Aus der Klinik für Augenheilkunde der Medizinischen Fakultät Charité –
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DISSERTATION

„Immune mediators in samples of aqueous humor in patients with primary and recurrent ocular toxoplasmosis“

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1. Abstract (English)

Toxoplasmosis is a worldwide endemic parasitic disease which is a common cause of visual impairment. Ocular infection with *Toxoplasma gondii (T. gondii)* leads to the production of antibodies, cytokines and chemokines as part of the immune response in the affected person's aqueous humor. We compared patterns of cytokines and chemokines in aqueous humor samples from patients with primary and recurrent ocular toxoplasmosis (OT). Our results point to a T helper (T_h) 1 cell-driven immune response in both primary and recurrent OT. Of 27 immune mediators (including cytokines, chemokines and growth as well as angiogenetic and wound-healing factors) analyzed, we found in pOT ten and in rOT six to be significantly elevated compared to the control group. Furthermore we could find noticeable correlations between clinical characteristics and immune mediator concentrations in aqueous humor. We detected a positive correlation between concentrations of IL-7 (p=0.026), IL-13 (p=0.045), VEGF (p=0.045) and IL-10/IFN-γ (p=0.003) and age at consultation. The number of recurrences of OT correlated negatively with the age at first manifestation (p=0.007). Interestingly and for the first time, we could observe that recurrent OT is characterized by reduced immune mediator concentrations of IL-7 (p=0.031) and IL-9 (p=0.046) in human aqueous humor. However, we could not find any correlation between size of active lesions and the 27 measured immune mediator concentrations.

According to our results, decreased concentrations of IL-7 and IL-9 may possibly be linked to reactivation of the infection. This may have therapeutic implications for secondary prophylaxis with prolonged administration of medications and monitoring of these patients in order to prevent further recurrences. However, further studies analyzing IL-7 and IL-9 concentrations in aqueous humor of OT patients are essential to substantiate these findings and may result in establishing biomarker panels for these two cytokines.

Abstract (Deutsch)

Die Toxoplasmose ist eine weltweit endemische parasitäre Erkrankung, die eine verbreitete Ursache für eine Visusbeeinträchtigung darstellt. Die okuläre Infektion mit *Toxoplasma gondii* (*T. gondii*) führt zu der Produktion von Antikörpern, Zytokinen und Chemokinen im Rahmen der Immunantwort im Kammerwasser der infizierten Patienten. Wir haben die Zytokin- und Chemokinmuster im Kammerwasser von Patienten mit primärer und rezidivierender okulärer Toxoplasmose (OT) verglichen. Unsere Ergebnisse weisen auf eine T_h–Zell-1-vermittelte Immunantwort sowohl bei primärer als auch rezidivierender OT hin. Von den analysierten 27 Immunmediatoren (Zytokine, Chemokine und Zellwachstumsfaktoren sowie wundheilungs- und gefäßwachstumsfördernde Faktoren) waren bei der pOT zehn und bei der rOT sechs im Vergleich zu den Kontrollen im Kammerwasser signifikant erhöht. Desweiteren konnten wir auffällige Korrelationen zwischen Klinik und Immunmediatorkonzentrationen im Kammerwasser feststellen. Wir konnten eine positive Korrelation zwischen den Konzentrationen von IL-7 ($p=0.026$), IL-13 ($p=0.045$), VEGF ($p=0.045$) und IL-10/IFN-γ ($p=0.003$) und Alter bei Vorstellung in der Klinik detektieren. Die Anzahl der Rezidive von OT korrelierte negativ mit dem Alter bei Erstmanifestation. Interessanterweise und erstmals haben wir beobachten können, dass die rezidivierende OT durch reduzierte Immunmediatorkonzentrationen von IL-7 ($p=0.031$) und IL-9 ($p=0.046$) im Kammerwasser charakterisiert ist. Bezüglich der Größe der aktiven Läsionen und der Immunmediatorkonzentrationen konnten wir allerdings keine Korrelation finden.

Unseren Ergebnissen zur Folge könnten reduzierte Konzentrationen von IL-7 und IL-9 mit einer Reaktivierung der Infektion verbunden sein oder könnten durch diese ausgelöst werden. Dies kann therapeutische Folgen für die Sekundärprophylaxe mit verlängerter Verabreichung von Medikamenten und engmaschigen Kontrolluntersuchungen der Patienten haben, um einem Rezidiv der OT vorzubeugen. Es sind jedoch weitere prospektive Studien zur Analyse der Konzentrationen von IL-7 und IL-9 Konzentrationen im Kammerwasser von OT Patienten notwendig, um diese Ergebnisse und Hypothesen zu untermauern, und diese könnten möglicherweise zur Etablierung von Biomarker Assays dieser beiden Zytokine führen.

2. Introduction

2.1. Systemic and ocular toxoplasmosis

2.1.1. Epidemiology and seroprevalence

T. gondii is the causative pathogen for the disease toxoplasmosis. Toxoplasmosis is a worldwide endemic parasitic disease with a geographically diverse seroprevalence in human beings, even within a single country. The prevalence of infection with *T. gondii* varies worldwide (Figure 1). The infection rate in Brazil is very high, with 50% of elementary school children and 50-80% of child-bearing women affected [1]. Figure 1 gives a worldwide overview of seroprevalence of *T. gondii* in people. In Germany, seroprevalence varied in different age groups: seroprevalence increased from 20% in women and men aged 18-29 years to 77% in women and men aged 70-79 years [2]. However, the Netherlands reported a decrease in seroprevalence of *T. gondii* from 40.5% in 1995/1996 to 26.0% in 2006/2007 in women of reproductive age [3].

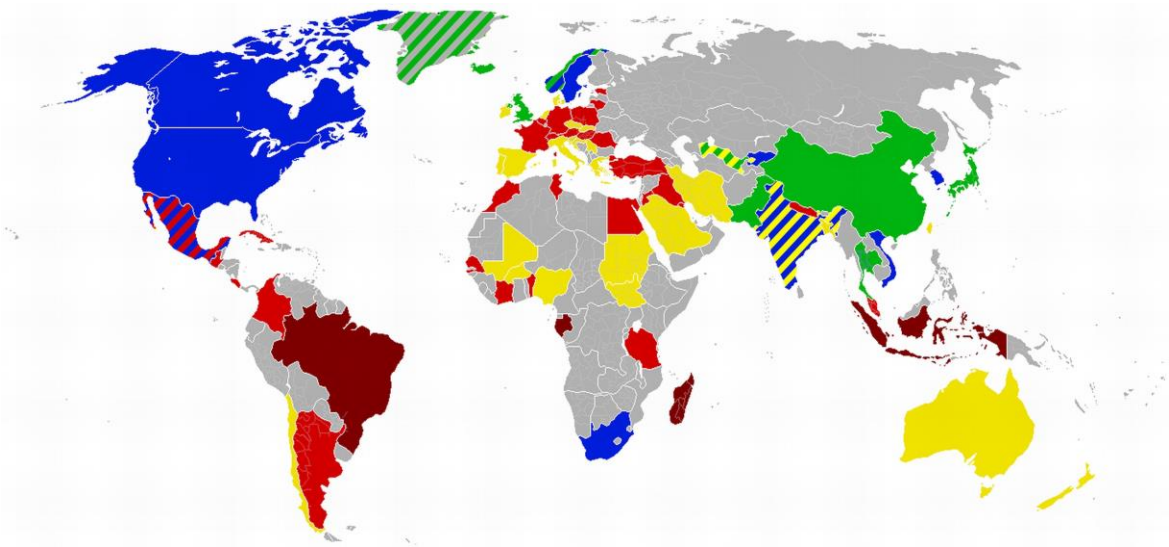


Figure 1: Simplified map of seroprevalence for T. gondii worldwide. Colour code: dark red: >60%; red: 40-60%; yellow: 20-40%; blue: 10-20%; green: <10%; grey: no data available; striated areas represent strong regional differences[4]

Interestingly, data from the United States of America also revealed a decline in seroprevalence in people over the age of 6 years, from 22.5% in 2000 to 13.2% in 2009-2010 [5]. However, north-eastern states of the United States of America have a higher seroprevalence than western, midwestern and southern states [5].

Ocular toxoplasmosis (OT) is the main cause of posterior uveitis in many countries and is responsible for up to 85% of infectious uveitis cases worldwide [6].

There are geographical differences in the prevalence of OT, with reported frequencies of 18% in southern Brazil [7, 8] 6.63% in Italy [9], 4.2% in Germany [10] and 2% in the United States of America [11]. The higher prevalence in Brazil has been attributed to infection with more virulent parasite strains. Previously, ocular involvement among people with post-natally acquired *T. gondii* infection was thought to be of lower prevalence than with congenital infection [12]. However, the analysis of the population in southern Brazil revealed a low prevalence of OT in young persons (e.g. 0.9% at the age of 1-9-year-olds and 1.4% of 9-12-year-olds), indicating that in this location, OT is more a sequela of a postnatally acquired infection than a congenital infection [7]. A Dutch study conducted 30 years ago by Koppe et al. (1986) revealed that retinochoroidal lesions could be detected in 82% of the prenatally infected children within the first 20 years after birth (11% of the children within the first 6 years after birth) [13]. A more recent study in France showed that the first retinal lesions could be found even within 2 years after birth in 75% of the children and first lesions could also be detected 12 years after birth [14]. Wallon et al. (2014) found similar results with children's median age of 3.1 years when first lesions were identified [15].

2.2. *Toxoplasma gondii*

The protozoan parasite *T. gondii* is an obligate intracellular eukaryotic pathogen of the phylum *Apicomplexa* which can cause toxoplasmosis in many warm-blooded animals, including humans as intermediate hosts [16]. In particular, one of its final hosts belongs to the family of cats. Sexual reproduction takes place in the cat intestine. Humans are an alternate host for *T. gondii*, with both congenital and postnatal infection possible. Human infection can occur through several pathways. One infection route is via the consumption of raw or insufficiently cooked meat and contaminated water in which the infectious cysts reside [16]. Infection can also occur through organ transplantation [17]. Additionally, another important infection route is vertical infection from mother to fetus, causing severe damage to the fetus/child such as mental retardation, epilepsy and blindness. This typically occurs because of a primary maternal infection during pregnancy. Infection of endothelial cells lining the placental blood vessels accounts for the main route of transmission to the fetus [18].

A study performed in a referral eye clinic in São José do Rio Preto in Brazil showed that there is an association between the consumption of raw or undercooked meat and contact with feces of dogs and cats on the one hand and the development of toxoplasmosis without particular ocular involvement on the other hand [19]. Moreover, *T. gondii* infection was also associated with low level of schooling/literacy and a low standard of living, although no association with OT was detected [19]. Nevertheless, immunocompetent persons rarely present with symptoms, but toxoplasmosis can cause a self-limited lymphadenopathic syndrome characterized by fever, malaise, fatigue, pharyngitis and cervical lymphadenopathy.

T. gondii infection can be most deleterious in two particular situations: during pregnancy and in immunocompromised patients. The most serious complications are ocular and cerebral toxoplasmosis.

It is known that the development of OT is dependent on the genotype of the parasite, the genetics of the host, and the immune status of the host when infection was acquired (congenitally or postnatally). The genetic make-up of the parasite shows geographical diversity. In Europe and North America, there are three clonal lineages, Types I, II and III, that dominate the majority of human infections [20]. In South America, an abundance of recombinant and atypical strains are more frequent [21, 22]. The low T_h1 response in Colombian patients found by de-la-Torre et al. (2013) could be explained by a modulation of immune response by South American strains [21]. It has been shown that strains of Types I and III inhibit the 'nuclear factor kappa-light-chain-enhancer of activated B cells' (NFκB) pathway which results in reduced IFN-γ production; on the contrary Type II strains induce the NFκB pathway [23].

In terms of the host's genetic, polymorphism in the gene *+874 T/A* encoding for IFN-γ and the gene *-108 G/A* encoding for IL-10 were found to enhance human susceptibility to OT in adult persons by low production of IFN-γ and IL-10 [24, 25]. Moreover, an IL-6 gene polymorphism (-174 G/C) was detected to be associated with toxoplasmic retinochoroiditis [26]. In infants, Meenken et al. (1995) could find an association between *HLA-Bw62* and serious congenital toxoplasmosis [27]. *ABCA4* encoding ATP-binding cassette transporter subfamily A member 4 and *COL2A1* encoding type II collagen are associated with susceptibility to congenital OT, whereas *P2RX7* is associated with susceptibility to both congenital and postnatally acquired OT [28, 29]. Class I MHC genes and $CD8^+$ T cells determine the cyst number in *T. gondii* infection [30].

Concerning the status of immune response of the host, immune deficiency of patients such as HIV positive individuals and organ transplant patients receiving immunosuppressants was

found to be a risk factor for complications like cerebral and disseminated toxoplasmosis [31-33].

There is evidence that the patient's age at the time of initial infection and the age at the most recent episode of active disease may correlate with the risk of recurrent *T. gondii* retinochoroiditis. Holland et al. (2008) found that Dutch patients of older age had a higher risk of recurrences than younger patients, but in a study by Garweg et al. (2008) in Switzerland, patients less than 30 years old had more recurrences in OT [34, 35].

Diagnosis of OT is primarily based on clinical findings. In unclear cases, invasive procedures can be performed by sampling aqueous humor through puncture for detection of *Toxoplasma* DNA by PCR and detecting antibodies by ELISA in aqueous humor.

2.3. Infection cycle of *Toxoplasma gondii*

Infection by *T. gondii* is a complex multistage process and, after intake by the host, comprises different development stages on microstructural level. *T. gondii's* life cycle comprises an asexual and sexual cycle: the asexual cycle occurs in a wide range of intermediate hosts and the sexual cycle exclusively in feline hosts whose feces contains oocysts with highly infectious sporozoites. Either food and water with feces contaminated or tissues of infected hosts with cysts can be ingested by intermediate hosts, which can be any warm-blooded animal including humans, and cause a chronic infection by rupturing, leading to release of sporozoites and bradyzoites (Figure 2). In the small intestine, the parasites transform into tachyzoites which can spread rapidly to all organs throughout the host including the brain, eye, heart, skeletal muscle, placenta and fetus [36]. Shortly after oral intake of the oocysts, parasites incorporated in macrophages can be found in the blood stream [36].

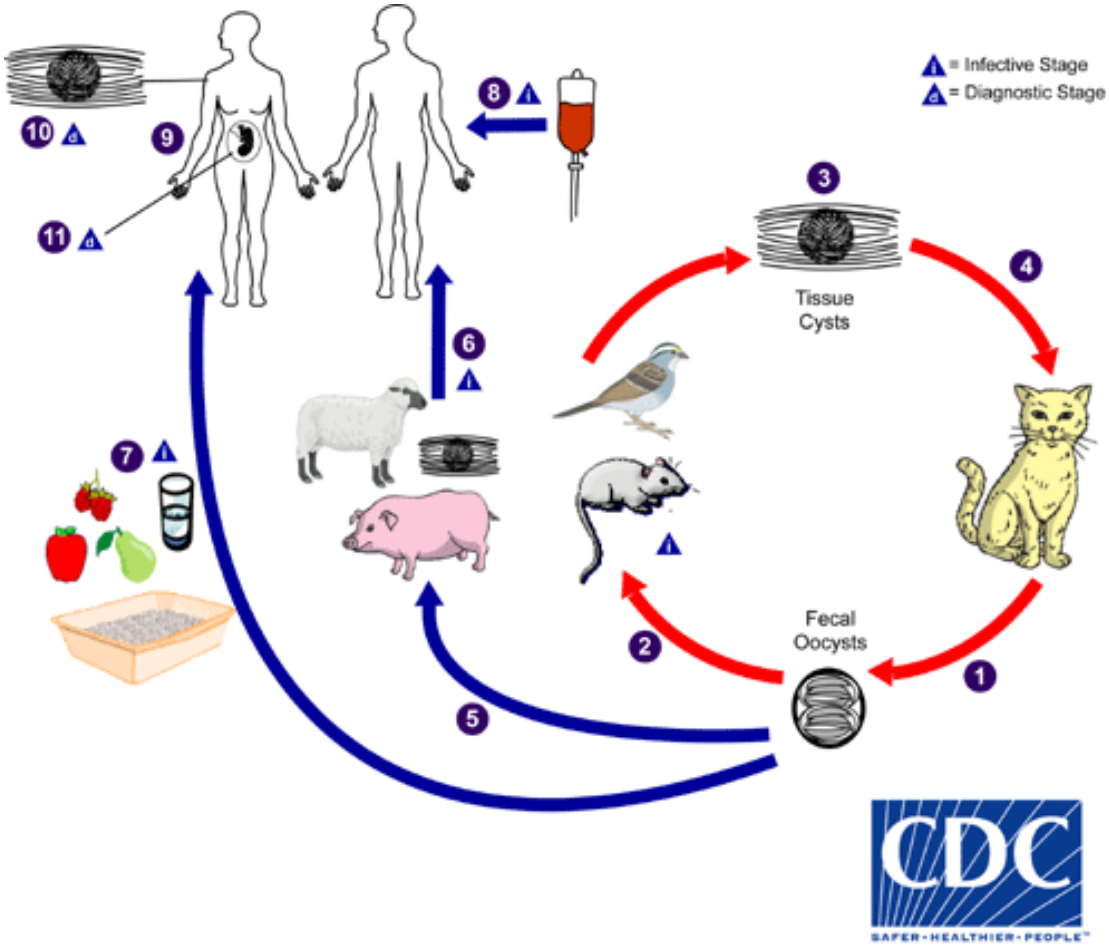


Figure 2: Life cycle of T. gondii, from www.cdc.gov/dpdx/toxoplasmosis/index.html
1.) Oocysts in cat's feces take 4-5 days to sporulate and develop to be infective. 2.) Intermediate hosts get infected by eating soil water or plant material which is contaminated with oocysts. Shortly after ingestion, oocysts develop into bradyzoites. 3. and 4.) The cat becomes infected either by eating tissue cysts in intermediate hosts or by ingesting sporulated oocysts. Game consumed by humans can also be infected by tissue cysts which develop after ingestion of sporulated cysts.
5.) Human infection occurs either via 6.) ingestion of tissue cysts in uncooked meat 7.) consumption of water or food contaminated with cat feces 8.) blood or organ transplantation 9.) transplacentally from mother to fetus
Diagnosis is made clinically and through serology but can also be made by 10.) biopsy samples 11.) or even for congenital toxoplasmosis by providing evidence for T. gondii DNA in amniotic fluid with e.g. PCR.

However, it is still unclear whether the entry point into the blood vessels is the intestine or the lymphatic system. It is known that *T. gondii* tachyzoites invade, replicate and traverse endothelial cells [18, 37, 38]. Depending on the *T. gondii* strain's virulence and its susceptibility, there are differences in migration of epithelial/endothelial barriers, vertical transmission efficiency and dissemination [39].

T. gondii can infect all types of cells. The life cycle of *T. gondii* is a multistage process including attachment to the host cell, sequential discharge of secretory organelles and

formation of an parasitophorous vacuole [40]. Toxoplasmosis is a self-limiting disease in an immunocompetent person, however in immunocompromised individuals, it often presents with cervical lymphadenopathy, sometimes accompanied by low-grade fever [41]. In immunocompromised individuals, the common cause of disease is the reactivation of a chronic infection, rather than a new acquired infection [42, 43].

Congenital or acquired toxoplasmosis infection can be followed by formation of tissue cysts in various organs such as the brain or the eye. Infection with *T. gondii* can lead to late and severe complications such as OT and cerebral toxoplasmosis (CT). A study found frequent concurrence of OT and CT [44]. Therefore, at presentation of congenital OT, an evaluation for CT (also vice-versa) is advisable. However, ocular involvement can also be an initial manifestation of *T. gondii* infection [45]. Congenital toxoplasmosis itself leads to ocular involvement in young age and therefore infection in older age, surmised to be caused by acquired toxoplasmosis, and prevalence in older age tends to be lower [46, 47].

