Elevated Levels of Serum IL-17A Secondary to Repeated Intravitreal Injections of Aflibercept in Treatment-Naive Patients with Neovascular Age-Related Macular Degeneration

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Abstract: We evaluated the effect of three monthly intravitreal injections of aflibercept on the serum concentration of interleukin 17A (IL-17A), monocyte chemoattractant protein 1 (MCP-1/CCL2), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in treatment-naive patients with neovascular age-related macular degeneration (nAMD). Twenty-two eyes of twenty-two patients with nAMD scheduled for the initial loading phase of intravitreal aflibercept (2 mg/0.05 mL) were included. Serum VEGF, PlGF, MCP-1/CCL2 and IL-17A levels were determined four times in each individual—just before the first injection, 2–3 days after the first injection, just before the third injection, and then 2–3 days after the third aflibercept injection. A statistically significant difference was found between the serum PlGF and IL-17A levels measured before the first injection and after the initial loading phase, with a mean value (MV) of 139.088 pg/mL vs. 151.233 pg/mL (p = 0.016) for IL-17A, respectively. There were no statistically significant differences for VEGF and MCP-1/CCL2 between any of the compared measurements. We reveal that repeated injections of aflibercept promote an increase in serum IL-17A concentration, which may lead to a systemic inflammatory response mediated by IL-17A, but not by MCP-1.

Keywords: aflibercept; interleukin-17; IL-17; monocyte chemoattractant protein 1; MCP-1/CCL2; vascular endothelial growth factor; VEGF; placental growth factor; PlGF; age-related macular degeneration; AMD

1. Introduction

Age-related macular degeneration (AMD) is a chronic neurodegenerative disease leading to loss of central vision due to the progressive degeneration of photoreceptor cells located in the central point of the retina—macula [1]. In developed countries, AMD is a major cause of central vision loss among the elderly population [2]. There are two main types of age-related macular degeneration—dry and wet (nAMD). AMD typically begins as a dry form, and then, in 10% of the affected population, transforms into a wet form (nAMD) [1], which is characterized by the presence of choroidal neovascularization (CNV)—abnormal and excessive growth of choroid vessels in the submacular area [2,3]. Despite the
high interest of researchers, the pathophysiology of AMD has not been fully understood; however, it is assumed that inflammation and abnormal immune regulation are focal factors leading to the development of AMD [1].

Inflammation is the body’s defense response precisely coordinated by complex immune processes. Inflammatory response can be initiated by non-infectious agents (e.g., cell damage, toxins and drugs) or infectious agents (e.g., bacteria and viruses) [4]. Acute inflammation usually leads to rapid neutralization of the causative agent; however, in the case of abnormalities in control mechanisms, it can become a chronic form and promote the development of atherosclerosis and neurodegenerative diseases [4,5], such as Parkinson disease, Alzheimer disease and AMD [2]. Previous studies have shown that macrophages and leukocytes—key cells for the inflammatory response—occur in 90–95% of removed CNV tissue [3]. It has been proven that monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and interleukin 17A (IL-17A) can participate in the development of nAMD by both regulating the inflammatory response and stimulating angiogenesis [6,7]. Vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are pivotal factors that stimulate angiogenesis and lead to the development of CNV; in addition, their ability to promote inflammation has also been proven [8,9].

Aflibercept—one of the newest approved monoclonal antibodies for the treatment of nAMD—is capable of binding the VEGF-A, VEGF-B and PlGF isoforms [10]. In vitro studies with vascular endothelial cells have shown that aflibercept, after binding to VEGF-A, can initiate an inflammatory response by the stimulation of toll-like receptor 4 (TLR 4), with subsequent activation of the nuclear factor kappa B (NF-kB) pathway [6]; meanwhile, in vivo studies have shown a decrease in the concentration of MCP-1 and other proinflammatory and proangiogenic factors in aqueous humor after intravitreal aflibercept treatment [11,12]. However, systemic levels of MCP-1 and IL-17A after intravitreal injection of aflibercept have not yet been studied. Therefore, the purpose of our study was to assess the effect of repeated intravitreal aflibercept injections on the systemic inflammatory response mediated by MCP-1 and IL-17A, as well as the concentration of VEGF and PlGF in the blood serum of patients with nAMD.

