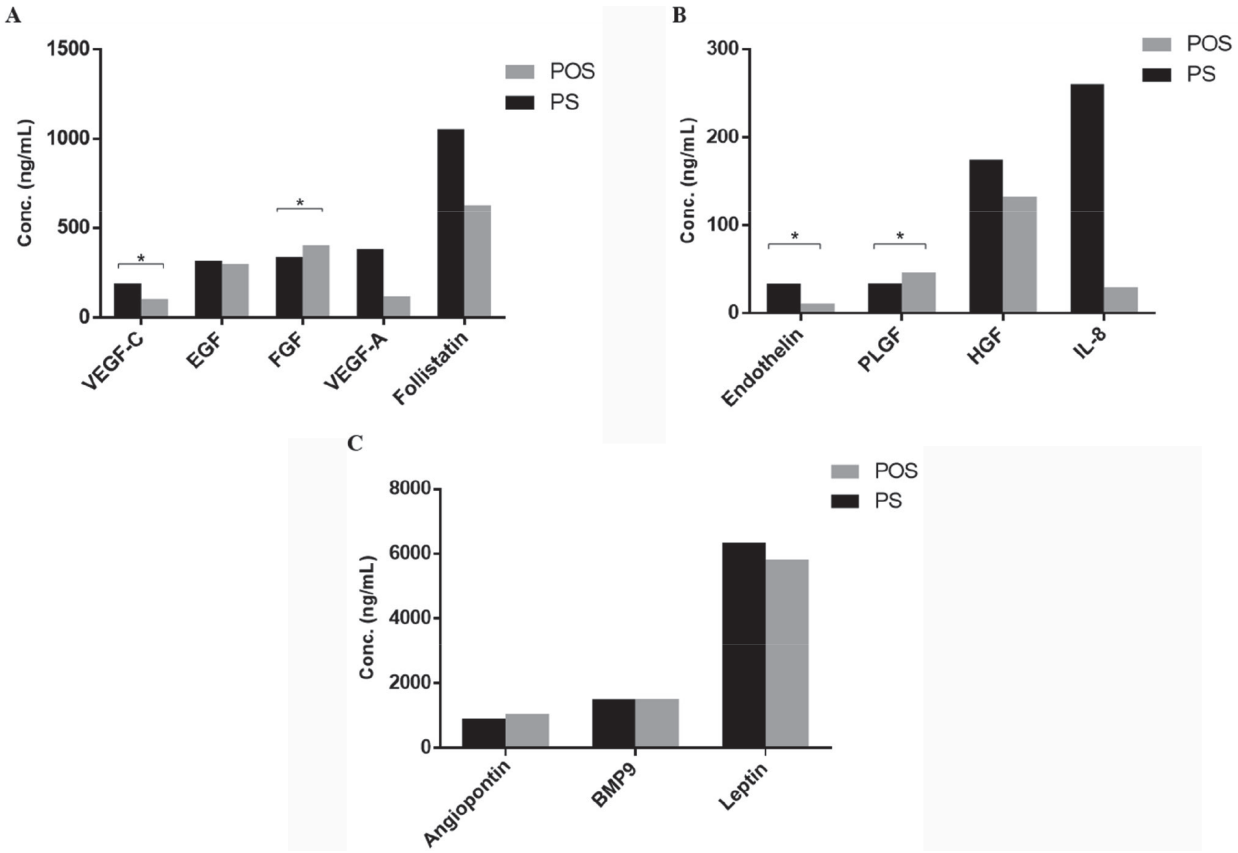


**Figure 1.** A, B, C) Effects of IVB on different proangiogenic factors. PS = Preinjection serum values, POS = postinjection serum levels.

and serum EGF (r = –0.553, P = 0.017). Serum endothelin had a negative correlation with vitreous HGF (r = –0.598, P = 0.009) and vitreous IL-8 (r = –0.515, P = 0.029). Serum FGF was negatively correlated with vitreous FGF (r = –0.598, P = 0.009), as shown in Table 2.

After IVB therapy, serum BMP9 was positively correlated with vitreous endothelin (r = 0.618, P = 0.014). Serum HGF was negatively correlated with vitreous EGF (r = –0.802, P = 0.030). Serum PLGF was negatively correlated with vitreous BMP9 (r = –0.528, P = 0.043). Serum angiopoietin 2 was negatively related with vitreous FGF (r = –0.802, P = 0.03) and positively correlated with VEGF-C (r = 0.762, P = 0.028), as shown in Table 2.

Spearman's correlation was also calculated between macular CAT (μm) and 12 proangiogenic factors before and after IVB therapy. Before IVB therapy, serum leptin was negatively correlated with cube volume (CV) (r = –0.746) and CAT (r = –0.0743). However, after IVB therapy, serum FGF was negatively correlated with CAT (r = –0.540, P = 0.021). Vitreous PLGF and vitreous endothelin were positively correlated with CAT (r = –0.546, P = 0.035 and r = –0.588, P = 0.021, respectively) (Table 2).