2.4. Risk factors for ocular toxoplasmosis and its clinical presentation

The clinical presentation of OT varies from subclinical manifestation to severe damage. Many patients notice an acute deterioration of visual acuity, in particular when the macula is affected. Other symptoms include blurry vision, metamorphopsia and pain [48]. OT presents as localized retinal necrosis which is commonly accompanied by vitreous inflammation. Often diffuse inflammation can be seen in the neighbouring tissue of the retina and choroid, which leads to the clinical finding of a retinochoroiditis. These lesions heal within 2-4 months in an immunocompetent patient, leaving a hyperpigmented scar because of a disruption of the retinal pigment epithelium. In congenital OT, destruction of all layers of the retina can be observed, including outer layers, retinal pigment epithelium (RPE) and choroid [49]. Moreover, it has been shown that macrophages destroy the outer segments of the photoreceptors. Strong inflammation correlates with advanced patient age, large size of retinal lesions and extramacular location [50]. A recent Brazilian study found that male gender and older age (above 40 years) are predominant risk factors for the development of *Toxoplasma* retinochoroiditis [45].

To distinguish between infection with *T. gondii* and infection with other microorganisms such as viruses, Elkins et al. (1994) described several clinical features in OT, including a thick and more densely yellow-white appearance of the lesions, as well as a lack of hemorrhages [51].

A study showed a relation between inflammation in the anterior chamber as well as aqueous humor, indicating that the visible inflammation in the anterior part reflects a severe development of ocular involvement [50]. Moreover, intraocular pressure was associated with an increase in anterior chamber inflammation and macular location of the lesion [50]. There are some reports about the occurrence of neuroretinitis which is characterized by optic nerve edema and hard exudates forming a star-shaped pattern. This condition is rarely seen in immunocompetent OT patients, whereas optic neuritis is an early finding in HIV-positive patients or individuals with severe course of congenital OT. In immunocompromised patients with OT, vitreous inflammation is apparent, whereas retinal inflammation is absent [52]. Most reports of HIV and OT describe extensive areas of full-thickness retinal necrosis and lesions often reactivate when treatment is terminated [51-54]. Similar findings are reported occur in patients who receive immunosuppressive therapy [55, 56].

2.5. Risk factors for recurrent ocular toxoplasmosis

In general, recurrences of OT are assumed to be caused by release of parasites from tissue cysts in the retina [11]. It is important to characterize risk factors for recurrences in order to establish an appropriate management of the OT patient. Several studies have analyzed whether certain conditions of the host influence recurrences rates. A retrospective analysis of a cohort of pregnant women in the Netherlands pointed out that there is no increase of recurrence rates of OT during pregnancy [57]. Several case reports of North American, Costa Rican, Brasilian and Colombian patients found several risk factors for recurrence in OT: immune suppression with systemic steroid therapy without any antibiotic treatment, subconjunctival or intravitreal injection of steroids and acquired immune deficiency syndrome [58-62].

A chemoprophylaxis for recurrences is advisable in the early post-transplant period of bone marrow transplanted patients whose risk of reactivation of OT can be high depending on $CD4^+$ cell counts [63]. In addition, age can also be considered a risk factor, as studies have shown that the risk for a recurrence of OT is higher in both younger (lower than 20.9 years) [35] and older age (more than 40 years) at first manifestation [34].

2.6. Diagnosis of ocular toxoplasmosis

In clinical practice, the diagnosis of OT is based on characteristic findings at clinical examination. In addition, in unclear cases but not routinely, a serum sample is taken for detection of IgM- or IgG-antibodies for *T. gondii* with the help of an immunofluorescence assay (IFA) or enzyme-linked immunosorbent Assay (EIA). However, a recent study demonstrated that testing of Toxoplasma IgG titres gives evidence for OT, but a differentiation between active and inactive chorioretinitis is not possible by reason of low specificity and sensitivity of the test [64]. Moreover, there is the possibility of obtaining samples of aqueous humor of selected patients by puncture to analyze for IgM- and IgG- antibodies with the same techniques which is not in clinical routine. Other methods include detecting DNA of *T. gondii* by means of PCR.

2.7. Treatment of toxoplasmosis

Usually, there is no drug treatment necessary because of the self-limiting nature of the systemic infection. In case of a severe development of the disease or presence of severe clinical findings such as myocarditis, meningoencephalitis, pneumonitis and polymyositis or ocular involvement, medical treatment is required. However, a treatment for eliminating the parasite is still not available and individuals carry a lifetime risk of a reactivation. There are several options for treatment. The most common treatment in Germany is the triple therapy with pyrimethamine in combination with sulfadiazine and folinic acid (48%), either with or without corticosteroids, the second common therapy in Germany is Clindamycin (38%) [65]. pyrimethamine inhibits the parasite's multiplication and causes tremendous changes in the parasite's structure like fragmentation of the nucleus due to an abnormal formation of the daughter membrane during endodyogeny [66]. Clindamycin is an antibiotic of the lincosamide family effecting not only bacteria but also anaerobics, *Chlamydia* and parasites like *T. gondii*. This antibiotic unfolds its parasiticidal effect by targeting mitochondrial ribosomes which are of prokaryotic origin [67]. The penetration of Clindamycin into the intracellular parasite's mitochondria is slow and can explain the delay of Clindamycin's impact [67]. The treatment duration varies between four to six weeks, however it can differ according to the clinical course of each patient's disease. Treatment of toxoplasmosis was found to reduce the size of

retinochoroidal scars or to limit the proliferation of the tachyzoite form of *T. gondii* in active disease [68, 69]. Short-term treatment of active OT does not prevent subsequent recurrence [70, 71]. However, antibiotics as a secondary prophylaxis may impede OT recurrence [72-74].

2.8. Immunological aspects of infection with *Toxoplasma gondii*

Whereas the adaptive immune system is responsible for the host's survival, the innate immune system has an effect on initial susceptibility and defence against the infection with *T. gondii* by means of T cell activation [75]. It has been shown that $CD8^+$ T lymphocytes can lyse extracellular *T. gondii* tachyzoites and *T. gondii*-infected target cells in both humans and mice in vitro [76, 77]. Moreover, $CD4^+$ T cells have an important role in the maintenance of $CD8^+$ T cell immunity against *T. gondii* [78].

In vitro studies found that human and rat Muller cells in the RPE guard neuron integrity in healthy and infected retina and in the case of animal's Muller cells it was found that they secrete mediators in response to *T. gondii* infection [79, 80].

The present "dogma" is presented as *T. gondii* infection being a T_h1- and T_h17-driven immune response (Figure 3) [81].

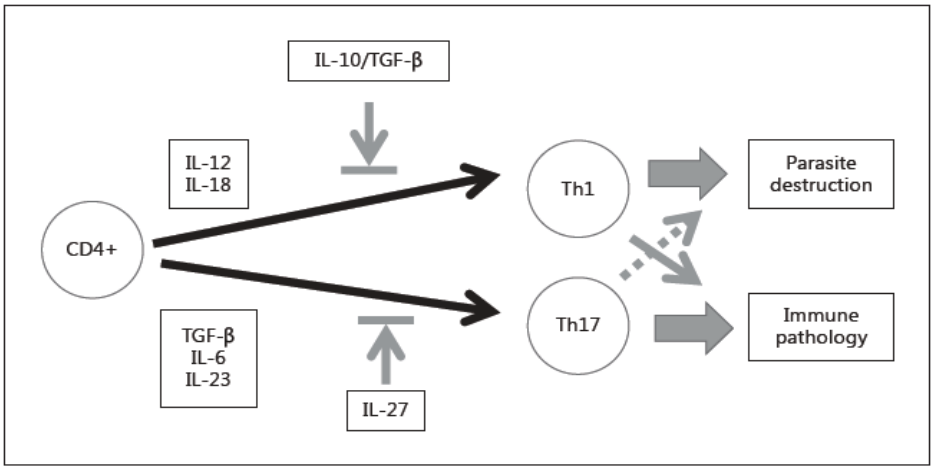


Figure 3: Simplified scheme of parasite destruction and immune pathology during T. gondii infection. The differentiation of $CD4^+$ T cells into T_h1 cells is promoted by IL-12 and IL-18 and impeded by IL-10 and TGF-ß [81].

It was been suggested that impaired neutrophil response during infection with *T. gondii* leads to recurrence [82, 83]. The inflammatory response is a mononuclear cell reaction and consists of lymphocytes and macrophages at the edge of the lesion. Secretion products of these cells are cytokines and chemokines, which are immune modulators with pro- and antiinflammatory

function during infection. We can distinguish between T_h1 cell-derived cytokines (IFN-γ, TNF-α and IL-2), T_h1 cell development-promoting cytokines (IL-12, IL-7 and IL-15), T_h2 cell-derived cytokines (IL-4, IL-5, IL-, IL-9, IL-10, IL-13), T_h17 cell development-promoting cytokines (IL-6, IL-1β), T_h17 cell-derived cytokines (IL-17, IL-1Ra), chemokines (IP-10, MIP-1α, MIP-1β, Eotaxin, IL-8, RANTES, PDGF-bb), as well as growth, angiogenetic and wound-healing factors (GM-CSF, MCP-1, FGF, G-CSF, VEGF). Cytokines and chemokines, especially IFN-γ and TNF-α, have an important role in resistance to *T. gondii* by activating macrophages. By contrast, IL-10 has an anti-inflammatory role by inhibiting MHC class II and co-stimulatory molecule (B7-1/B7-2) expression on monocytes and macrophages and limiting the production of proinflammatory cytokines and chemokines [84]. Moreover, IL-10 enhances the function of natural killer (NK) cells and cytotoxic $CD8^+$ T cells as well as regulates growth and differentiation of T helper cells, B cells, mast cells, granulocytes, dendritic cells, keratinocytes and endothelial cells [84]. In particular, IL-10 suppresses the T_h1-response in order to prevent overproduction of IL-12, IFN-γ and TNF-α [85].

According to current knowledge, in mice *T. gondii* incorporation into macrophages induces production of pro-inflammatory IL-12 in macrophages and dendritic cells in response to toll-like receptor-(TLR) 11 and 12 mediated recognition of *T. gondii* profilin [86-88]. A mouse experiment found that IL-1ß induces secretion of IL-12, which in turn activates NK cells to secrete IFN-γ [89]. Neutrophil leukocytes are a potential source of IL-12 and therefore may play an influential role in the generation of a T_h1-response [90]. In humans as well as in mice, neutrophils provide an important source of IFN-γ as well, however, mechanisms leading to neutrophil-derived IFN-γ production are not yet fully understood: there is evidence that neutrophil-derived IFN-γ can be induced by IL-12, but it can also be IL-12 independent [91, 92]. Neutrophil-derived IFN-γ is regulated by means of TNF-α and IL-1ß as is natural killer cell derived IFN-γ, but is TLR independent [92]. Unlike mouse experiments, the IL-12 response of human dendritic cells and monocytes is stimulated by phagocytosis of live tachyzoites rather than host cell invasion [93].

IFN-γ plays an important role in the host's resistance towards *T. gondii*. It promotes the intracellular elimination of *T. gondii* through interferon-regulated GTPases, induction of reactive nitrogen intermediates, tryptophan degradation in human cells and autophagy. A mouse experiment performed in the 1980s found that antibodies neutralizing IFN-γ *in vivo* result in induction of reactivated and exacerbated encephalitis [94]. Another study group revealed that IFN-γ together with perforin-mediated immune response is essential by

controlling tachyzoite proliferation during both acute acquired and recurrent toxoplasmosis in the mouse brain [95]. Suzuki et al. (1994) found that particularly in toxoplasma encephalitis (TE) there are reduced serum levels of IFN-γ as well as a high cyst burden and brain inflammation apparent in mice [96]. Another study confirmed these findings in humans, showing that patients with CT and OT have low levels of IFN-γ in serum, which is produced by peripheral blood mononuclear cells (PBMC), indicating a reduced ability of the organism to resist to the parasite's infection [97].

Furthermore, there are experimental studies in mouse and human cells showing synergy of IFN-γ with TNF-α and TNF-ß in activating macrophages to produce reactive oxygen species [98] and reactive nitrogen intermediates [99]. It has been confirmed that reactive oxygen species and reactive nitrogen intermediates are effective reagents in the control of *T. gondii* infection [98, 100].

Both a mouse and a human cell study showed that IFN-γ induced killing of *T. gondii* is partly related to a TNF-mediated pathway [101, 102]. However, IFN-γ can also induce the transcription of TNF and IL-1 genes [103]. In addition, lipopolysaccharides on *T. gondii*'s surface promote TNF release [104]. Findings in mice reveal that TNF-α itself enhances the anti-toxoplasmic activity of IFN-γ primed macrophages [99]. In mice, CT TNF-neutralization leads to reduced expression of inducible nitric oxide synthase which is a marker for macrophage activity leading to progressive parasite growth and tissue damage [105].

Moreover, production of anti-inflammatory IL-10 in disseminated toxoplasmosis infection is upregulated, supporting the parasite to persist in the host by down-regulating the immune system [97, 106]. It is not clear whether IL-27 influences IL-10 production. There are studies on mice which revealed that differentiation into IL-10-producing $CD4^+$ T cells is mediated by IL-27 but another mouse experiment analyzing glycoprotein 130 (gp130)-mediated pathway of IL-27 could not confirm this [107-109]. In both mouse and human experiments, it was found that IL-10, TNF-α and IL-4 inhibit the protective effect of IFN-γ and therefore support *T. gondii's* growth in the organism [110-112]. Several experimental studies on mice found that IFN-γ and IL-10 are produced simultaneously in immune response to infection with *Mycobacterium avium*, *Listeria monocytogenes* and *Trypanosoma cruzi* leading to the assumption that IFN-γ is responsible for parasite control and IL-10 curtails an excessive inflammatory response [113-115]. Thus, IFN-γ and IL-10 counterbalance each other in parasite infection. Similar results were found in IL-10-deficient mice infected with *T. gondii* leading to a lethal immune response by $CD4^+$ T cells characterized by overproduction of IL-12, IFN-γ and TNF-α [85]. Serological tests on humans revealed different results with elevated IL-10 levels in

patients with systemic *T. gondii* (without ocular or cerebral symptoms but not tested for OT or CT) and low levels for IFN-γ and IL-10 in OT as well as CT patients [97].

In *T. gondii* infection, IL-6 has pro- as well as anti-inflammatory functions. For instance, a mouse experiment showed that IL-6 deficiency leads to inability to initiate a rapid pro-inflammatory response to *T. gondii* which results in increased parasite growth and as brain cyst development, and subsequently, mortality is high due to augmented parasite burden and excessive inflammatory response several weeks after infection [116, 117]. In other words, there is evidence that IL-6 is part of a regulatory loop that is important to initiate inflammation, but can also act to limit this response in a chronic set, i.e. basically although IL-6 is often pro-inflammatory, it can also be anti-inflammatory depending on interactions with other signaling molecules [109]. Several more studies on mouse and human cells revealed the anti-inflammatory aspect of IL-6 [118-121]. Furthermore, mouse studies found that IL-6 and TGF-β can mediate the production of IL-17 and IL-10 and restrain T_h17 cell influence [122, 123]. Together with TNF-α, IL-6 can also enhance proliferation and differentiation of B lymphocytes [124].

In recent years, IL-17 in aqueous humor was seen as a possible indicator for infection with *T. gondii*. Lahmar et al. (2009) analyzed aqueous humor from individuals with either viral infections or *T. gondii* infection and concluded that IL-17 was elevated in *T. gondii*-infected samples [125]. This was confirmed by another study on humans which additionally showed that intravitreal injection with IL-17 led to decrease in intraocular inflammation measuring cytokine concentrations in aqueous humor at day 1, 3 and 5 after injection [126]. Therefore, anti-IL-17-detergents were regarded as potential therapeutic options for treating OT. However, de-la-Torre et al. (2014) could not detect elevated IL-17 levels in OT [127].

There are several mouse studies investigating the role of chemokines during *T. gondii* infection. Analyzing chemokine concentrations in cerebrospinal fluid of mice during TE, a study reported elevated concentrations of RANTES, MIP-1α, MIP-1β and MCP-1 [128]. Experiments on *T. gondii*-infected human epithelial cells revealed that expression of IL-8-specific mRNA is increased but secretion of IL-8 is dependent on soluble host cell factors like IL-1β and additional factors in fibroblasts [129]. An investigation of rat retinal vascular endothelial cells has shown that MCP-1 is upregulated at the very beginning of an acute infection to direct the traffic of inflammatory cells into the infected area [130].

IP-10 is expressed in many T_h1-type human inflammatory diseases like psoriasis [131], multiple sclerosis [132], atherosclerosis [133], rheumatoid arthritis [134], transplant rejection [135] and inflammatory bowel disease [136]. IP-10-deficient mouse models have a more than 100-fold increase in *T. gondii* parasite tissue burden and a marked increase in mortality [137, 138]. It seems to differ from most other CXC (CXC = two N-terminal cysteines separated by one amino acid) chemokines in that it does not interact with neutrophil leukocytes [139] but targets lymphocytes specifically [140]. IP-10 plays a role in the recruitment of T effector cells to inflammatory sites. Additionally, IP-10 has been shown to block tumor cell growth in mouse models [141, 142] and inhibit neovascularization [143, 144]. Another study found that IP-10 is also relevant for regulating cytokine synthesis by augmenting IFN-γ release by PBMC in human blood samples and promoting T_h1-immune response dominance [145].

So far, animal studies have analyzed the intraocular immune response in recurrent OT by provoking tachyzoite release by reactivation of tissue cysts residing in the retina with general immune depression by total lymphoid irradiation and application of antilymphocyte serum in monkeys and rabbits [146, 147] or by administration of neutralizing antibodies against T cells and cytokines in mice [148]. Recently, Rochet et al. (2015) conducted a mouse experiment without general immune depression of the mice, giving an insight into the murine immune reaction to *T. gondii* reinfection. In this study, two types of mice (Swiss-Webster and C57Bl/6J mice) were infected intraperitoneally at the age of 5 weeks with cysts of *T. gondii* type II obtained from brains of Swiss-Webster mice infected perorally. Again after 4 weeks, the intraperitoneally infected mice were administered an intravitreal injection containing cysts of *T. gondii* type II [149]. The study by Rochet et al. (2015) found that Swiss-Webster mice, which are known to be resistant to primary infection, have a much lower intraocular production of IL-6 and IFN-γ than C57Bl/6J mice susceptible to *T. gondii* infection. On the one hand, further experiments in Rochet's same study on C57Bl/6J mice with neutralization of IFN-γ resulted in high parasite burden showing the protective role of IFN-γ in *T. gondii* parasite load control; on the other hand, neutralization of IL-6 led to lower *T. gondii* parasite load and ameliorated retina structure revealing its deleterious role [149].

Studies by de-la-Torre et al. (2009, 2013, 2014) investigating the intraocular immune response to OT in humans analyzed intraocular cytokine/chemokines in patients of different nationalities (French and Colombian) but they did not distinguish between primary and recurrent OT within the groups [21, 58, 127]. Colombian OT patients are characterized by decreased intraocular

IFN-γ and IL-17 levels and increased intraocular IL-6 and IL-13 levels in relation to French OT patients, associated with a more severe course of OT in Colombian patients, including macular involvement, vitreous inflammation, strabismus, bilateral involvement and synechiae [21]. De-la-Torre et al. (2013) suggested that there is a geographical difference in prevalence and virulence of *T. gondii* parasite strains, as Colombian OT patients appear to be infected by the I/III strain and an atypical strain with demonstrably high intraocular *T. gondii* parasite load, whereas French OT patients seem to be susceptible to infection with the type II strain and have a lower intraocular parasite load [21].