2. Materials and Methods

2.1. Subjects

The study was conducted at the Department of Ophthalmology, Collegium Medicum, Nicolaus Copernicus University in Bydgoszcz, Poland. Each participant was informed about the rules of the experiment and gave written consent to participate in the study. Our study was designed and conducted in accordance with the principles of the Helsinki Declaration, and then received a positive opinion from the Local Bioethics Committee. Twenty-two eyes of twenty-two treatment-naive patients with nAMD scheduled to be given the initial loading phase of intravitreal aflibercept were included in our study. Exclusion criteria included baseline elevated serum C-reactive protein (CRP) levels, cardiovascular disease, uncontrolled hypertension, history of stroke or myocardial infarction, chronic liver and kidney dysfunction, nicotinism, alcoholism, and chronic use of anti-inflammatory drugs (NSAIDs or steroids). Demographic characteristics of the participants are presented in Table 1. During our experiment, intravitreal aflibercept injections were well tolerated and there were no local or systemic side effects in any of the qualified patients.
Table 1. Characteristics of included patients.

| N (Eyes) | Gender | Age (Years) | Baseline Visual Acuity (BVA) | Final Visual Acuity (FVA) |
|----------|--------|-------------|-----------------------------|--------------------------|
| 22       | 13F/9M | 77.95       | SE 1.644                    | SE 0.2399                |

|       | SE     | MV         | SE                | Mean LogMAR | SE     | Mean LogMAR |
|-------|--------|------------|-------------------|-------------|--------|-------------|
| 1.644 | F:78.69| M:76.89    | 0.2944            | 0.040       | 0.2399 |

*—measured with the use of Snellen eye test charts and converted to LogMAR; a—before the first IVA injection; b—after the third IVA injection; c—comparison of the baseline and final visual acuity of individuals in the study group.

Abbreviations: IVA—intravitreal aflibercept; F—female; M—male; MV—mean value; SE—standard error; LogMAR—logarithm of the minimal angle of resolution.

2.2. Study Design

This was a prospective longitudinal clinical study. Prior to the first intravitreal injection of aflibercept, serum CRP levels were determined in each participant to exclude the effect of primary inflammation processes on the results obtained. Due to abnormally high serum CRP levels, three of the initially qualified twenty-five individuals did not meet the inclusion criteria. Before the first administration of aflibercept, each of the enrolled individuals was subjected to an ophthalmological examination, including a visual acuity test using Snellen charts, intraocular pressure (IOP) measurement and anterior eye segment assessment in a slit-lamp, as well as an examination of the posterior segment of the eye using spectral domain optical coherence tomography (SD-OCT) and fluorescein angiography (FA). Patients received three monthly intravitreal injections of aflibercept (Eylea, Bayer, HealthCare, Berlin, Germany) at a dose of 2 mg/0.05 mL. Serum VEGF, PIGF, MCP-1 and IL-17A levels were determined four times in each individual—just before the first injection, 2–3 days after the first injection, just before the third injection, and then 2–3 days after the third aflibercept injection. Intravitreal aflibercept injections were performed under sterile conditions in the operating room. After puncturing a cubital vein, blood was collected in sterile biochemical tubes. Within 30 min of collection in tubes, the obtained blood samples were centrifuged at 2000–3000 rpm for 20 min, then the supernatant was frozen at −80 °C until specific ELISA determinations were carried out for the each tested parameter using dedicated kits produced by Shanghai Sunred Biological Technology Co., Ltd. (Shanghai, China).

2.3. Statistical Analysis

Statistical analysis was performed using the STATISTICA v.7.1 software (StatSoft Inc., Tulsa, OK, USA). The Shapiro–Wilk test showed normal distribution of the variables. The differences between the continuous normally distributed variables were analyzed by the by one-way analysis of variance (ANOVA) with post-hoc analysis using Tukey’s honestly significant difference (HSD) test. Pearson’s correlation coefficient (r) was used to examine the dependencies between select continuous variables. The results are presented as mean values (MV) and standard error (SE). p value < 0.05 was considered as statistically significant.