## 4. Discussion

This particular study showed that VEGF-A is not the only factor affected by IVB; there are other angiogenic

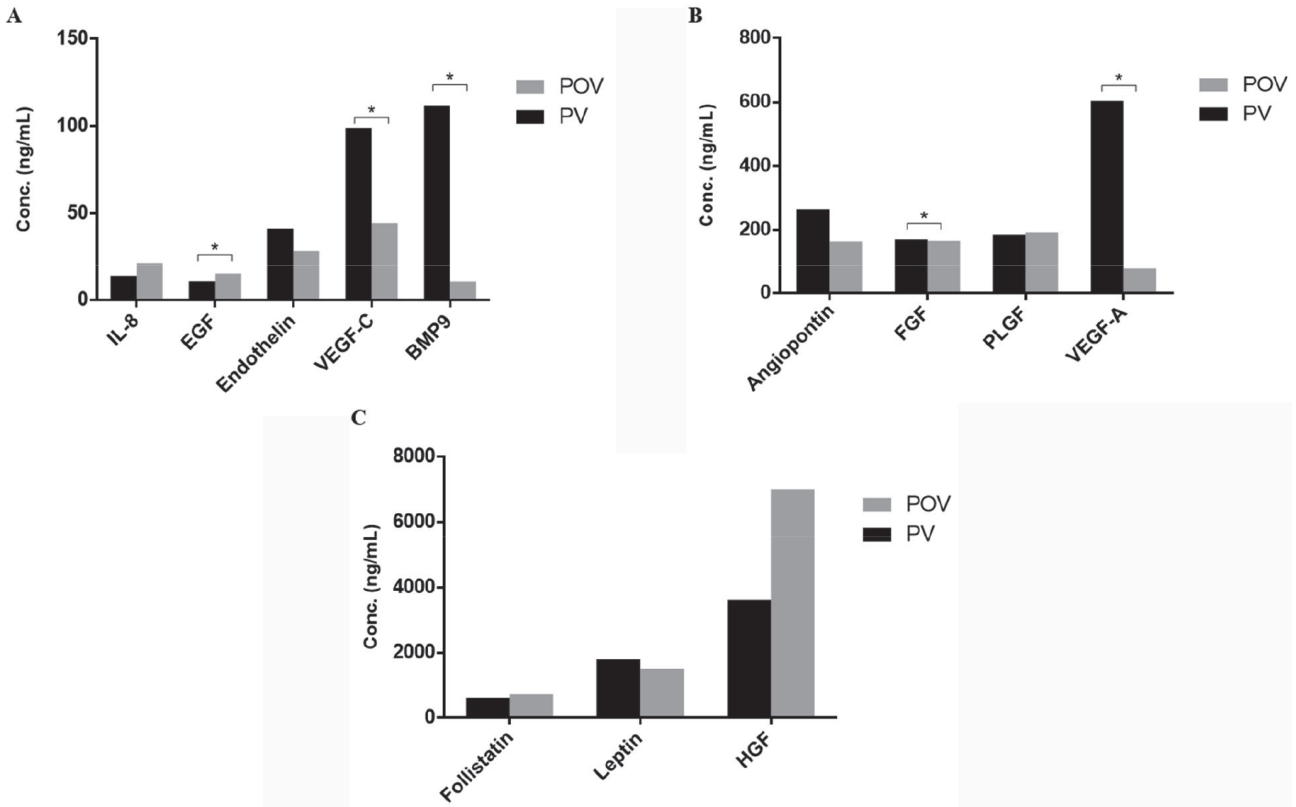


**Figure 2.** A, B, C) Effects of IVB on different proangiogenic factors. PV = Preinjection vitreous values, POV = postinjection vitreous levels.

**Table 2.** Correlations among different proangiogenic factors and cube average thickness.

| Name | Factors correlated with (P < 0.05) |
|---|---|
| Before therapy | |
| Serum Endothelin | Vitreous IL-8 (–0.515) and HGF (0.598**) |
| Serum FGF | Vitreous FGF (0.598**) |
| Vitreous BMP9 | Serum EGF (–0.553), PLGF (–0.499), and FGF (–0.515) |
| Serum leptin | CV (–0.746), CAT (–0.0743) |
| After therapy | |
| Serum BMP9 | Vitreous endothelin (0.618) |
| Serum HGF | Vitreous EGF (–0.802) |
| Serum PLGF | Vitreous BMP9 (–0.528) |
| Serum Angiopoietin | Vitreous FGF (–0.802) and VEGF-C (0.762) |
| CAT | Serum FGF (–0.540), vitreous PLGF (0.546), and endothelin (0.588) |
| | Parentheses contain value of correlation coefficient, ** = highly significant at <0.01 level. |

biomarkers that are also significantly affected by IVB. These factors are separately addressed in the following discussion.