2.9. Measurement of immune mediators with Multiplex-Immunoassay

Multiplex Bead Assays were first introduced in the late 1970s and developed for commercially available instruments in the late 1990s [150].

Figure 4: Bio-Plex Pro™ Human Cytokine 27-plex Assay: 1 x 96 well, including coupled magnetic beads, detection antibodies, standards, reagents and diluents for detecting 27 human immune mediators [151]

The advantage of this method compared to ELISA is the possibility of measuring concentrations of numerous immune mediators in one sample with a small volume of the test sample at the same time. Only 12.5 µl volume is required for investigating serum or plasma whereas a cell culture medium volume of 50µl is compulsory for testing immune mediator concentrations. An average of 3 hours is required for getting results.In principle, colour-coded beads coated with capture antibodies are mixed together with a sample of interest, e.g. aqueous

humor. After washing, beads are incubated with a solution containing fluorescently-labeled detection antibodies which bind to the captured analyte molecules (Figure 5).

This conglomerate is sent through a standard flow cytometer in which a laser identifies the bead size, bead and reporter tags (Figure 5).

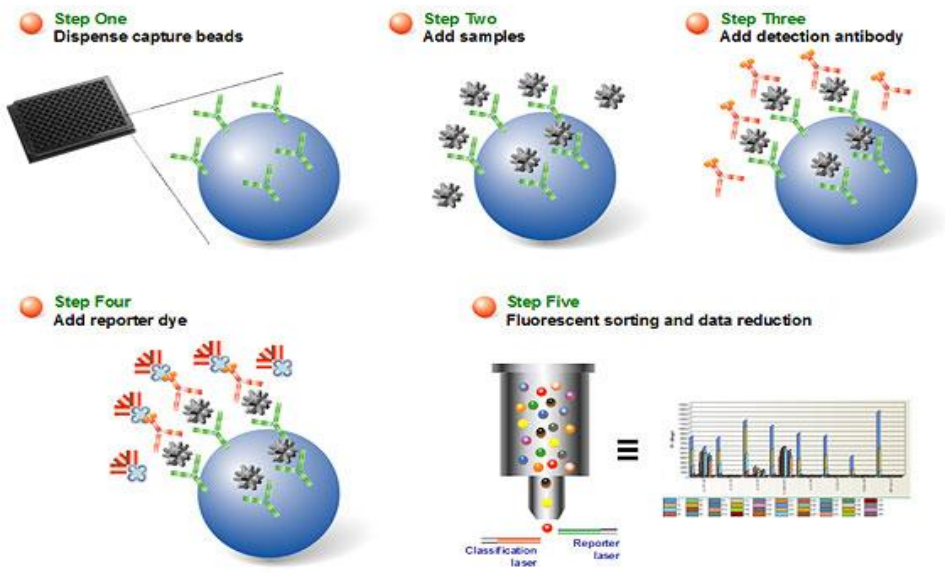


Figure 5: Aqueous humor molecules are bound to colour-coded beads which are conjugated to antibodies themselves and sent through a flow cytometer. A laser detects bead size, bead and reporter tags on the conglomerates [152].

There are Multiplex-Immunoassays which make it possible to test up to 64 immune mediators in one sample with about 100 different assays including 100 distinctly coloured bead sets. However, the more assays will be measured at a time, the greater the chance of inaccuracy of the measurements. Based on internal standard curves, Bioplex Pro Human 27-plex Immunoassay is an effective method to simultaneously quantify immune mediator levels with high accuracy and precision.

2.10. Purpose of the study

A comprehensive analysis of immune mediator patterns in human aqueous humor may facilitate clinical management of OT. OT severity and recurrences may be more predictable. Consequently, patient care management could be improved in terms of appropriate medication duration, secondary chemoprophylaxis and scheduling of follow-up consultations.

In the present study, we analyzed concentrations of 27 immune mediators in aqueous humor of 51 patients infected with *T. gondii* and 11 individuals as controls and marked out clinical correlations. To date, this is the largest cohort so far characterizing the immune mediator profile of OT patients in aqueous humor and differentiating between primary and recurrent OT. Our study focuses on the relation of aqueous humor immune mediator profiles in OT and the following clinical parameters: 1. Age at consultation 2. Age at first manifestation 3. Number of recurrences and 4. Size of active lesions. It is also taken into account whether systemic therapy with steroids alters immune mediator profiles.

In the present study, we discuss the following five hypotheses:

1. Are there characteristic differences in immune mediator concentrations in aqueous humor in patients with primary and recurrent OT?
2. Are immune mediator levels in aqueous humor dependent on the patient's age? Is the expression of immune mediators decreased in older age and higher in younger age?
3. Does younger age at first manifestation correlate with a higher number of recurrences in OT?
4. Do levels of immune mediators in aqueous humor change with the number of recurrences in OT?
5. Are concentrations of immune mediators in aqueous humor elevated in samples with larger size of active lesions?

3. Patients and Methods

3.1. Infected and control patient groups

In the study, we included 51 patients with a proven intraocular infection as confirmed with *T. gondii* antibodies in the aqueous humor (Goldmann/Witmer – Desmonts coefficient ≥ 3) and 11 individuals as controls [153].

The samples of aqueous humor of the infected patients were taken between December 2005 and April 2014 at the Department of Ophthalmology, Campus Virchow, Charité Berlin, Germany. We subdivided the infected group into patients with primary and recurrent OT. Aqueous humor was obtained at variable times after initial presentation (Range: day of presentation to 14 days after presentation).

The control group consisted of 11 individuals who had undergone routine cataract surgery in April 2014 at the Department of Ophthalmology, Campus Virchow, Charité in Berlin, Germany. These patients had no other intraocular pathology at preoperative ophthalmological examination including funduscopy. The samples of aqueous humor were obtained at the beginning of the cataract surgery procedure.

The age distribution of both OT groups is similar, however, compared to the control groups, they show a difference due to the older age of cataract patients.

Some patients in the OT groups were treated with Clindamycin due to its lower complication rate compared to the triple therapy with pyrimethamine, sulfadiazine and folinic acid and proven parasiticidal effect against *T. gondii* [67, 68]. Some of these patients additionally received corticosteroids in addition when the clinical findings revealed a high level of inflammation in the vitreous fluid and/or anterior chamber as well as central and large retinal lesions.

Depending on the degree of inflammation, some patients received systemic antibiotic treatment combined with or without steroids before aqueous humor puncture was performed (16 individuals were administered Clindamycin, 3 individuals were treated with daraprim, 11 individuals received systemic steroids).

Four patients with primary OT and seven patients with recurrent OT received systemic steroid medication prior to aqueous humor sampling. None of the patients had a previous medical history of chemotherapeutics in terms of cytostatics or were known to be immunodeficient.

3.2. Surgical procedure for obtaining aqueous humor

Following each patient's informed consent, the procedure for obtaining aqueous humor was performed. After disinfection, sterile covering of the area and putting the eyelid retractor in position and anterior chamber paracentesis, approximately 100-300µl of aqueous humor was aspirated with a tuberculin syringe and a 31-gauge needle. The samples were immediately stored and maintained at -80°C to prevent degradation.

3.3. Clinical parameters

We identified the level of inflammation at presentation of cells in the anterior chamber according to the Standardization of Uveitis Nomenclature (SUN) grading system [154] and the vitreous haze referred to the National Eye Institute Grading Scheme [155] by chart review of the medical records of the Department of Ophthalmology, Campus Virchow, Charité, Berlin, Germany. Data could not be collected from all patients due to incomplete medical records (Table 2).

In addition, further clinical characteristics included age at consultation and age at first manifestation of infection. Information about the patients' age at consultation and age at first manifestation was obtained from medical histories of the patients and the medical records of the Department of Ophthalmology, Charité, Berlin, Germany. Furthermore, we noted the number of active and old lesions as well as scars with the fundus photographs and written documentation in the medical records of the patients. Retinochoroidal lesion size was measured by disc diameter. We also obtained data about the treatment the patients received and the start of the treatment in relation to obtaining the aqueous humor through puncture.

3.4. Measurement of immune mediators in aqueous humor

We used the Bio-Plex Pro Human 27-plex Panel Immunoassay (Bio-Rad) and the LUMINEX ® 200™ to measure immune mediator concentrations in 50µl volume of sample from patients in the groups with primary OT and recurrent OT as well as the control group according to the manufacturer's recommendations as described in Table 1. Measurements are done on 96-well-plate formats. We measured the concentrations of the following immune mediators: IFN-γ, IL-1ß, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IP-10, MCP-1, MIP-α, PDGF-bb, MIP-1ß, RANTES,

TNF-α and VEGF. If the sample volume was less than 50 µl, it was diluted to the required volume and dilution was taken into account accordingly. The assay's layout consisted of eight standards in duplicate, two blank wells, and 62 aqueous humor samples.

Concentrations were calculated using standard curves of known concentrations and levels of cytokines and chemokines expressed in pg/ml for each immune mediator. Data analysis was performed using Bio-Plex Manager™ software 6.1.

Table 1: Performance of Bioplex pro Human Cytokine 27-plex Immuno Assay (Biorad)	
1.) Preparing standards	Reconstitution of Bioplex standard diluent in a single vial of standard in 500 µl (vortex performed and stored on ice (30min). Out of the single standard an eight point standard dilution series and blank are prepared
2.) Preparing samples	Samples are diluted with Bioplex sample diluent
3.) Preparing the Coupled Beads	Adding 575 µl beads to 5.175 µl assay buffer
4.) Running the Assay	a) Moistening filter plate with 100 µl assay buffer
	b) 50 µl of beads are added to the assay plate
	c) Washing 2 times with 100 µl wash buffer
	d) Covering and incubation in the dark at room temperature with shaking at 300 RPM für 30 Min
	e) When 10 min remain, adding 300 µl of detection diluent to 2.700 µl detection antibodies
	f) Washing 3 times with 100 µl wash buffer
	g) Adding 25 µl of detection antibody
	h) Covering everything and incubating in the dark at room temperature with shaking at 300 RPM for 30 min
	i) Meanwhile preparing the software protocol by entering normalized standard S1 values
	j) In the remaining 10 min, preparation of Streptavidin-Phycoerythrin (SA-PE) by adding 60 µl to 5.940 µl assay buffer in the dark
	k) Washing 3 times with 100 µl wash buffer
	l) Adding 50 µl of SA-PE
	m) Covering and incubation in the dark at room temperature with shaking at 300 RPM for 10 min
	n) Washing 3 times with 100 µl wash buffer
	o) Beads are resuspended in 125 µl assay buffer and shaken at 110 RPM for 30 sec

Table 1: The procedure of preparing the samples and standards when performing the Bioplex Pro Human Cytokine 27-Plex Immuno Assay (Biorad) Source: Bio-Plex Pro™ Assays, Cytokine, Chemokine and Growth Factors Instruction Manual (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10014905.pdf)

3.5. Statistical analysis

The software R version 3.1 was used for the statistical analysis to compare immune mediator levels of the 3 groups (primary OT, recurrent OT and control group) by Mann-Whitney test, and to detect correlations between clinical parameters and immune mediator levels by Spearman rank correlation. We performed the Chi-Square-Test to quantify the age distribution of the different groups [156]. Moreover, we formed cytokine ratios of the measured concentrations in aqueous humor for each patient and compared the two groups with primary and recurrent OT using the Mann-Whitney test. A statistically significant result was defined as $p \leq 0.05$ after adjusting for multiple comparisons by controlling the false discovery rate at the same nominal level [157].

The following cytokine quotients were formed: IL-4/IFN-γ, IL-10/IFN-γ, IL-17/IFN-γ, TNF-α/IL-10 and IL-12p70/IL-10. Boxplots and graphs were created with the software Graph Pad Prism Version 6.07 [158].

4. Results

In this study, we analyzed aqueous humor samples of 62 individuals. The cohort of OT patients consisted of 22 patients with *primary OT* and 29 patients with *recurrent OT*. 11 individuals served as controls. Aqueous humor samples were obtained from 20 male and 31 female patients. The mean age of the patients together in *primary* and *recurrent OT* group was 38 ± 14 years and of the control group 75 ± 7 years.

4.1. Clinical characteristics of the study cohort

The age distribution of patients *in primary* and *recurrent OT* as well as the control groups is illustrated in Figures 6 and 7.

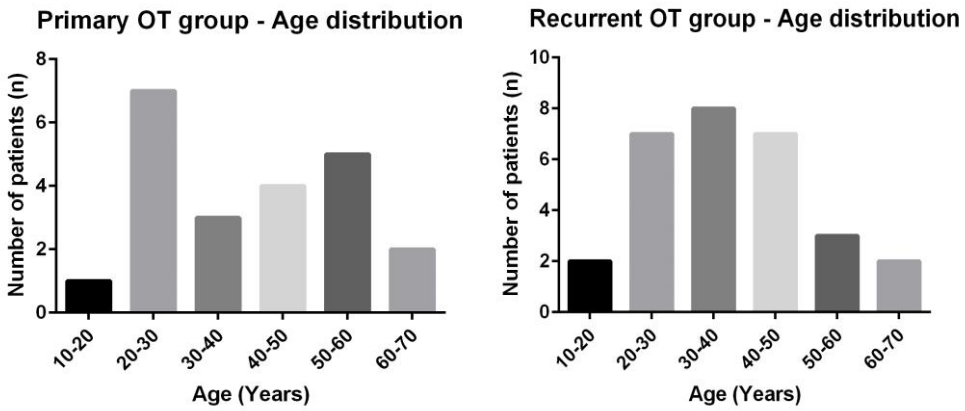


Figure 6: Age distribution of the patients with primary and recurrent OT. There was no statistical difference between the age distribution of the primary OT and recurrent OT group (p= 0.932). The mean age of the primary OT group: 39 ± 15 years (n=22), mean age of recurrent OT group: 38 ± 13 years (n=29); 10-20 years: primary OT n=1, recurrent OT n= 2; 20-30 years: primary OT n=7, recurrent OT n= 7; 30-40 years: primary OT n=3, recurrent OT n=8; 40-50 years: primary OT n=4, recurrent OT n= 7; 50-60 years: primary OT n= 5, recurrent OT n=3; 60-70 years: primary OT n= 2, recurrent OT n=2)

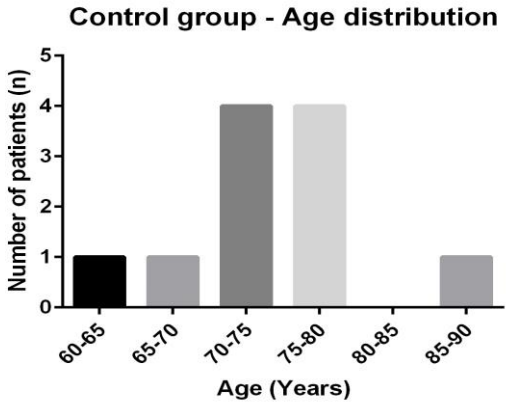


Figure 7: Age distribution in the control group. The age distribution is older compared to the primary and recurrent OT group, as samples are taken from patients during cataract surgery (mean: 75 ± 7 years; 60-65 years: n=1, 65-70 years: n=1, 70-75 years: n=4, 75-80 years: n=4, 80-85 years n=0, 85-90 years: n=1=

The information about patients' clinical characteristics was acquired from the medical records and is summarized in Table 2.

Table 2: Clinical characteristics of the patients

	Primary OT	Recurrent OT	Control group
Number of patients (n)	22/62 (35.5%)	29/62 (46.8%)	11/62 (17.7%)
Gender	male: 40.91% (9/22)	male: 37.93 (11/29)	male: 63.63% (7/11)
Mean age at first episode (years ± SD)	39 ± 15	25 ± 14[a]	
Mean age at aqueous humor sampling (years ± SD)	39 ± 15	38 ± 13[a]	75 ± 7
Median age at first episode (years, Min.-Max.)	41 (15-64)	24 (0-66)[a]	
Median age at sampling of aqueous humor sampling (years, Min.-Max.)	41 (15-64)	35 (19-68)[a]	74 (60-89)
vitreous haze (National Eye Institute Grading Scheme)	(0; 1+;2+,3+)[b]	(0;1+;2+;3+)[c]	
	0: 1/22	0: 6/29	
	1+: 4/22	1+: 6/29	
	2+: 5/22	2+: 1/29	
	3+: 1/22	3+: 0/29	
anterior chamber cells (SUN classification)	(0;0,5+;1+;2+)[d]	(0;0,5+;1+;2+)[e]	
	0: 8/22	0: 12/29	
	0,5+: 1/22	0,5+: 0/29	
	1+: 4/22	1+: 3/29	
	2+: 2/22	2+: 2/29	
Mean number of active lesions (n)	1.66 ± 1.5 (1-6)[f]	2 ± 1.03 (1-4)[g]	
Mean size of active lesions (PD)	1.65 ± 1.72 (0.3-6.5)[h]	2.08 ± 0.95 (0.3-4.5)[i]	
Mean number of scars (n)		1.3 ± 0.72[j]	
Bilateral OT involvement	4.5% (1/22)	10.3% (3/29)	
Mean number of recurrent active episodes (n)		2.68 ± 3.61	
		age <40 years old: 3.57 ± 4.43	
		age >40 years old: 2.1 ± 2.8	
Macular involvement	9.1% (2/22)	13.79% (4/29)	

Table 2: A summary of the patients' clinical characteristics in the pOT and rOT groups as well as control group. OT = ocular toxoplasmosis, SD = standard deviation, PD = diameter of the papilla, [a]Data obtained from the medical records of 24/29 patients, [b]Data obtained from the medical records of 11/22 patients, [c]Data obtained from the medical records of 13/29 patients, [d]Data obtained from the medical records of 15/22 patients, [e]Data obtained from the medical records of 17/29 patients, [f]Data obtained from the medical records of 18/22 patients, [g]Data obtained from the medical records of 22/29 patients, [h]Data obtained from the medical records of 11/22 patients, [i]Data obtained from the medical records of 16/29 Patients. [j]Data obtained from the medical record of 15/29 patients.

4.2. Influence of steroid medication on immune mediator concentrations prior to aqueous humor sampling

The medical records show that 4 out of 22 patients with primary OT and 7 out of 29 patients with recurrent OT received systemic steroid medication combined with Clindamycin prior to aqueous humor sampling.

Table 3 shows that there is no significant difference between any of those groups ($p>0.05$) for all 27 immune mediators. Therefore, we could not detect any significant differences of immune mediator levels in aqueous humor between those obtained from OT patients who were administered steroids and those who were not.