3. Results

The results of our study are presented in Table 2.
Table 2. Results of determinations of PIGF, VEGF, MCP-1/CCL2 and IL-17 concentrations in tested blood samples.

| Pairs of Compared Measurements | Determined Parameter |
|-------------------------------|---------------------|
|                              | VEGF-A              | PLGF               | MCP-1/CCL2          | IL-17A              |
| MV (pg/mL)                    | SE                  | p Value            | MV (ng/L)           | SE                  | p Value            | MV (pg/mL) | SE      | p Value |
| 1 vs. 2                       | 271.819             | 38.721             | 0.086              | 440.884             | 101.786             | 0.151             | 349.082  | 39.306  | 0.797   | 139.088  | 53.938  | 0.653   |
| 1 vs. 3                       | 271.819             | 38.721             | 0.969              | 356.953             | 305.707             | 0.216              | 349.082  | 39.306  | 0.461   | 139.088  | 53.938  | 0.888   |
| 1 vs. 4                       | 271.819             | 38.721             | 0.320              | 440.884             | 101.786             | 0.023              | 349.082  | 39.306  | 0.428   | 139.088  | 53.938  | 0.016   |
| 2 vs. 3                       | 264.608             | 38.411             | 0.722              | 360.349             | 105.293             | 0.996              | 366.747  | 46.699  | 0.694   | 146.329  | 55.155  | 0.971   |
| 2 vs. 4                       | 270.760             | 35.376             | 0.348              | 356.953             | 103.707             | 0.839              | 366.747  | 46.699  | 0.683   | 146.329  | 55.155  | 0.216   |
| 3 vs. 4                       | 270.760             | 35.376             | 0.747              | 356.953             | 103.707             | 0.737              | 386.029  | 48.258  | 0.987   | 143.556  | 52.383  | 0.091   |

MV—mean value; SE—standard error; VEGF—vascular endothelial growth factor; PIGF—placental growth factor; MCP-1/CCL2—monocyte chemoattractant protein 1/chemokine (C-C motif) ligand 2; IL-17A—interleukin 17A—IVA, intravitreal aflibercept. *Measurement: 1—just before the first IVA injection; 2—2–3 days after the first IVA injection; 3—immediately before the third IVA injection; 4—2–3 days after the third IVA injection.

3.1. VEGF

At baseline, the mean VEGF serum concentration was 271.819 pg/mL. There was no significant difference between the values measured at baseline and during follow up. No correlation was found between the initial VEGF concentration and the age of the patients (r = 0.009; p value = 0.962), nor the baseline visual acuity (BVA) (r = −0.297; p value = 0.180); however, we found a strong positive correlation between the VEGF and PIGF (r = 0.835; p value < 0.001) serum concentrations, as well as a moderate positive correlation with the IL-17A (r = 0.752; p value < 0.001) concentration at baseline. Moreover, after the initial loading phase, the PIGF and IL-17A levels positively correlated, to a moderate extent, with the VEGF serum concentration (r = 0.762; p value < 0.001 for PIGF, as well as r = 0.703; p value < 0.001 for IL-17A, respectively).

3.2. PLGF

In subsequent measurements, we observed a downward trend in PIGF serum concentration of 440.884, 360.349, 356.953, and 302.151 ng/mL, respectively. A statistically significant difference was found between the serum PIGF levels before the first injection and after the initial intravitreal aflibercept (IVA) loading phase (p value = 0.023). There were no statistically significant differences between the PIGF levels at other compared study time points (Figure 1). Similarly, no correlation was found between the age of the patients (r = 0.209; p value = 0.351), or the BVA (r = −0.274; p value = 0.217) and the initial serum PIGF concentration.

3.3. MCP1/CCL2

In subsequent measurements, the concentration of MCP-1/CCL2 increased; however, there were no statistically significant differences between the compared measurements. Analysis of the correlation showed no relationship between the initial serum concentration of MCP-1/CCL2 and the age of the patients (r = −0.066; p value = 0.774) or the BVA (r = 0.356; p value = 0.104). Serum MCP-1/CCL2 concentration did not correlate significantly with the concentrations of other parameters at the baseline and after the initial loading phase.
3.4. IL-17A

Our results demonstrated that the IL-17A level was elevated by IVA treatment—we found a statistically significant difference between the initial and final measurements of the IL-17A concentration ($p$ value = 0.016). There were no significant differences in IL-17A concentration in the other pairs of compared results (Figure 2). Initial IL-17A levels did not correlate with the age ($r = 0.375; p$ value = 0.085) or the BVA ($r = -0.078; p$ value = 0.730) of the patients studied. A moderately positive correlation was found between both the initial and final IL-17A and PlGF concentrations ($r = 0.662; p$ value < 0.001, as well as $r = 0.605; p$ value = 0.003, respectively).
4. Discussion