Vitreous BMP9 was significantly decreased after IVB (P = 0.01), indicating some common pathway in causing DME. We did not find any reference in the literature that could show the effect of IVB on vitreous BMP9. After IVB, FGF was significantly reduced in vitreous fluid of DME patients, but its level was significantly raised in serum. This paradoxical effect of IVB on serum FGF could be due to

uncontrolled diabetes, but the vitreous FGF was decreased due to the local effect of IVB in the vitreous. Although the literature shows that by injecting intravitreal FGF inhibitor (dobesilate), there was rapid reduction in macular edema (7), our study showed that IVB also results in a decrease in vitreous FGF levels, which in turn influences macular thickness. Some studies have shown that FGF is involved in the production of VEGF (8). Thus, its action is direct as well as indirect through activation of VEGF.

The effect of growth factors on diabetic retinopathy is not a new concept. Earlier, it was seen that diabetic retinopathy progression stopped after pituitary ablation. Similarly, it was also observed that there was an increase in diabetic retinopathy at the start of puberty, indicating a role of growth factors in diabetic retinopathy (9–12). We analyzed VEGF-A and -C in this particular study. Values of both factors were significantly reduced in the vitreous after IVB but the effect on serum VEGF-A was not significant. Prior studies have shown that after IVB serum VEGF-A was considerably reduced and the effect was also seen on the other eye, with a significant decrease in macular edema of the other eye (13).

PLGF has structural similarity with VEGF-A (14). In prior research, PLGF when injected in rat eyes induced retinal pigment epithelium (RPE) tight junction abnormalities and hence retinal edema (15). It acts by activating vascular endothelial growth factor receptor (VEGFR-1) (16). This common pathway and structural similarity with VEGF-A may explain the significantly decreased levels of PLGF in serum after IVB. However, vitreous PLGF was raised after IVB. This might be caused by rebound increase in the vitreous PLGF due to the anti-VEGF effect of IVB. Contrary to this, there were some previous studies that showed that when PLGF was injected into rat and mouse vitreous, there was no effect on retinal vessels, suggesting that PLGF had no clear proangiogenic activity (17). Cao et al. found that VEGF and PLGF affect pericytes through the ERK signaling pathway mediated by VEGFR-1, resulting in breakdown of the inner blood retinal barrier (18,19).

EGF has limited proangiogenic activity when compared with FGF but they act synergistically (20). This was supported by our study, where no considerable effect was seen on serum EGF after injecting IVB.

In this particular study no significant effect of IVB was seen on the serum and vitreous HGF in patients with DME, indicating different pathways for its action. Earlier research showed that vitreous HGF was raised independently of serum HGF levels (21,22).

Among inflammatory cytokines, IL-8 is produced by endothelial cells and glial cells of the retina in response to ischemia (23). Its levels are also increased in diabetic retinopathy patients as compared to normal controls (24,25). In another study it was found that IL-8 elevation occurred as a consequence of VEGF expression (26). Contrary to that, no significant effect of IVB on serum and vitreous IL-8 was seen in our study, indicating different pathways for its activity.

Reduced retinal perfusion is related with upregulation of endothelin (27). Studies have shown that increased endothelin was associated with reduced total retinal blood flow in patients with diabetes (28). Endothelin is raised in early diabetic retinopathy but it plays no role in proliferative diabetic retinopathy and DME. It acts as a vasoconstrictor and results in ischemia of the retina. In this study, vitreous endothelin was not affected after IVB, but serum endothelin was significantly reduced.

RPE contains receptors for leptin, which has proangiogenic activity and is seen in higher concentrations in the serum of diabetic patients (29–31). No effect of IVB on vitreous and serum leptin of patients with DME in our study indicated separate proangiogenic pathways for leptin in DME.

Follistatin is a protein that is high in the plasma of diabetic patients (32). However, in this particular study, no significant effect of IVB was seen on the serum and vitreous of patients with DME.

Angiopoietin 1 and 2 are natural antagonists of each other. Angiopoietin 1 has antipermeability activity (33,34). Their levels depend on the stage of diabetic retinopathy. Angiopoietin 2 increases the body's response to VEGF and results in increased vascular permeability. Angiopoietin 2 in the serum and vitreous of our patients was independent of IVB.

In this study, the effect of IVB on proangiogenic factors indicates that there must be certain signaling pathways that overlap in the mechanism of action of different vascular endothelial growth factors and inflammatory biomarkers.

A similar correlation study was conducted after intravitreal implanting of dexamethasone and a complex relationship was found among three angiogenic factors: angiopoietin 2, VEGF, and HGF (35). Although new treatment modalities are directed towards specific VEGF receptors, further research is needed to target other proangiogenic factors in DME.

In conclusion, the correlations among different factors indicated that there exists a complex interplay of proangiogenic factors and there is a multifactorial molecular mechanism that controls vascular permeability of the retina in patients with DME. These factors do not act independently of each other, making management of DME a difficult and complicated procedure. This further explains why there are certain patients who do not respond to IVB whereas response in other patients is quite promising. Further research is needed to find out the role of these agents, which will help in defining new strategies for treatment of macular edema.

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