Table 3: Influence of the application of systemic steroid medication before aqueous humor sample was taken

Immune mediator	Primary OT with steroids (n=4) vs recurrent OT with steroids (n=7) p-values (not corrected)	Primary OT with steroids (n=4) vs primary OT without steroids (n=18) p-values (not corrected)	Recurrent OT with steroids (n=7) vs recurrent OT without steroids (n=22), p-values (not corrected)
IL-1β	0.969	0.489	0.198
IL-1Ra	0.018	0.953	0.536
IL-2	0.020	0.232	0.943
IL-4	0.021	0.306	0.747
IL-5	0.037	0.634	0.379
IL-6	0.099	0.406	0.476
IL-7	0.045	0.619	0.310
IL-8	0.066	0.227	0.990
IL-9	0.128	0.938	0.758
IL-10	0.106	0.591	0.657
IL-12p70	0.167	0.743	0.657
IL-13	0.026	0.957	0.359
IL-15	0.031	0.375	0.773
IL-17	0.095	0.672	0.545
Eotaxin	0.017	0.549	0.843
FGF basic	0.227	0.743	0.739
G-CSF	0.236	0.495	0.434
GM-CSF	0.063	0.253	0.175
IFN-γ	0.015	0.542	0.608
IP-10	0.021	0.059	0.578
MCP-1	0.067	0.134	0.735
MIP-1α	0.423	0.552	0.174
PDGF-bb	0.016	0.319	0.847
MIP-1β	0.408	0.618	0.178
RANTES	0.510	0.766	0.131
TNF-α	0.26	0.388	0.822
VEGF	0.211	0.249	0.820

Table 3: Influence of the application of systemic steroid medication before aqueous humor sample was taken. Prior to aqueous humor sampling, 4 patients with primary OT and 7 patients with recurrent OT were given oral corticosteroid medication. There is no difference of immune mediator concentrations when comparing each of these groups to patients who did not receive any corticosteroids ($p>0.05$)

4.3. Profile of immune mediators in aqueous humor from patients with primary and recurrent ocular toxoplasmosis versus controls

Table 4a: Cytokine concentrations in aqueous humor of a control group (n=11) and patients with primary (n=22) and recurrent (n=29) ocular toxoplasmosis

Immune mediator	Median concentration in patients with primary ocular toxoplasmosis [pg/ml]	Mean concentration in patients with primary ocular toxoplasmosis [pg/ml]	Median concentration in patients with recurrent ocular toxoplasmosis [pg/ml]	Mean concentration in patients with recurrent ocular toxoplasmosis [pg/ml]	Median concentration in patients in control group [pg/ml]	Mean concentration in patients in control group [pg/ml]	p-value (not corrected) primary OT vs control group	p-value (corrected*) primary OT vs control group	p-value (not corrected) recurrent OT vs control group	p-value (corrected*) recurrent OT vs control group	p-value (not corrected) primary vs recurrent OT	p-value (corrected*) primary vs recurrent OT
T cell development-promoting cytokine												
IL-2	187.150	206.458	117.439	129.517	0.840	0.477	0.517	0.636	0.819	0.929	0.319	0.443
T_h1-cytokines												
IFN-γ	807.805	9086.212	348.170	806.781	2.130	9.476	0.010	0.041	0.398	0.671	0.076	0.391
TNF-α	57.160	67.962	22.064	40.777	15.483	10.600	0.015	0.054	0.051	0.793	0.073	0.391
T_h1 cell development-promoting cytokines												
IL-12p70	251.537	410.240	131.790	221.758	191.754	263.051	0.331	0.435	0.022	0.144	0.263	0.417
IL-7	670.925	1065.744	448.470	550.294	771.610	722.931	0.953	0.953	0.068	0.272	0.031	0.391
IL-15	83.308	122.052	45.300	80.547	1.690	12.102	0.469	0.125	0.334	0.639	0.090	0.391
T_h2-derived cytokines												
IL-4	30.005	34.038	15.330	21.493	0.270	0.153	0.036	0.104	0.599	0.852	0.131	0.391
IL-5	61.030	138.487	35.520	91.731	0.000	1.128	0.079	0.195	0.182	0.485	0.347	0.463
IL-9	144.895	272.175	89.020	120.152	0.620	3.506	0.158	0.265	0.612	0.852	0.046	0.391
IL-10	67.920	156.754	15.420	104.923	1.988	24.489	0.827	0.913	0.079	0.280	0.205	0.403
IL-13	374.020	699.347	175.880	346.989	230.280	202.504	0.122	0.243	0.918	0.929	0.069	0.391
T_h17-derived cytokines												
IL-17	72.848	201.459	0.000	144.450	1.780	1.011	0.935	0.953	0.248	0.568	0.192	0.403
IL-1Ra	281.030	517.045	120.490	251.410	4.270	4.658	0.111	0.243	0.893	0.929	0.146	0.391
T_h17 cell development-promoting cytokines												
IL-6	2888.395	4942.919	1047.470	3057.601	149.530	205.876	<0.001	0.003	0.023	0.144	0.110	0.391
IL-1β	4.353	4.981	1.140	3.881	5.220	3.766	0.131	0.247	0.859	0.929	0.214	0.403

Table 4a: Cytokine concentrations in aqueous humor of a control group (n=11), patients with primary (n=22) and recurrent (n=29) ocular toxoplasmosis (OT). Comparing primary OT and control group, the following cytokines are elevated: IFN-γ, TNF-α, IL-4 and IL-6. Comparison of recurrent OT and control group shows elevated concentrations of the following cytokines: IL-12p70 and IL-6. Contrasting primary OT with recurrent OT, there are high concentrations of IL-7 and IL-9. A value of $p \leq 0.05$ is considered statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

Table 4b: Chemokine, growth, angiogenetic and wound-healing factor concentrations in aqueous humor of a control group (n=11), patients with primary (n=22) and recurrent (n=29) ocular toxoplasmosis

Immune mediator	Median concentration in patients with primary ocular toxoplasmosis [pg/ml]	Mean concentration in patients with primary ocular toxoplasmosis [pg/ml]	Median concentration in patients with recurrent ocular toxoplasmosis [pg/ml]	Mean concentration in patients with recurrent ocular toxoplasmosis [pg/ml]	Median concentration in patients in control group [pg/ml]	Mean concentration in patients in control group [pg/ml]	p-value (not corrected) primary OT vs control group	p-value (corrected*) primary OT vs control group	p-value (not corrected) recurrent OT vs control group	p-value (corrected*) recurrent OT vs control group	p-value (not corrected) primary vs recurrent OT	p-value (corrected*) primary vs recurrent OT
chemokines												
MIP-1α	260.480	264.694	130.480	242.212	0.000	13.564	0.002	0.014	0.020	0.144	0.666	0.688
MIP-1β	402.710	491.673	278.125	383.333	218.032	244.390	0.027	0.088	0.851	0.929	0.205	0.403
IP-10	597354.600	601495.708	106136	349955.109	5748.012	7563.385	0.001	0.001	0.001	0.001	0.129	0.391
Eotaxin	951.210	1074.703	726.525	661.366	1.950	104.250	0.340	0.435	0.871	0.929	0.252	0.417
IL-8	336.763	551.696	109.880	413.577	56.530	55.860	0.006	0.029	0.377	0.670	0.174	0.403
RANTES	231.333	233.588	81.750	246.664	10.298	14.418	0.003	0.016	0.053	0.241	0.361	0.463
PDGF-bb	309.915	342.921	154.160	255.612	2.230	10.001	0.117	0.243	0.520	0.793	0.298	0.434
growth, angiogenetic and wound-healing factors												
GM-CSF	0.620	76.578	0.000	51.890	1.240	12.424	0.203	0.325	0.118	0.364	0.487	0.578
G-CSF	658.189	4192.988	186.410	1832.356	2.290	181.211	0.151	0.265	0.929	0.929	0.233	0.414
MCP-1	1971.770	3094.140	1536.110	1984.948	957.642	933.948	<0.001	0.005	0.009	0.138	0.139	0.391
VEGF	1184.940	1937.783	904.160	1176.945	1294.330	1195.950	0.266	0.388	0.200	0.492	0.546	0.623
FGF basic	40.765	45.818	26.710	35.643	1.300	24.093	0.634	0.751	0.043	0.231	0.273	0.417

Table 4b: Chemokine, growth factor and angiogenetic and wound healing factor concentrations in aqueous humor of a control group (n=11) as well as patients with primary (n=22) and recurrent (n=29) ocular toxoplasmosis (OT). Comparing primary OT (pOT) with the control group, the following immune mediators are elevated: MIP-1α, MIP-1ß, IP-10, IL-8, RANTES and MCP-1. The following parameters are increased when looking at recurrent OT (rOT) and the control group: MIP-1α, IP-10 and FGF basic. The comparison of immune mediator concentrations of pOT and rOT shows no difference. A value of $p \leq 0.05$ is considered statistically significant.

**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

Tables 4a and 4b give an overall view of the concentrations that were measured for all 27 immune mediators in primary and recurrent OT and in the control group. In the following, the presentation of the results is ordered according to the immune mediator classification:

<u>T cell development-promoting cytokine:</u>

Concerning IL-2, there is no difference of concentrations in aqueous humor detectable when comparing *primary OT* to the control group (p=0.517) and the *recurrent OT* group to the control group (p=0.819). When comparing the concentrations of the *primary OT* to the *recurrent OT* group, concentrations of IL-2 are not different (p=0.319) (Figure 8).

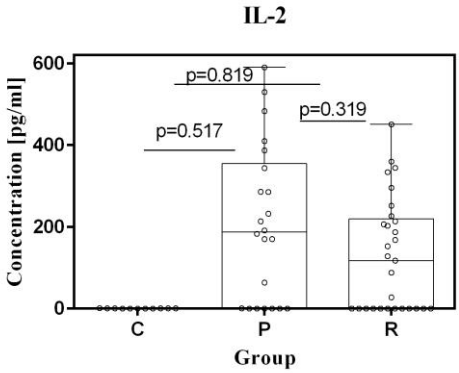


Figure 8: Box plot depicting the cytokine profile of IL-2 in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). Comparing pOT and rOT to the control group and pOT to rOT group, IL-2 does not show any difference in concentrations (p>0.05).

T-helper (T_h) 1-derived cytokines:

Primary OT was characterized by high levels of IFN-γ (p=0.010) and TNF-α (p=0.015) in contrast to the control group. Comparing *recurrent OT* to the control group, TNF-α showed a tendency to be elevated, but it was not statistically significant (p=0.051). Levels of IFN-γ (p=0.398) are not elevated in this context (Figure 9).

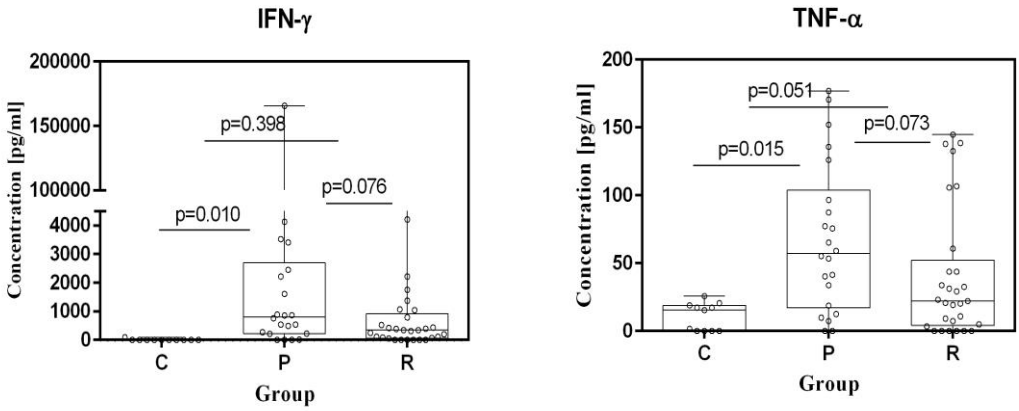


Figure 9: Boxplots showing the cytokine patterns of IFN-γ and TNF-α in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). IFN-γ (p=0.010) and TNF-α (p=0.015) are elevated in pOT when compared to the control group. However, in this relation, IFN-γ shows no raised concentration in rOT (p=0.398), whereas TNF-α reveals a tendency to differ in rOT in terms of an elevated concentration, however, it was not statistically significant (p=0.051). A value of p ≤ 0.05 is defined as statistically significant.

T_h1 cell development-promoting cytokines:

Comparing *primary OT* with the control group, IL-12p70 (p=0.331), IL-7 (p=0.953) and IL-15 (p=0.469) were not elevated. Comparing *recurrent OT* with the control group, IL-12p70 demonstrated an increased concentration in aqueous humor (p=0.022).

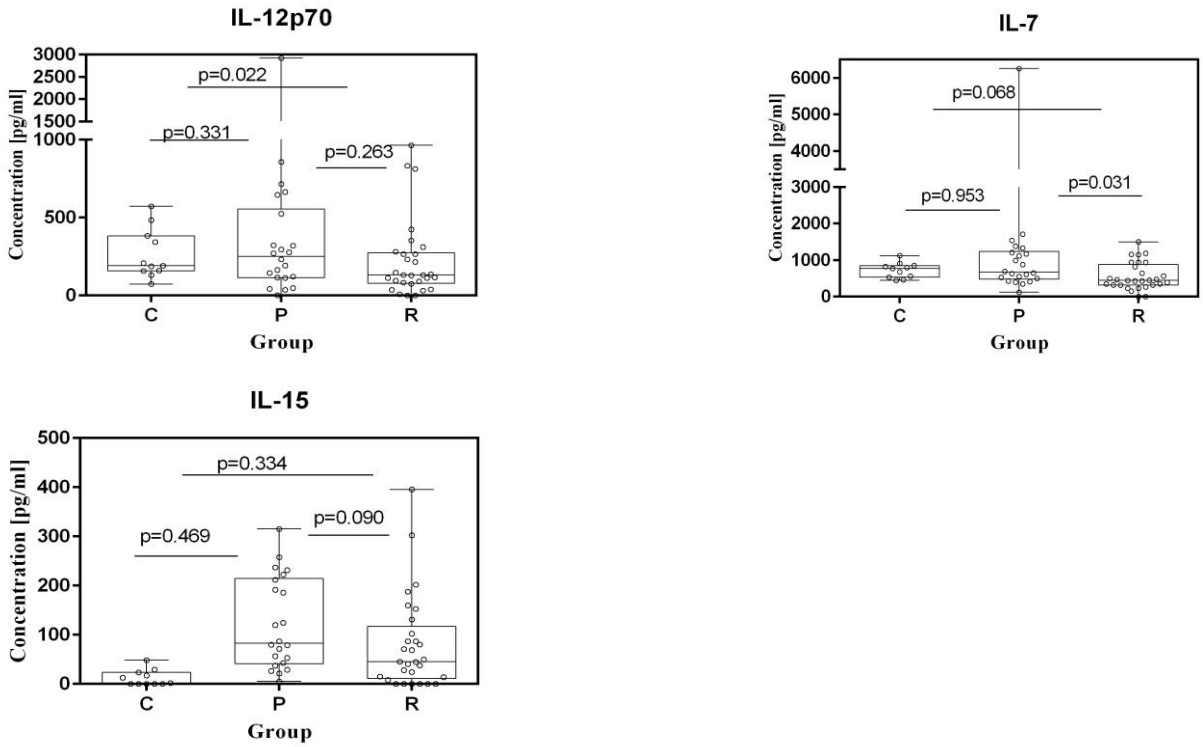


Figure 10: Box plots depicting the cytokine patterns of IL-12p70, IL-7 and IL-15 in aqueous humor comparing results of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). Comparing to the control group, IL-12p70 shows an increased concentration in rOT (p=0.022), showing no elevation in pOT (p=0.331). Contrasting primary and recurrent OT, there is no difference in concentrations of IL-12p70 (p=0.263).
Comparing either the pOT group with controls (p=0.953) or the rOT with controls (p=0.068), there is no difference in concentration of IL-7. Contrasting pOT with rOT, IL-7 is increased in pOT (p=0.031). IL-15 shows no elevation in pOT (p=0.469) and rOT (p=0.334) compared to the control group, and when contrasting pOT with rOT (p=0.090). A value of $p \leq 0.05$ is defined as statistically significant.

Levels of IL-7 (p=0.068) and IL-15 (p=0.334) were not elevated in this connection. Comparing the groups of *primary and recurrent OT*, the concentration of IL-7 (p=0.031) was elevated in the *primary OT* group (Figure 10).

T_h2 – derived cytokines:

Primary OT, in contrast with the control group, was characterized by an elevated concentration of IL-4 (p=0.036). However, IL-5 (p=0.079), IL-9 (p=0.158), IL-10 (p=0.827) and IL-13 (p=0.122) were not elevated in this group (Figure 11).

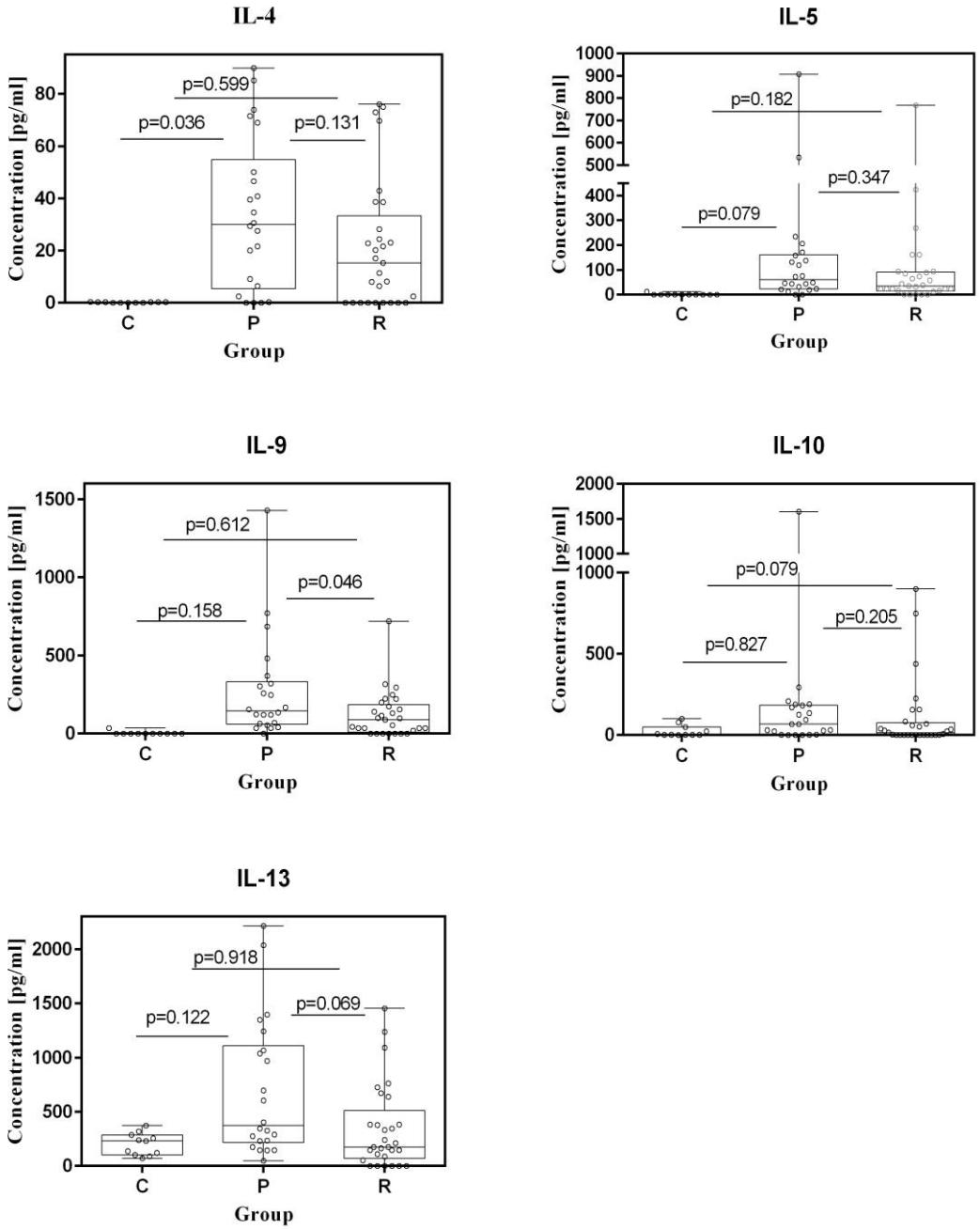


Figure 11: Cytokine patterns of the T_h2-cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 comparing cytokine concentrations in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). IL-4 is elevated in pOT (p=0.036), but shows no difference in rOT (p=0.599) when contrasting with the control group. Moreover, concentrations of IL-5, IL-10 and IL-13 demonstrate no difference comparing pOT and rOT to the control group and the pOT to rOT group (p>0.05). A value of $p \leq 0.05$ is defined as statistically significant.