Aflibercept, also known as “VEGF-Trap Eye”, is a human recombinant glycoprotein designed by combining portions of VEGF receptor (VEGFR) and human IgG1 Fc region \[10\]. Due to its characteristic molecular structure, aflibercept has the ability to bind VEGF-A, VEGF-B, PlGF-1, and PlGF-2, whereas the other three anti-VEGF agents approved for the treatment of nAMD—ranibizumab, bevacizumab and brolucizumab—can only bind VEGF-A \[13,14\]. It has been proven that repeated intravitreal injections of aflibercept can lead to its accumulation in serum. Avery et al. revealed that the maximum serum concentration \(C_{\text{max}}\) of aflibercept was 0.45 nM after the first dose of 2 mg/0.05 mL, and then after the third monthly injection \(C_{\text{max}}\) this increased to 0.58 nM \[15\]. Two main routes for drug entry into systemic circulation after intravitreal injection are known. The first pathway is based on the mechanism of the diffusion of drug molecules into the anterior chamber of the eye and the outflow, along with the aqueous humor, further into the systemic circulation \[16\]; the second pathway involves the interaction between drug molecules with fragments of Fc molecules in their structure and Fc receptors located in the blood–retina barrier (BRB). According to current knowledge, this interaction is responsible for the penetration into the systemic circulation of aflibercept and bevacizumab, anti-VEGF agents with an Fc domain fragment in their structure, as well as a much smaller systemic exposure of intravitreal ranibizumab lacking the Fc domain in its structure \[8\].

VEGF-A, along with PlGF, belongs to the platelet-derived growth factor (PDGF) supergene family \[10\]. Pathological conditions, such as inflammation, hypoxia, ischemia and injury, result in increased VEGF expression \[6,10\]. It has been proven that both VEGF and PlGF are key factors regulating the process of abnormal angiogenesis, and also play an important role in inflammatory \[8\] and autoimmune \[9\] processes—these three components are suspected of being the main contribution to the still not fully understood pathophysiology of nAMD \[1\]; hence, the current therapeutic strategies for nAMD are based on monoclonal antibodies inactivating VEGF-A (bevacizumab, ranibizumab) or VEGF-A, VEGF-B and PlGF (aflibercept) \[15\]. Our study showed a significant decrease in systemic PlGF levels after the third monthly intravitreal administration of aflibercept in patients with nAMD, compared to the serum PlGF levels determined before the first injection. Zehetner et al. reported a counterregulatory increase in systemic PlGF, but not VEGF-A, one week and four weeks after a single intravitreal injection of aflibercept \[17\]. An increase in systemic PlGF after a week, but not after four weeks, associated with only a single intravitreal injection of aflibercept, was observed by Sugimoto et al. in patients with diabetic macular edema (DME) \[18\]. Interestingly, in our study, negligible changes in serum VEGF-A levels were observed. Contrary to our results, in previous studies a significant decrease in systemic VEGF levels measured after 3 h \[15,19\], 1 day \[20\] or 7 days \[21–23\] was observed, which persisted over one month after intravitreal aflibercept administration \[15,19–23\]. In studies that also evaluated the effect of three monthly aflibercept injections on systemic VEGF levels, the results were comparable with those obtained after the first injection \[15,19\]. We suggest that further studies with longer monitoring of serum VEGF-A concentration are necessary to fully assess the possibility of the occurrence of long-term VEGF-A-mediated pro-angiogenic response after the initial loading phase of intravitreal aflibercept.

Among the IL-17 family, six specific cytokines have been distinguished—IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, respectively. These cytokines affect target cells through specific receptors located on the surface of the cell membrane \[24\]. IL-17A is mainly produced by T\(_{h}\)17 cells and is a well-known factor mediating the inflammatory response in inflammatory and autoimmune diseases, and also in AMD \[7\]. In addition, IL-17A may co-stimulate the angiogenic effect of VEGF \[7\]. Furthermore, results of animal studies on laser-induced CNV have shown that IL-17A has the ability to stimulate neovascularization in choroid tissue by a mechanism independent of VEGF \[7,25\]. MCP-1/CCL2 is a protein molecule belonging to the CC chemokine family and can be produced by a variety of cell types, including fibroblasts, lymphocytes, macrophages and vascular endothelial cells \[26\]. MCP-1/CCL2 is an important factor in both acute inflammatory response by recruiting monocytes to the site of inflammation and chronic inflammatory response by regulating the penetration
of monocytes and macrophages into target tissues [27]. Wojtkowska et al. revealed that IL-17A stimulation of human brain epithelial cells is associated with an increase in Th17 cell adherence, as well as an increase in MCP-1/CCL2 expression, leading to an increase in blood–brain barrier (BBB) permeability for inflammatory cells [28]. In this study, we observed a significant increase in serum IL-17A concentration in patients with nAMD after the initial loading phase of IVA, compared to the initial measurement. There were no significant differences between any of the MCP-1/CCL2 concentration measurements. The study conducted by Arnott et al. on vascular endothelial cell culture has shown that aflibercept, after binding to VEGF-A, can promote an inflammatory response by the stimulation of TLR 4 with subsequent activation of the NF-κB signaling pathway [6], while other studies revealed a decrease in the concentration of MCP-1 and other proinflammatory and proangiogenic cytokines in aqueous humor after intravitreal administration of aflibercept [11,12]. To the best of our knowledge, our study is the first in which the effect of intravitreal anti-VEGF drugs on systemic levels of IL-17A and MCP-1/CCL2 was evaluated.