Comparing *recurrent OT* and control group, IL-4 (p=0.599), IL-5 (p=0.182), IL-9 (p=0.612), IL-10 (p=0.079) and IL-13 (p=0.918) were not increased.

Interestingly, the concentration of IL-9 (p=0.046) was elevated when *primary* and *recurrent OT* are compared (Figure 10). In this context, concentrations of IL-4, IL-5, IL-10 and IL-13 were not risen (p>0.05).

T_h17 cell development-promoting cytokines:

Both *primary* and *recurrent OT* are characterized by an increased concentration of IL-6 (*primary OT*: p<0.001; *recurrent OT*: p= 0.023) compared to the control group (Figure 12). Though, IL-1β does not reveal an elevated concentration in this context (*primary OT* p=0.131; *recurrent OT*: p=0.214). When comparing *primary* and *recurrent OT*, IL-6 and IL-1β show no difference in concentration (IL-6: p= 0.110; IL-1β: p=0.859).

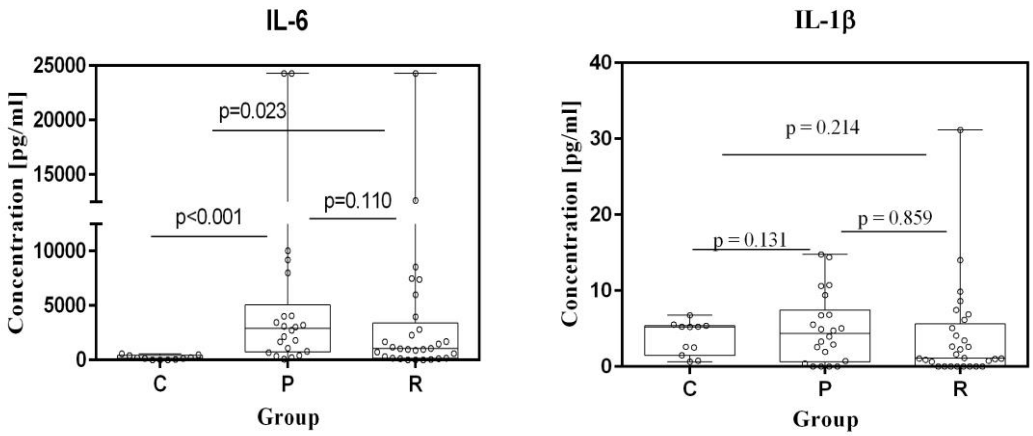


Figure 12: Boxplots for cytokines concentrations of IL-6 and IL-1β with a comparison of their concentrations in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). The concentration of IL-6 is increased in pOT (p<0.001) as well as in rOT (p=0.023) in contrast with the control group. Concentrations of IL-1β reveal no difference comparing pOT to the control group, rOT to the control group and pOT to rOT, respectively (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

T_h17 - derived cytokines:

In *primary OT*, IL-17 (p=0.935) and IL-1Ra (p=0.111) did not differ from concentrations in the control group. Concerning *recurrent OT* in this relation, IL-17 (p=0.248) and IL-1Ra (p=0.893) were not increased (Figure 13).

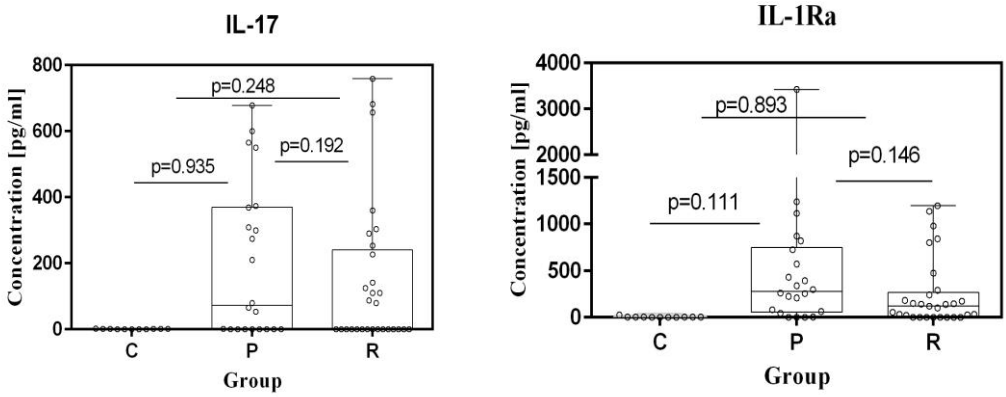
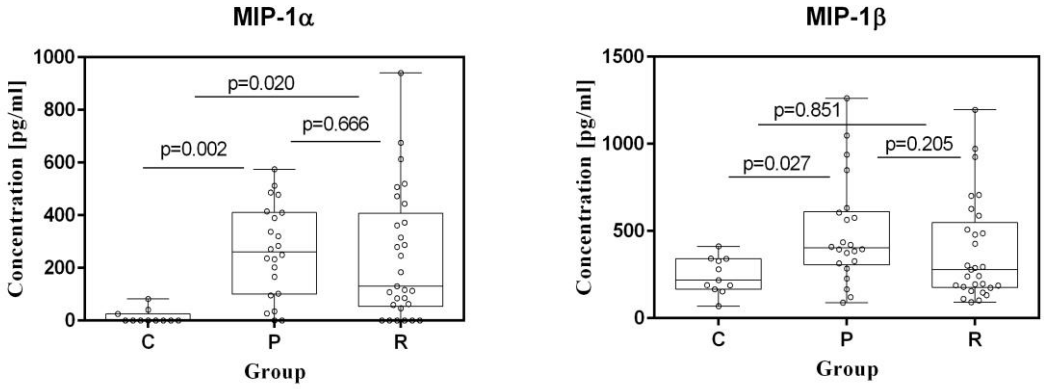


Figure 13: Boxplots with cytokine concentration patterns of IL-17 and IL-1Ra a the comparison of their concentrations in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). Concentrations of IL-17 and IL-1Ra show no difference comparing between pOT and the control group, between rOT and the control group and between pOT and rOT (p>0.05). A value of $p \leq 0.05$ is defined as statistically significant.

Chemokines:

Compared to the control group, our results indicate increased concentrations of MIP-1α (p=0.002) in *primary OT* whereas concentrations of Eotaxin (p=0.340) and PDGF-bb (p=0.117) are not elevated.

There is no difference in concentrations between *primary* and *recurrent OT* for MIP-1α (p=0.666), MIP-1ß (p=0.205), Eotaxin (p=0.252), IP-10 (p=0.129), IL-8 (p=0.174), RANTES (p=0.361), and PDGF-bb (p=0.298) (Figures 14 and 15).



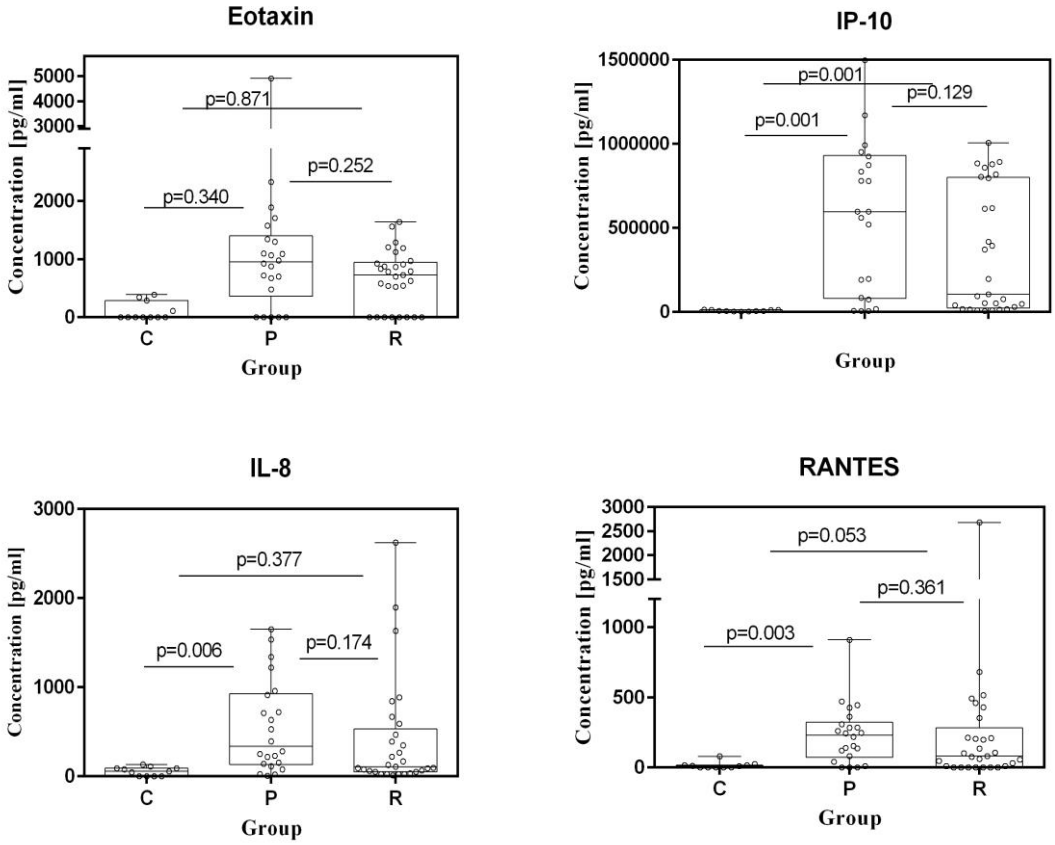


Figure 14: Boxplots with the chemokine concentrations of MIP-1α, MIP-1β, Eotaxin, IP-10, IL-8 and RANTES in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). The concentration of MIP-1α is increased in pOT (p=0.002) and rOT (p=0.020) when contrasted with the control group. In the same relation, concentrations of IP-10 also reveal a distinct elevation in pOT (p<0.001) and rOT (p<0.001). However, there is no difference in IP-10 concentration between pOT and rOT (p=0.129). MIP-1ß and IL-8 are elevated in pOT (MIP-1ß, p=0.027; IL-8, p=0.006) but show no difference in rOT (MIP-1ß: p=0.851; IL-8: p=0.377). Unlike the control group, RANTES is elevated in pOT (p=0.003) and shows a tendency to differ from the control group in rOT in terms of an elevated concentration, however it is not statistically significant (p=0.053). Eotaxin in pOT (p=0.034) and rOT (p=0.871) is not elevated in contrast with the control group. Comparing pOT and rOT, MIP-1a, MIP-1ß, Eotaxin, IL-8 and RANTES are not elevated (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

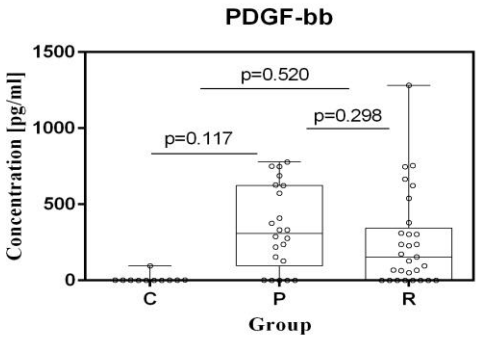


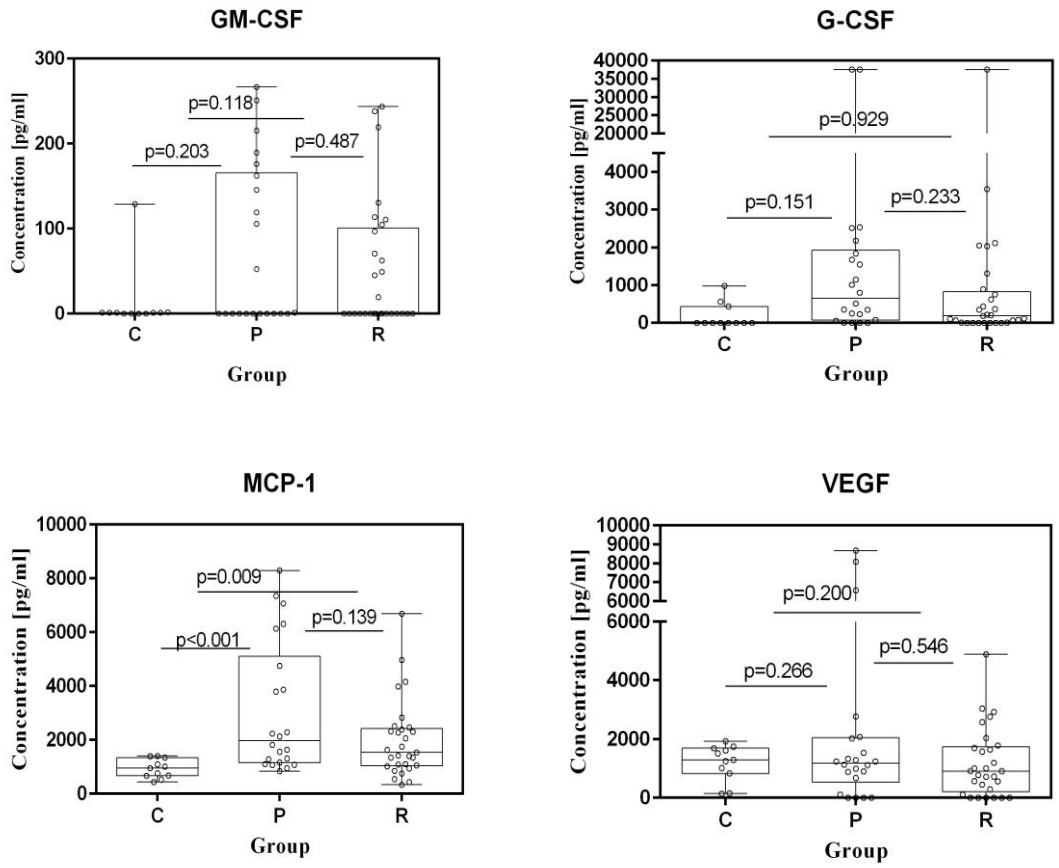
Figure 15: Boxplots showing concentrations of the chemokine PDGF-bb in aqueous humor in a comparison between patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). PDGF-bb in pOT (p=0.117) and rOT (p=0.520) is not elevated compared with the control group. Comparing pOT and rOT, the concentration of PDGF-bb is not elevated (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

Recurrent OT is characterized by elevated concentrations of MIP-1α (p=0.020) and IP-10 (p=0.001). In *recurrent OT*, RANTES shows a tendency to differ from the control group in terms of an elevated concentration, however it is not statistically significant (p=0.053). Moreover, MIP-1ß (p=0.851), Eotaxin (p=0.871), IL-8 (p=0.377), and PDGF-bb (p=0.520) show no difference in concentration between recurrent OT and control group.

Growth, angiogenetic and wound-healing factors:

Contrasting *primary OT* with the control group, concentrations of MCP-1 (p<0.001) are elevated. In this relation, GM-CSF (p=0.203), G-CSF (p=0.151), VEGF (p=0.266) and FGF basic (p=0.634) exhibit no increased concentration.

Results for *recurrent OT* compared to the control group reveal increased concentrations of MCP-1 (p=0.009) and FGF basic (p=0.043). In this context, concentrations of GM-CSF (p=0.118), G-CSF (p=0.929) and VEGF (p=0.200) showed no difference from the control group. Comparing *primary* and *recurrent OT*, GM-CSF (p=0.487), G-CSF (p=0.233), MCP-1 (p=0.139), VEGF (p=0.546) and FGF basic (p=0.273) show no elevated concentration (Figure 16).



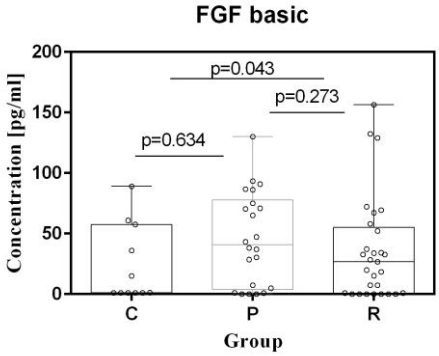


Figure 16: Boxplots showing concentration patterns of the growth, angiogenetic and wound-healing factors GM-CSF, G-CSF, MCP-1, VEGF and FGF basic by comparing concentrations in aqueous humor of patients with pOT (P, n=22) and rOT (R, n=29) and the control group (C, n=11). MCP-1 presents elevated concentrations in pOT (p<0.001) and rOT (p=0.009). But there is no difference in concentrations of MCP-1 when comparing pOT and rOT (p=0.139) In addition, the concentration of FGF basic is increased in rOT (p=0.043) with no difference in pOT (p=0.634) when contrasting with the control group and in the comparison of pOT and rOT (p=0.273). GM-CSF, G-CSF, VEGF and FGF basic exhibit no difference between pOT, rOT and control group (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

4.4. Cytokine ratios do not reveal any shift in the T-helper-cell response when comparing primary and recurrent ocular toxoplasmosis

Ratios of cytokine concentrations were formed for each patient in both groups (*primary* and *recurrent OT*) in order to point out the weighting of T helper (T_h)1, T_h2 and T_h17 cell response among the groups themselves and whether there is a shift of T_h cell response when comparing primary and recurrent OT.

Table 5: Cytokine ratios

		primary OT	recurrent OT	p-value (uncorrected)	p-value (corrected*)
IL-4/IFN-γ	mean	0.029	0.032	0.057	0.628
	median	0.024	0.033		
	standard deviation	0.029	0.029		
IL-10/IFN-γ	mean	0.235	0.010	0.961	0.961
	median	0.050	0.039		
	standard deviation	0.515	0.144		
IL-17/IFN-γ	mean	0.153	0.018	0.637	0.680
	median	0.062	0		
	standard deviation	0.230	0.274		
TNF-α/IL-10	mean	2.676	2.129	0.036	0.391
	median	0.798	0.161		
	standard deviation	8.632	8.632		
IL-12p70/IL-10	mean	10.700	7.998	0.439	0.541
	median	2.838	1.382		
	standard deviation	28.709	20.507		

Table 5: The cytokine ratios indicate a predominant T_h1-response (IL-4/IFN-γ =0.029; IL-10/IFN-γ = 0.670; IL-17/IFN-γ = 0.018; TNF-α /IL-10 = 2.129; IL-12p70/IL-10 = 7.998). There is no shift of T_h-response when comparing primary and recurrent OT (IL-4/IFN-γ: p=0.057, IL-10/IFN-γ: p=0.961, IL-17/IFN-γ: p=0.637; IL-12p70/IL-10: p=0.439. However, TNF-α/IL-10 shows a dominant T_h2-response in recurrent OT in this context (p=0.036). OT = ocular toxoplasmosis; $p \leq 0.05$ is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

Our results in Table 5 reveal that there is predominance of T_h1 cell-response (IFN-γ, TNF-α and IL-12p70) compared to T_h2 cell-response (IL-4, IL-10) and T_h17 cell-response (IL-17) looking at *pOT* (IL-4/ IFN-γ = 0.029 ; IL-10/IFN-γ = 0.235; IL-17/IFN-γ ratio= 0.153 TNF-α/IL-10 = 2.676 and IL-12p70/IL-10 = 10.700) and *recurrent OT* (IL-4/IFN-γ = 0.032; IL-10/IFN-γ = 0.010; IL-17/IFN-γ = 0.018; TNF-α/IL-10 = 2.129 and IL-12p70/IL-10 = 7.998) separately. This data does not reveal a T_h cell response shift for IL-4/IFN-γ (p=0.057), IL-10/IFN-γ (p=0.961); IL-17/IFN-γ (p= 0.637) and IL-12p70/IL-10 (p= 0.439) in *rOT*. However, the cytokine ratio of TNF-α/IL-10 demonstrates that the immune response seems to be shifted to a "more" T_h2 and "less" T_h1 dominated immune mediator pattern in *rOT* (p=0.036).

4.5. Immune mediator profiles of selected patients

The foregoing results give an overall view of selected patients of the study. Examining each patient's immune mediator pattern in correlation with their medical history and clinical findings one by one gives a closer insight into human immune response by means of immune mediator secretion. In the following, a selection of patients with postnatally acquired pOT, recurrent congenital and postnatally acquired OT with few and numerous recurrences is displayed.

4.5.1. 20-year-old female patient with primary ocular toxoplasmosis

The 20-year-old female (patient 1) presented with a "dot" appearing in her visual field on the right eye with accompanying pain, feeling of pressure and noticeable temporal visual defect for six days. There was no apparent redness of the eye. Visual acuity was 1.0 in both eyes. There was no general medical or ophthalmological history of a previous OT episode. A juxtapapillary lesion was visible in indirect ophthalmoscopy (Figure 17). An anterior chamber paracentesis was performed demonstrating antibody synthesis against
T. gondii. However, serologically there was no evidence of antibody synthesis. The patient was administered Clindamycin 4 x 300mg daily systemically.
At a follow-up visit 2 weeks later, the patient did not complain about any visual problems. Still, a 30° visual field test in the further course revealed a juxtapapillary absolute scotoma in the right eye.

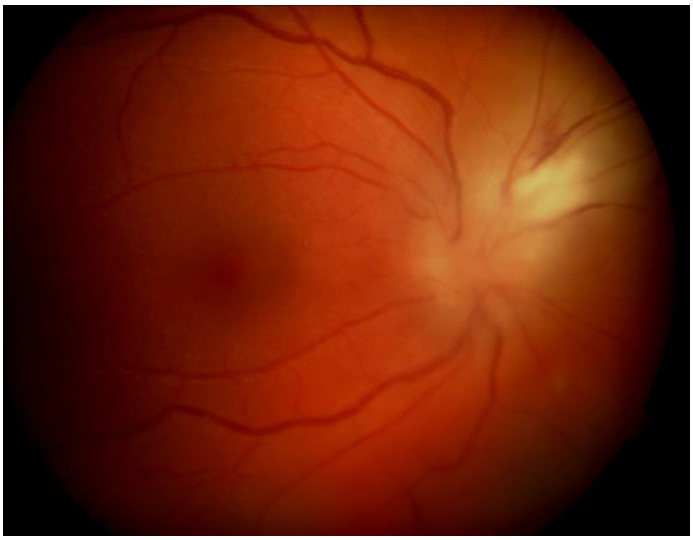


Figure 17: Fundus photograph of the right eye of patient 1 with juxtapapillary active lesion of 1 disc diameter dimension at initial presentation

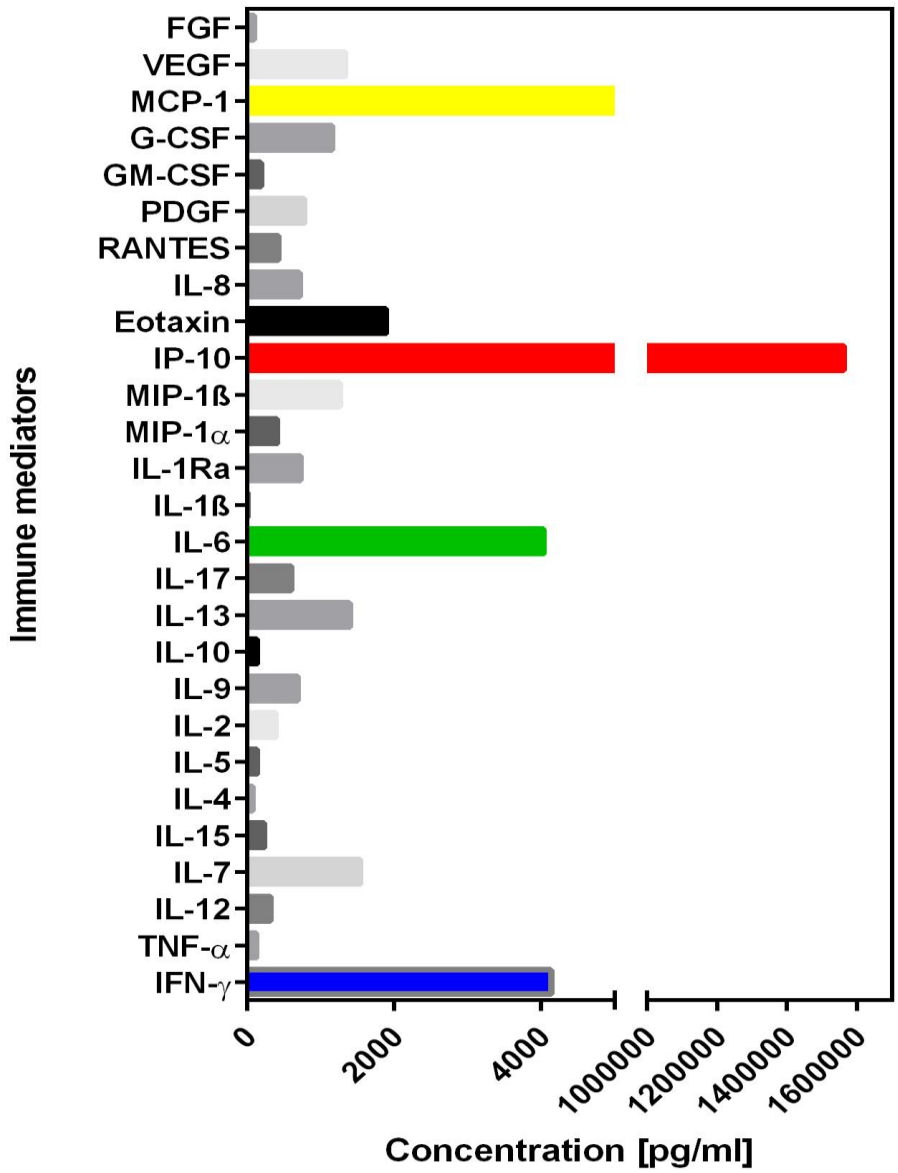


Figure 18: Bar chart showing 27 immune mediators in aqueous humor of patient 1 with acquired primary infection and displaying remarkably high levels of IP-10, IFN-γ, MCP-1 and IL-6.

The immune mediator profile in the patient's aqueous humor (Figure 18) displays elevated levels of IP-10 (1,562,532.883 pg/ml), IFN-γ (4133.217 pg/ml), MCP-1 (6312.167 pg/ml) and IL-6 (4031.367 pg/ml).

4.5.2. 28-year-old male patient with numerous recurrences of ocular toxoplasmosis

The 28-year-old male (patient 2) of Russian origin initially presented with deteriorated vision on both eyes 10 days after a febrile infection. Past ophthalmological history revealed initial retinochoroiditis of the right eye at the age of 10 and regular recurrences each year. The patient had received several intravitreal triamcinolone injections before. Large central scars could be detected via indirect ophthalmoscopy (Figures 19 and 20). Visual acuity was 0.05 on both eyes. A QuantiFERON ® -test was negative. The patient was administered Clindamycin systemically 4 x 300mg daily.

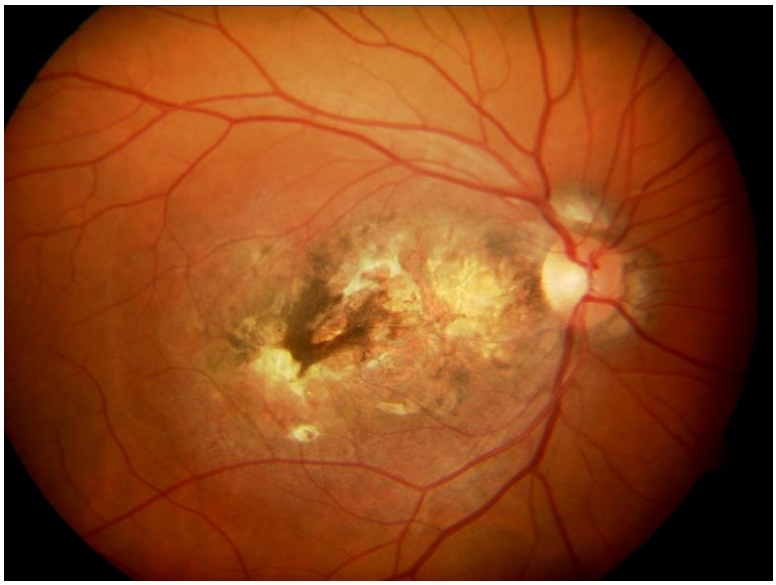


Figure 19: Fundus photograph of the right eye of patient 2 with a centrally located scar.

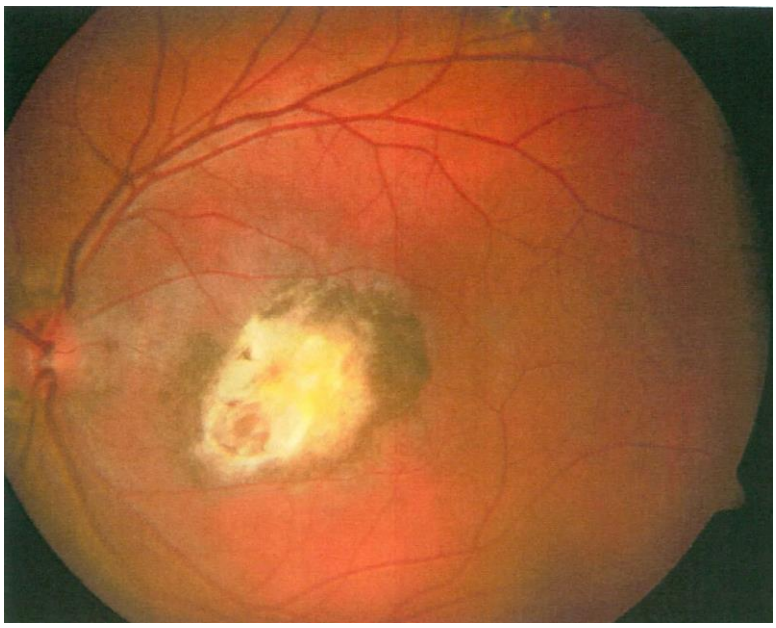


Figure 20: Fundus photograph of the left eye of patient 2 with a centrally located scar

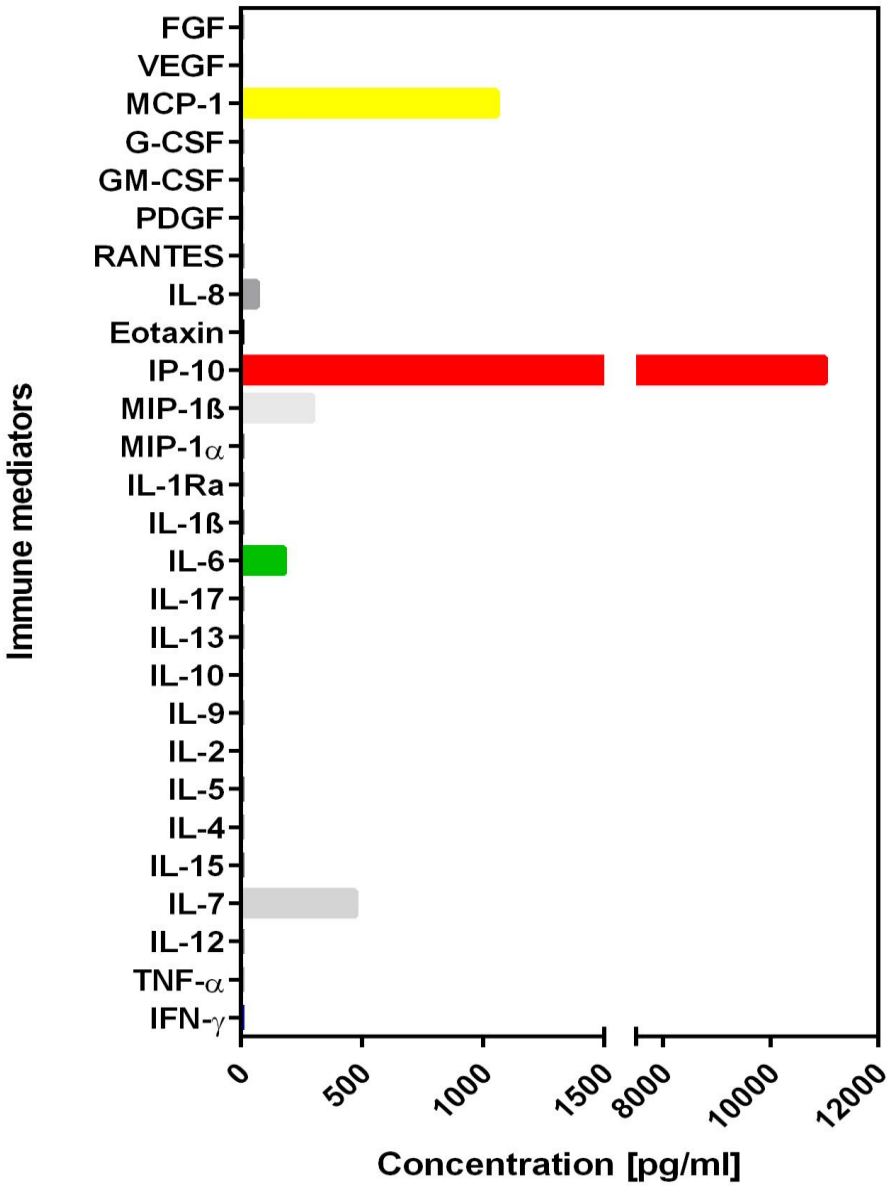


Figure 21: Bar chart showing the pattern of immune mediators in aqueous humor of patient 2, who had 18 recurrences, with generally low immune mediator levels in aqueous humor and elevated levels of IP-10 and concentrations of MCP-1, MIP-1ß , IL-7 and IL-6 are detectable.

This patient's immune mediator pattern in aqueous humor (Figure 21) is remarkable for general low secretion. There are detectable concentrations of MCP-1 (1055.56 pg/ml), MIP-1ß (294.1 pg/ml), IL-6 (178.36 pg/ml) and IL-7 (471.64 pg/ml). T_h1 and T_h2 cytokines are not apparent. A number of immune mediators are not detectable such as IFN-γ (aqueous humor concentration: 0 pg/ml; limit of detection (LOD): 6.4pg/ml), IL-9 (aqueous humor concentration: 0 pg/ml; LOD: 2.5pg/ml) and IL-12p70 (aqueous humor concentration: 0 pg/ml; LOD: 3.5 pg/ml). In contrast, levels of IP-10 are greatly elevated (11,013.06 pg/ml).

4.5.3. 36-year-old male patient with two recurrences of ocular toxoplasmosis

The 36-year-old patient (patient 3) initially presented with pain in the right eye for 5 days. In his past ophthalmological history, a chorioretinitis juxtapapillaris was documented without finding the cause. The patient had no general medical history. Visual acuity was 0.8 on the right eye. An initially performed 30° visual field test displayed an arc-shaped scotoma inferior. Indirect ophthalmoscopy revealed an active lesion 1.5 disc diameter with surrounding scar tissue (Figure 22). The patient was administered Clindamycin systemically 4 x 300mg daily and Prednisolone beginning with a dosage of 75mg followed by a tapering scheme followed. Moreover, he received NSAR-drops and mydriatica. 4 weeks later visual acuity improved to 1.0.

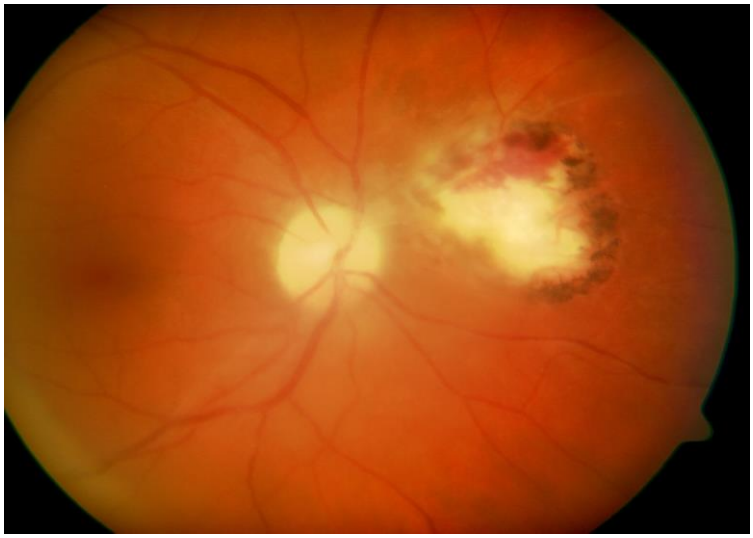


Figure 22: Fundus photograph of the right eye of patient 3 displaying a retinochoroidal active focus of 1.5 disc diameter with a surrounding scar area.

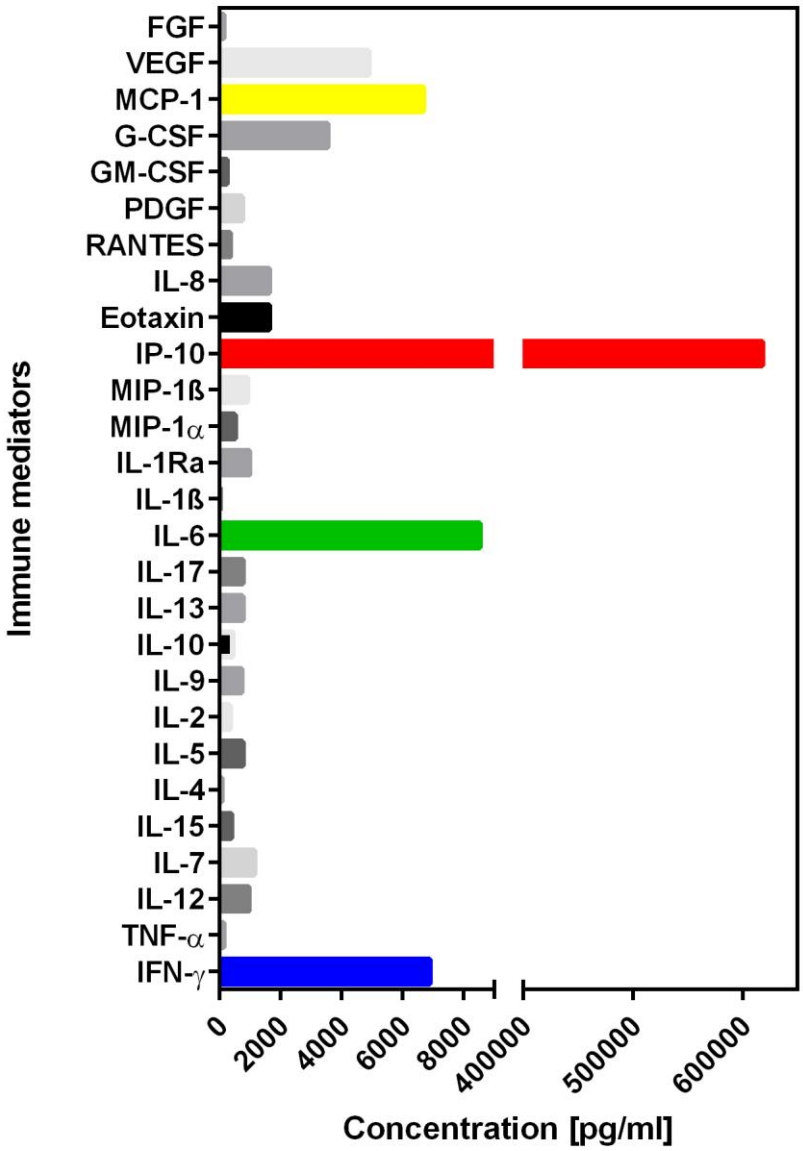


Figure 23: This bar chart displays the immune mediator patterns in aqueous humor of patient 3 with recurrent OT showing high levels of IP-10, IFN-γ, IL-6, VEGF and MCP-1.