The results of previous studies suggest that our findings, showing a significant increase in IL-17A concentration, secondary to repeated intravitreal injections of aflibercept, may have several important therapeutic implications associated with anti-VEGF treatment. Sigurdardottir et al. showed that retinal cells, including retinal vascular endothelial cells, photoreceptors and Muller cells, have the ability to express both IL-17A receptor components—IL-17RA and IL-17RC. In addition, an increase in retinal vascular permeability was correlated with elevated IL-17A concentration. Interestingly, it was observed that IL-17A systemic ablation in mice was associated with significant changes in retinal tissue concentration of factors that are strongly involved in the development of retinal degenerative changes—reduction in MCP-1/CCL2, CCL5, CXCL1, CX3CL1 and CXCL5, as well as an increase in TIMP-1 [29]. Furthermore, animal studies using Akita mice with induced diabetic retinopathy have shown that intravitreally injected IL-17A causes activation of the Act1 signaling pathway and subsequent IL-17A dose-dependent Muller cell dysfunction and promotion of retinal ganglion cell apoptosis, as well as both increased leukocyte adherence and retinal vascular permeability, which leads to enhanced BRB breakdown [30]. It was also proven that an increase in IL-17A leads to the stimulation of Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT) signaling and, as a result, increased VEGF expression, which can accelerate CNV progression [31]. In vitro studies with tumor models have shown that IL-17A may be responsible for resistance to anti-VEGF antibody therapy in a VEGF-independent mechanism by stimulating the NF-κB and extracellular signal-regulated kinase (ERK) pathways, resulting in increased expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), a factor involved in inflammation and tumorigenesis [32,33] (Figure 3). Thus, immunomodulation of the paracrine function of Th17 cells should be investigated as a possible factor influencing the efficacy of intravitreal aflibercept therapy in patients with nAMD.

Therefore, other than reported in previous in vitro studies, the indirect effects of aflibercept on pathways modulating the inflammatory response leading to the stimulation of the NF-κB or other pro-inflammatory pathways in vivo cannot be excluded; however, further research is necessary.
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Figure 3. An overview diagram showing the potential mechanisms associated with the IL-17A- and MCP-1/CCL2-mediated inflammatory response, secondary to repeated aflibercept injections. The results of this study are highlighted in red. The numbers enclosed in square brackets indicate the relevant reference placed in the text. Abbreviations: VEGF—vascular endothelial growth factor; PlGF—placental growth factor; IL-17A—interleukin-17A; MCP-1/CCL2—monocyte chemoattractant protein-1/C-C motif chemokine ligand 2; TLR 4—toll-like receptor 4; ERK—extracellular signal-regulated kinase; NF-κB—nuclear factor-κB; GM-CSF—granulocyte-macrophage colony-stimulating factor; CCL5—C-C motif chemokine ligand 5; CX3CL1—C-X3-motif chemokine ligand 1; VCAM-1—vascular cell adhesion molecule 1; ICAM-1—intercellular adhesion molecule 1.

5. Conclusions

In conclusion, our results reveal that repeated injections of aflibercept result in an increase in IL-17A serum concentration, which potentially can lead to a systemic inflammatory response mediated by IL-17, but not by MCP-1, the concentration of which did not change. In addition, we have shown that intravitreal aflibercept causes a significant decrease in serum PlGF concentration after the third monthly injection, compared to the initial measurement, whereas VEGF-A concentration remains stable.

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