In Figure 23, the analysis of aqueous humor of patient 3 revealed elevated levels for IP-10 (617,882 pg/ml), IFN-γ (6,892.685 pg/ml), IL-6 (8539.875 pg/ml), VEGF (4885.75 pg/ml) and MCP-1 (6688.72pg/ml).

4.5.4. 33-year-old female patient with congenital ocular toxoplasmosis

The 33-year-old patient (patient 4) presented for clarification of unspecific visual field defects on both eyes, but predominantly on the right eye. Past ophthalmological history comprises a retinal laser treatment either for retinal degenerations or foramina and extraction of the lens and insertion of lens implants in both eyes. Through indirect ophthalmoscopy a large scar of 8 disc diameter at 12 o'clock with central atrophic area and hyperpigmented edges on the right eye (Figure 24) and a hyperpigmented scar of 0.4 disc diameter at 12 o'clock on the left eye (Figure 25). The patient was administered Clindamycin systemically 3 x 400mg daily.

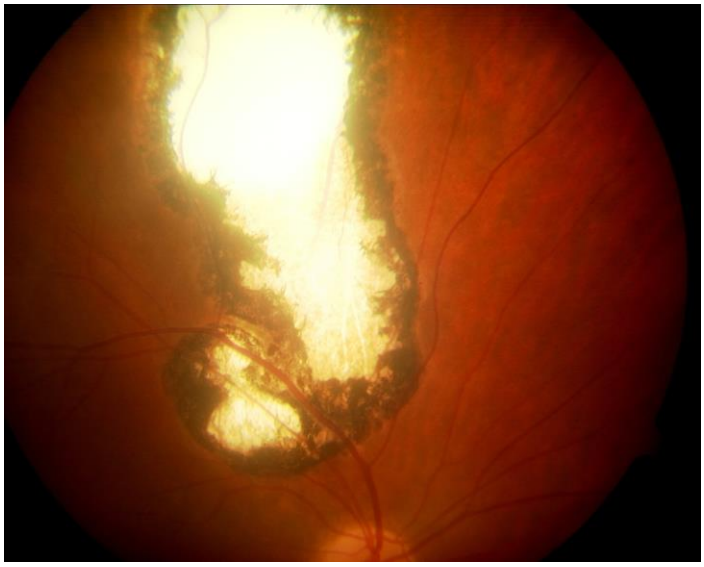


Figure 24: Fundus photograph of the right eye of patient 4 with a large scar of 8 disc diameter at 12 o'clock.

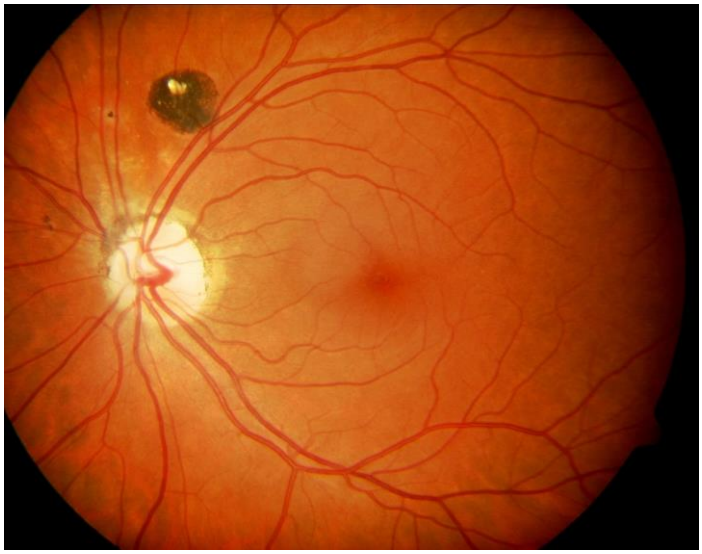


Figure 25: Fundus photograph of the left eye of patient 4 with pigmented scar of 0.4 disc diameter superior to the optic nerve.

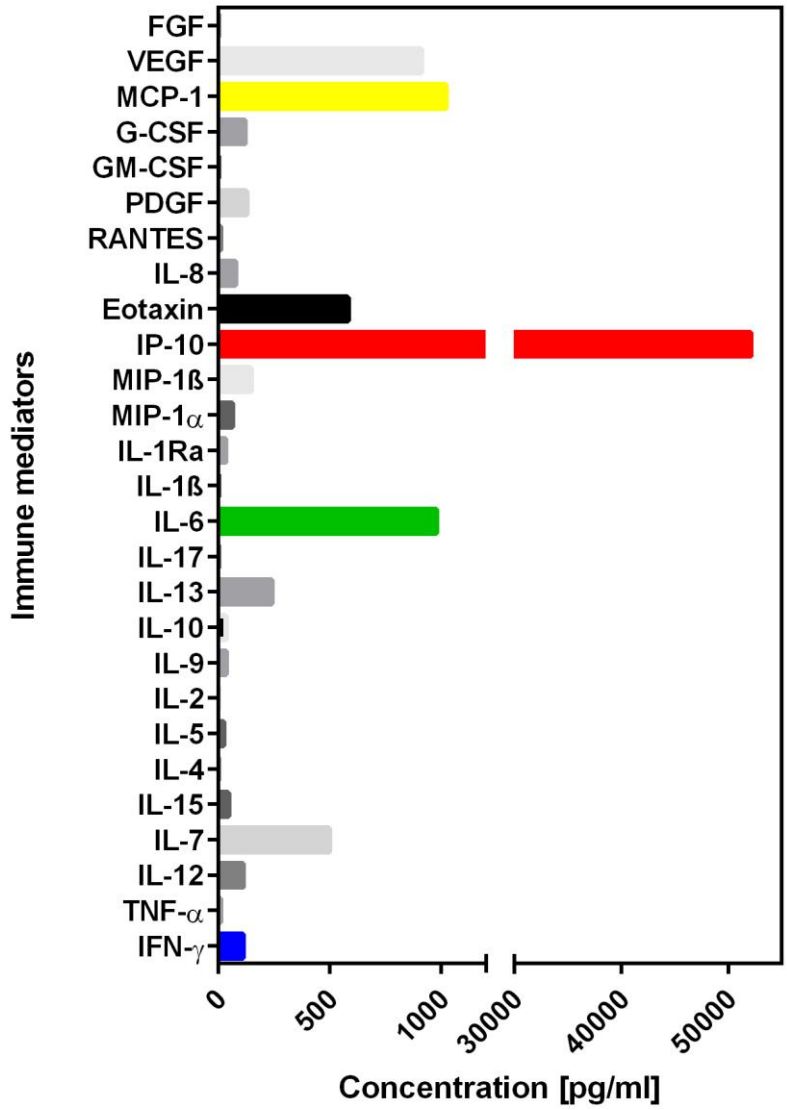


Figure 26: Bar graph showing the profile of immune mediators in aqueous humor of patient 4 with congenital, recurrent OT with low IFN-γ levels, but high levels of IP-10, IL-6, MCP-1 and VEGF.

In Figure 26, the immune mediator profile in aqueous humor of this patient (patient 4) reveals high concentration of IP-10 (52,124.57 pg/ml), but IFN-γ levels are low (111.86 pg/ml). Furthermore, IL-6 (978.6 pg/ml), MCP-1 (1020.61 pg/ml) and VEGF (911.5 pg/ml) are increased.

Although inter-individual variations are evident, certain immune mediator patterns can be observed. The chemokine IP-10 is predominantly present in all of the four patient profiles. Moreover, MCP-1 and IL-6 show high levels in all four patients as well. IFN-γ is absent in the patient with numerous recurrences. Concentrations of IL-7 and IL-9 are detectable in the patient with 2 recurrences (patient profile 3), however, IL-9 is low in patient profile 2 with numerous recurrences.

4.6. Correlations between immune mediator concentrations in human aqueous humor and clinical parameters

In the following, we analyzed whether there are correlations between immune mediator concentrations in aqueous humor and clinical parameters of our study cohort group with *primary* and *recurrent OT* (n=51 OT patients). As clinical parameters we defined age at consultation, age at first manifestation, number of recurrences and size of active lesions.

4.6.1. Correlation between immune mediator concentrations and age at consultation

As age implies changes in the innate and adaptive immune systems, it is of interest to compare immune mediator concentrations in aqueous humor with the patient's age. Table 6 shows the results for concentration of 27 immune mediators in human aqueous humor in correlation with patients' age at consultation at the time of aqueous humor sample collection.

Human aqueous humor concentrations of VEGF (p=0.031), IL-13 (p=0.030), IL-7 (p=0.018) and the IL-10/IFN-γ ratio (p=0.002) correlate positively with age at consultation of patients with pOT and rOT (n=51), though there are no correlations detectable between any of the other tested immune mediators and age at consultation (Table 6). So, with age, these immune mediator levels rise.

Table 6: Correlation between immune mediator concentrations and age at consultation			
	Correlation coefficient	p-value (not corrected)	p-value (corrected)*
IL-4	-0.006	0.968	0.968
MCP-1	-0.008	0.956	0.968
IL-2	0.010	0.947	0.968
Eotaxin	-0.011	0.937	0.968
PDGF-bb	-0.019	0.896	0.968
IL-17/IFN-γ	-0.027	0.849	0.968
TNF-α	0.034	0.811	0.968
IFN-γ	-0.047	0.742	0.950
FGF basic	-0.058	0.685	0.914
IL-9	0.204	0.664	0.914
IL-12p70/IL-10	0.064	0.653	0.914
IL-15	0.305	0.603	0.914
IL-1Ra	0.077	0.591	0.914
TNF-α/IL-10	-0.085	0.554	0.914
MIP-1β	0.087	0.543	0.914
IP-10	-0.088	0.539	0.914
MIP-1α	0.111	0.438	0.877
IL-5	-0.127	0.374	0.798
RANTES	0.128	0.371	0.798
IL-17	-0.131	0.361	0.798
GM-CSF	-0.133	0.351	0.798
G-CSF	0.141	0.325	0.798
Il-1β	0.170	0.234	0.748
IL-6	0.177	0.213	0.748
IL-4/IFN-γ	0.199	0.161	0.646
IL-8	0.204	0.151	0.646
IL-10	-0.062	0.105	0.560
IL-12p70	0.230	0.077	0.496
VEGF	0.302	0.031	0.252
IL-13	0.249	0.030	0.252
IL-7	0.330	0.018	0.252
IL-10/IFN-γ	0.428	0.002	0.056

Table 6: Correlation of immune mediator concentrations in aqueous humor and age at consultation of patients with pOT and rOT (n=51). This table displays that the concentrations of VEGF (p=0.031), IL-13 (p=0.030), IL-7 (p=0.018) and the ratio of IL-10/IFN-γ (p=0.002) are positively correlated to the patients' age at consultation. Correlations for IL-5, GM-CSF, IL-17, IP-10, TNF-α/IL-10, IL-9, FGF basic, IFN-γ, PDGF-bb, Eotaxin, IL-17/IFN-γ, IL-4, MCP-1, IL-2, TNF-α, IL-1Ra, IL-15, IL-12p70/IL-10, MIP-1ß, MIP-1α, RANTES, G-CSF, IL-6, IL-1ß, IL-8, IL-10, IL-4/IFN-γ, IL-12p70 and IL-13 are not statistically significant (p>0.05). A value of p ≤ 0.05 is defined as statisticall y significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

Analyzing pOT separately (n=22), there is a positive correlation between the IL-10/IFN-γ ratio and age at consultation (p=0.025) (Table 6a).

Table 6a Correlation between age at consultation and immune mediator concentrations in the primary OT group

	correlation coefficient	p-value (not corrected)	p-value (corrected)*
TNF-α/IL10	-0.010	0.962	0.962
IL-1β	0.012	0.957	0.962
IL-6	-0.026	0.905	0.962
VEGF	0.041	0.852	0.940
G-CSF	0.045	0.837	0.940
IL-13	0.047	0.833	0.940
MIP-1β	-0.049	0.826	0.940
IL-12p70	0.053	0.810	0.940
RANTES	-0.054	0.808	0.940
IL-10	-0.068	0.759	0.940
MIP1α	-0.117	0.595	0.865
IL-17/IFN-γ	0.131	0.551	0.840
TNF-α	-0.144	0.512	0.819
IL-12p70/IL-10	0.147	0.503	0.819
IL-8	0.158	0.471	0.819
IL-1Ra	-0.160	0.467	0.819
IL-7	0.170	0.439	0.819
FGF basic	-0.171	0.435	0.819
GM-CSF	-0.176	0.421	0.819
IL-15	-0.181	0.408	0.819
IL-2	-0.198	0.366	0.819
Eotaxin	-0.206	0.346	0.819
MCP-1	-0.243	0.265	0.819
IL-17	-0.277	0.201	0.715
IL-4	-0.277	0.200	0.715
PDGF-bb	-0.279	0.197	0.715
IL-9	-0.294	0.173	0.715
IL-5	-0.345	0.107	0.685
IFN-γ	-0.354	0.098	0.685
IP-10	-0.394	0.063	0.672
IL-4/IFN-γ	0.403	0.057	0.672
IL-10/IFN-γ	0.466	0.025	0.672

Table 6a: Correlation of immune mediator concentrations in aqueous humor and age at consultation of patients with pOT (n=22). This table displays that the IL-10/IFN-γ ratio is positively correlated with age at consultation (p=0.025). Correlations for IL-5, GM-CSF, IL-17, IP-10, TNF-α/IL-10, IL-9, FGF basic, IFN-γ, PDGF-bb, Eotaxin, IL-17/IFN-γ, IL-4, MCP-1, IL-2, TNF-α, IL-1Ra, IL-15, IL-12p70/IL-10, MIP-1ß, MIP-1α, RANTES, G-CSF, IL-6, IL-1ß, IL-8, IL-10, IL-4/IFN-γ, IL-12p70 and IL-13, VEGF, IL-13, IL-7 and age at consultation are not statistically significant (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

Analyzing rOT separately (n=29), there is a positive correlation between age at consultation and IL-9 (p=0.041), MIP-1α (p=0.024), IL-1Ra (p=0.021), MIP-1β (p=0.010), RANTES (p=0.010), IL-15 (p=0.009), IL-8 (p=0.007), IFN-γ (p=0.003), IL-6 (p=0.002), IL-12p70 (p=0.002), Eotaxin (p=0.001), G-CSF (p=0.001), IL-7 (p<0.01), IL-13 (p<0.01), IP-10 (p<0.01), MCP-1 (p<0.01), VEGF (p<0.01) and the ratio of IL-10/IFN-γ (p=0.008) (Table 6b).

Table 6b Correlation between age at consultation and immune mediator concentrations in the recurrent OT group

	correlation coefficient	p-value (not corrected)	p-value (corrected)*
IL-1β	0.168	0.322	0.468
IL-17/IFN-γ	-0.015	0.930	0.930
IL-17	-0.021	0.904	0.930
IL-12p70/IL-10	0.043	0.828	0.883
FGF basic	0.050	0.768	0.847
PDGF-bb	-0.058	0.731	0.835
IL-4	0.072	0.673	0.798
IL-10	0.089	0.601	0.739
TNF-α/IL10	0.092	0.587	0.739
TNF-α	-0.121	0.475	0.633
IL-5	-0.126	0.459	0.633
GM-CSF	-0.244	0.146	0.223
IL-4/IFN-γ	0.247	0.141	0.223
IL-2	0.399	0.14	0.223
IL-9	0.337	0.041	0.070
MIP-1α	0.369	0.024	0.045
IL-1Ra	0.378	0.021	0.042
MIP-1β	0.416	0.010	0.021
RANTES	0.420	0.010	0.021
IL-15	0.423	0.009	0.021
IL-10/IFN-γ	0.427	0.008	0.021
IL-8	0.439	0.007	0.020
IFN-γ	-0.479	0.003	0.010
IL-6	0.489	0.002	0.007
IL-12p70	0.485	0.002	0.007
Eotaxin	0.509	0.001	0.005
G-CSF	0.530	0.001	0.005
IL-7	0.562	<0.01	<0.01
IL-13	0.642	<0.01	<0.01
IP-10	0.643	<0.01	<0.01
MCP-1	0.639	<0.01	<0.01
VEGF	0.657	<0.01	<0.01

Table 6b: Correlation of immune mediator concentrations in aqueous humor and age at consultation of patients with rOT (n=29). There is a positive correlation between IL-9 (p=0.041), MIP-1α (p=0.024), IL-1Ra (p=0.021), MIP-1β (p=0.010), RANTES (p=0.010), IL-15 (p=0.009), IL-8 (p=0.007), IFN-γ (p=0.003), IL-6 (p=0.002), IL-12p70 (p=0.002), Eotaxin (p=0.001), G-CSF (p=0.001), IL-7 (p<0.01), IL-13 (p<0.01), IP-10 (p<0.01), MCP-1 (p<0.01), VEGF (p<0.01) and the ratio

of IL-10/IFN-γ (p=0.008) and age at consultation. Levels of IL-1β, IL-17/IFN-γ, IL-17, IL-12p70/IL-10, FGF basic, PDGF-bb, IL-4, IL-10, TNF-α/IL-10, TNF-α, IL-5, GM-CSF, IL-4/IFN-γ, IL-2 are not statistical significant (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

4.6.2. Correlation between immune mediator concentrations and age at first manifestation

In addition to age at consultation, the focus was also on the patient's age at first manifestation of OT in both groups pOT and rOT (n=51, see Table 2). Our analysis reveals no correlation between the 27 immune mediator concentrations and age at first manifestation (Table 7).

Table 7: Correlation between immune mediator concentration and age at first manifestation

	correlation coefficient	p-value (not corrected)	p-value (corrected)*
IL-15	-0.008	0.957	0.957
TNF-α	0.008	0.956	0.957
IL-1Ra	0.013	0.930	0.957
TNF-α/IL-10	0.016	0.917	0.957
IL-9	0.016	0.914	0.957
FGF basic	-0.022	0.886	0.957
IL-2	-0.030	0.844	0.957
MCP-1	-0.035	0.817	0.957
IL-4	-0.038	0.802	0.957
IFN-γ	-0.048	0.751	0.957
IL-12p70/IL-10	0.051	0.736	0.957
Eotaxin	-0.058	0.704	0.957
IL-17/IFN-γ	-0.067	0.656	0.957
IP-10	-0.067	0.656	0.957
RANTES	0.069	0.648	0.957
IL-5	-0.091	0.550	0.957
MIP-1α	0.091	0.546	0.957
GM-CSF	-0.101	0.503	0.957
PDGF-bb	-0.102	0.501	0.957
G-CSF	0.107	0.477	0.957
IL-10	0.125	0.409	0.957
IL-6	0.127	0.400	0.957
MIP-1β	0.129	0.392	0.957
IL-12p70	0.144	0.340	0.957
Il-1β	0.157	0.298	0.957
IL-8	0.168	0.266	0.957
IL-4/IFN-γ	0.169	0.263	0.957
VEGF	0.190	0.206	0.957
IL-17	-0.199	0.184	0.957
IL-7	0.214	0.154	0.957
IL-13	0.235	0.117	0.957
IL-10/IFN-γ	0.245	0.101	0.957

Table 7: Correlation between immune mediator concentrations in aqueous humor and age at first manifestation in pOT and rOT (n=51). There is no correlation between immune mediator concentrations and age at first manifestation for GM-CSF, IL-17, IL-5, IP-10, PDGF-bb, MCP-1, IFN-γ, IL-2, FGF basic, IL-9, TNF-α/IL-10, Eotaxin, IL-4, IL-15, TNF-α,IL-17/IFN-γ, IL-12p70/IL-10, IL-1Ra, MIP-1ß, MIP-1α, IL-10, IL-6, RANTES, G-CSF, IL-12p70, IL-8, IL-4/IFN-γ, IL-1ß, VEGF, IL-13, IL-7, IL-10/IFN-γ (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

In pOT separately analyzed (n=22), the ratios of IL-4/IFN-γ (p=0.44) and IL-10/IFN-γ (p=0.030) are elevated with age at first manifestation, however TNF-α (p=0.029) shows a negative correlation to age at first manifestation (Table 7a).

Table 7a Correlation between age at first manifestation and immune mediator concentrations in the primary OT group

	correlation coefficient	p-value (not corrected)	p-value (corrected)*
TNF-α/IL-10	0.015	0.948	0.948
IL-6	-0.017	0.940	0.948
IL-1β	0.026	0.910	0.948
IL-12p70	0.029	0.899	0.948
VEGF	0.029	0.898	0.948
G-CSF	0.043	0.848	0.948
MIP-1β	-0.050	0.824	0.948
RANTES	-0.053	0.814	0.948
IL-13	0.062	0.785	0.948
IL-10	-0.084	0.712	0.948
MIP-1α	-0.119	0.597	0.868
IL-12p70/IL-10	0.147	0.515	0.785
IL-17/IFN-γ	0.159	0.480	0.768
IL-1Ra	-0.160	0.476	0.768
IL-7	0.168	0.455	0.768
IL-8	0.169	0.451	0.768
IL-15	-0.171	0.447	0.768
GM-CSF	-0.179	0.424	0.768
FGF basic	-0.181	0.421	0.768
IL-2	-0.191	0.393	0.768
Eotaxin	-0.201	0.369	0.768
MCP-1	-0.252	0.257	0.748
IL-4	-0.268	0.227	0.726
IL-17	-0.270	0.225	0.726
IL-9	-0.274	0.216	0.726
PDGF-bb	-0.278	0.210	0.726
IFN-γ	-0.348	0.113	0.603
IL-5	-0.367	0.093	0.595
IP-10	-0.379	0.082	0.595
IL-4/IFN-γ	0.434	0.044	0.469
IL-10/IFN-γ	0.464	0.030	0.469
TNF-α	-0.133	0.029	0.469

Table 7a: Correlation between immune mediator concentrations in aqueous humor and age at first manifestation in pOT (n=22). Concentration of the ratios IL-4/IFN-γ (p=0.044) and IL-10/IFN-γ (p=0.30) are positively correlated with age at first manifestation, however, TNF-α (p=0.029) is negatively correlated with age at first manifestation. TNF-α/IL-10, IL-6, IL-1β, IL-12, VEGF, G-CSF, MIP-1β, RANTES, IL-13, IL-10, MIP-1α, IL-12p70/IL-10, IL-17/IFN-γ, IL-1Ra, IL-7, IL-8, IL-15, GM-CSF, FGF basic, IL-12p70, Eotaxin, MCP-1, IL-4, IL-17, IL-9, PDGF-bb, IFN-γ, IL-5 and IP-10 are not statistically significant (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

In rOT separately analyzed (n=24), IL-6 (p=0.044), IL-12p70 (p=0.022), IL-7 (p=0.017), IL-10 (p=0.013), VEGF (p=0.011) and IL-13 (p=0.002) and the ratio of IL-10/IFN-γ (p=0.016) are positively correlated with age at first manifestation (Table 7b).

Table 7b Correlation between age at first manifestation and immune mediator concentrations in the recurrent OT group			
	correlation coefficient	p-value (not corrected)	p-value (corrected)*
GM-CSF	0.002	0.991	0.991
TNF-α/IL-10	-0.023	0.916	0.946
IL-12p70/IL-10	0.062	0.775	0.827
FGF basic	0.074	0.730	0.806
IL-4/IFN-γ	0.089	0.678	0.775
Eotaxin	0.122	0.571	0.677
IL-5	0.127	0.553	0.677
TNF-α	0.141	0.510	0.653
IL-17	-0.144	0.503	0.653
PDGF-bb	0.149	0.488	0.653
IFN-γ	0.173	0.418	0.608
IL-1Ra	0.190	0.374	0.570
IL-9	0.192	0.369	0.570
IP-10	0.196	0.360	0.570
IL-4	0.199	0.350	0.570
IL-2	0.224	0.292	0.550
IL-1β	0.255	0.229	0.458
IL-15	0.259	0.223	0.458
RANTES	0.263	0.215	0.458
G-CSF	0.263	0.215	0.458
MIP-1β	0.311	0.140	0.373
IL-8	0.332	0.113	0.329
IL-17/IFN-γ	-0.335	0.110	0.329
MIP-1α	0.353	0.090	0.320
MCP-1	0.375	0.071	0.284
IL-6	0.414	0.044	0.201
IL-12p70	0.466	0.022	0.117
IL-7	0.483	0.017	0.109
IL-10/IFN-γ	0.488	0.016	0.109
IL-10	0.501	0.013	0.109
VEGF	0.512	0.011	0.109
IL-13	0.591	0.002	0.064

Table 7b: Correlation between immune mediator concentrations in aqueous humor and age at first manifestation in rOT (n=24). IL-6 (p=0.044), IL-12p70 (p=0.022), IL-7 (p=0.017), IL-10 (p=0.013), VEGF (p=0.011) and IL-13 (p=0.002) and the ratio of IL-10/IFN-γ (p=0.016) are positively correlated with age at first manifestation. GM-CSF, TNF-α/IL-10, IL-12p70/IL-10, FGF, IL-4/IFN-γ, Eotaxin, IL-5, TNF-α, IL-17, PDGF-bb, IFN-γ, IL-1Ra, IL-9, IP-10, IL-4, IL-2, IL-1β, IL-15, RANTES, G-CSF, MIP-1β, IL-8, IL-17/IFN-γ, MIP-1α and MCP-1 are not statistically significant (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.

**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

4.6.2.1. Correlation between age and number of recurrences

We could obtain consistent information about both age at first manifestation in 24 out of 29 patients in the rOT group. In this group patients under 40 years of age have a higher number of recurrences than patients over 40 years of age (Patients under 40 years of age: Mean= 3.57 ± 4.43 recurrences, Median: 2 recurrences, patients over 40 years of age: Mean= 2.1 ± 2.8 recurrences, Median: 1 recurrence; p=0.032).

According to the incidence rate ratio, patients less than 40 years old have a 2.34 times higher risk for recurrence than patients above 40 years old in the rOT group (95%CI 1.38-4.11). The recurrence rate in the group of patients who are less than 40 years old is 0.385 per person and year; which means that in three years, a patient in this group has one recurrence. But the recurrence rate in the group with patients above 40 years old is 0.164 per person and year leading to the conclusion that in ten years a patient in this group has one recurrence.

4.6.3. Correlation between immune mediator concentrations and number of recurrences of ocular toxoplasmosis

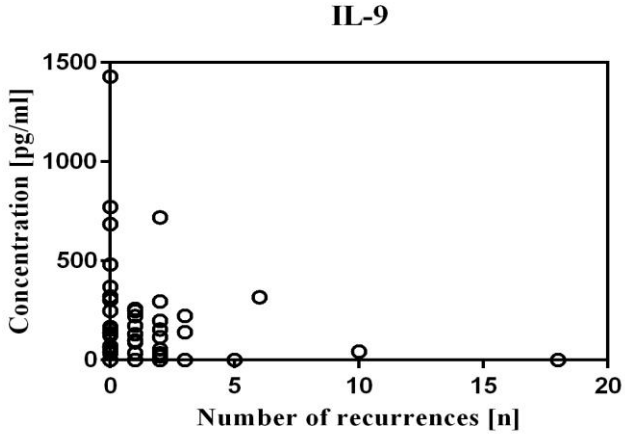
In order to know whether immune mediator concentrations in aqueous humor have an influence on the number of recurrences of OT and have another possible tool to distinguish between the immune mediator profiles in *primary* and *recurrent OT*, we analyzed the correlation between immune mediator concentrations and number of recurrences of patients with pOT and rOT (n=51).

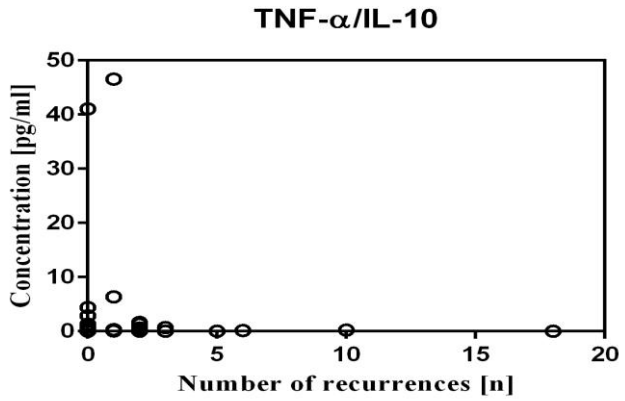
Table 8 : Correlation between immune mediator concentrations and number of recurrences

	Correlation coefficient	p-value (not corrected)	p-value (corrected)*
IL-10/IFN-γ	0.001	0.994	0.994
IL-4/IFN-γ	-0.005	0.972	0.994
IL-17/IFN-γ	-0.019	0.896	0.956
VEGF	-0.037	0.798	0.881
GM-CSF	-0.048	0.738	0.843
IL-12p70	-0.100	0.486	0.576
IL-17	-0.103	0.471	0.576
MIP-1α	-0.132	0.357	0.457
IL-1ß	-0.139	0.332	0.442
IL-2	-0.141	0.323	0.442
IL-10	-0.148	0.303	0.441
PDGF-bb	-0.148	0.301	0.441
IL-12p70/IL-10	-0.150	0.296	0.441
RANTES	-0.165	0.249	0.419
Eotaxin	-0.176	0.217	0.386
MIP-1ß	-0.199	0.162	0.305
FGF basic	-0.203	0.152	0.305
IL-5	-0.205	0.150	0.305
IL-8	-0.214	0.132	0.302
MCP-1	-0.216	0.128	0.302
IP-10	-0.219	0.123	0.302
IL-4	-0.219	0.122	0.302
IL-1Ra	-0.220	0.121	0.302
G-CSF	-0.226	0.111	0.302
IL-15	-0.246	0.082	0.302
IL-13	-0.249	0.078	0.302
IL-6	-0.252	0.074	0.302
TNF-α	-0.255	0.070	0.302
IFN-γ	-0.273	0.052	0.302
IL-7	-0.284	0.043	0.302
TNF-α/IL-10	-0.314	0.025	0.302
IL-9	-0.321	0.022	0.302

Table 8: Correlation between immune mediator concentrations in aqueous humor and number of recurrences in our patients with pOT and rOT (n=51). This table shows negative correlations between number of recurrences of OT and IL-9 (p=0.022), the ratio of TNF-α/IL-10 (p=0.025) and IL-7 (p=0.043). A value of $p \leq 0.05$ is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

The negative correlation between IL-7 as well as IL-9 and immune mediator concentration reveals that higher numbers of recurrences are correlated with diminished immune mediators concentrations. Our results also show that the balance between T_h1 cell response and T_h2 cell response (illustrated by the ratio of TNF-α/IL-10) has a significant negative correlation with recurrences of OT (Figure 28). In our study, we could also detect a significant decrease in immune mediator concentrations in aqueous humor for IL-7 and IL-9 with remarkably low immune mediator concentration at the first recurrent episode (Figure 28).





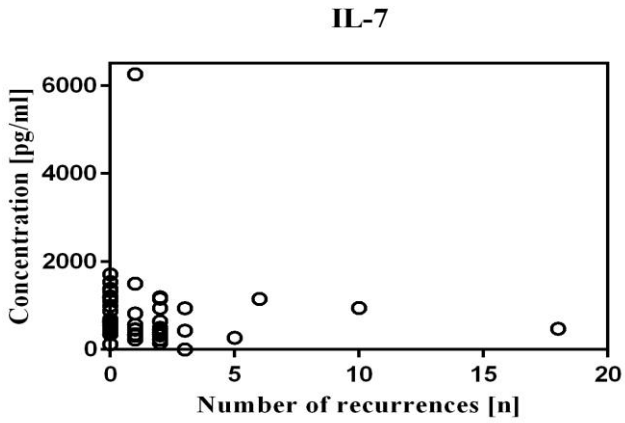


Figure 28: Correlation between cytokine levels and number of recurrences for IL-9 (p=0.022), the ratio of TNF-α/IL-10 (p=0.025) and IL-7 (p=0.043) in aqueous humor of patients with pOT and rOT (n=51). A value of $p \leq 0.05$ is defined as statistically significant.

4.6.4. Correlation between immune mediator concentrations and size of active lesions in the retina

Our study does not show any correlation between the 27 immune mediator concentrations and size of active lesions in the retina of patients with pOT and rOT (n=27, Table 9).

Table 9: Correlation between immune mediator concentrations and size of active lesions

	correlation coefficient	p-value (not corrected)	p-value (corrected)*
TNF-α/IL-10	0.002	0.993	0.993
IL-4/IFN-γ	0.009	0.963	0.993
MCP-1	0.023	0.910	0.969
IL-13	0.027	0.894	0.969
IP-10	0.051	0.799	0.910
IL-2	0.054	0.789	0.910
MIP-1β	0.056	0.781	0.910
PDGF-bb	0.062	0.757	0.910
RANTES	0.065	0.748	0.910
TNF-α	0.065	0.746	0.910
IL-10/IFN-γ	-0.066	0.743	0.910
IL-9	0.067	0.738	0.910
IL-12p70/IL-10	-0.081	0.687	0.910
IL-15	0.081	0.687	0.910
Il-1β	0.083	0.680	0.910
IL-4	0.084	0.676	0.910
IL-1Ra	0.085	0.675	0.910
MIP-1α	0.092	0.648	0.910
IFN-γ	0.098	0.625	0.910
IL-10	-0.100	0.619	0.910
Eotaxin	0.110	0.584	0.910
GM-CSF	0.116	0.564	0.910
IL-17	0.144	0.475	0.910
IL-7	0.147	0.465	0.910
IL-6	0.152	0.448	0.910
G-CSF	0.162	0.420	0.910
IL-17/IFN-γ	0.164	0.415	0.910
IL-5	0.172	0.392	0.910
IL-8	0.185	0.354	0.910
FGF basic	0.190	0.343	0.910
IL-12p70	-0.215	0.281	0.910
VEGF	-0.257	0.196	0.910

Table 9: Correlation between immune mediator concentrations in aqueous humor and size of active lesions in our patients with pOT (n=11) and rOT (n=16). This table shows no correlation between size of active lesions and MCP-1, IL-13, IP-10, IL-2, MIP-1β, PDGF-bb, RANTES, TNF-α, IL-9, IL-15, IL-1β, IL-4, IL-1Ra, MIP-1α, IFN-γ, IL-10, Eotaxin, GM-CSF, IL-17, IL-7, IL-6, G-CSF, IL-5, IL-8, FGF basic, IL-12p70, VEGF and the ratios of TNF-α/IL-10, IL-4/IFN-γ, IL-10/IFN-γ, IL-12p70/IL-10 and IL-17/IFN-γ (p>0.05). A value of p ≤ 0.05 is defined as statistically significant.
**corrected p-value = p-value adjusted for multiple comparisons by controlling the false discovery rate at the same nominal level according to Benjamini Hochberg*

5. Discussion

T. gondii is absorbed in the small intestine and reaches the eye through the lymphatic and vascular system by overcoming the blood/lymphatic retina barrier, leading to the formation of cysts in the retinal tissue. Aqueous humor sampling is becoming increasingly important, not only to supporting the diagnosis and detecting *T. gondii* antibodies, but also allows examining immune mediator levels. Our study included 51 patients with OT and 11 individuals as controls, which is the largest study population to date in which immune mediators have been analyzed in aqueous humor of patients infected with *T. gondii*.

Within this study, we characterized differences between immune mediator patterns in primary and recurrent OT and discussed correlations between immune mediator profiles in aqueous humor and clinical characteristics such as age, number of recurrences and size of active lesions.

In particular, we were interested in the following hypotheses:

5.1. Are there characteristic differences between immune mediator concentrations in aqueous humor samples of patients with primary and recurrent ocular toxoplasmosis?

5.1.1. Primary ocular toxoplasmosis: pathways of IFN-γ activation

In our study, IFN-γ and TNF-α are elevated in aqueous humor samples in patients with primary OT. Concordantly, a study by Meira et al. (2014) measured cytokines in blood samples of patients with CT and OT, finding that blood sera of patients with OT and CT contained high levels of TNF-α compared to patients with chronic toxoplasmosis [97].

Experiments with mice by Sher et al. (1993) provided more precise evidence that TNF-α is important for inducing secretion of IFN-γ, though they admit that TNF-α alone is not sufficient for initiating the secretion of IFN-γ [159]. Another study outlined the importance of TNF-α for initiating macrophages to release reactive nitrogen intermediates [160]. Dealing with the question of how IFN-γ production is induced particularly in pOT, Scharton-Kersten et al. (1996) found in a vaccination experiment that when infection of mice with the strain ts-4 of *T. gondii* occurs, IFN-γ can be produced in both IL-12-dependent and independent pathways [161]. Another *in vitro* study on mice from Denkers et al. (1996) has also shown that there are IL-12 independent pathways for production of IFN-γ by $CD8^+$ T cells interacting with responding cells through the Vβ5 chain as a result of the so-called superantigen activity of *T. gondii* [162]. Apart from the ability of TNF-α to activate IFN-γ in a murine model as mentioned above, two *in vitro* studies on human peripheral blood lymphocytes (PBL) and PBMC have pointed out that IFN-γ production can be initiated

either through IL-12, IL-2 or natural killer cell stimulatory factor (NKSF) [163, 164]. Our results showed low levels of IL-12 in pOT and high levels of IL-12 in rOT which would also point to different mechanisms of IFN-γ activation. In a study with human PBMC cultures, Micallef et al. (1996) proposed that a cytokine named interferon-gamma-inducing factor (IGIF) enhances production of IFN-γ by NK cells [165]. The same study also found that IGIF acts in an IL-2-dependent pathway which is not corroborated by our study which did not reveal elevated IL-2 levels in *primary* or *recurrent OT*. Moreover, Micallef et al. (1996) determined that there is a synergistic effect between IGIF and IL-12 on the production of IFN-γ which is not concordant with our findings [165]. Taken together, in concordance with previous reports, elevated levels of IFN-γ play a major role in the host's immune response to *T. gondii*. The pathways of activation are of current interest and vary considerably.

5.1.2. Decreased IL-7 and IL-9 in aqueous humor of samples with recurrent ocular toxoplasmosis

We found decreased concentrations of IL-7 and IL-9 in the aqueous humor of patients with rOT. A mouse experiment revealed that IL-7 augments $CD8^+$ cell responses against *T. gondii* to survive lethal infection [166]. It was shown that IL-7 specifically targets effector memory $CD8^+$ T cells in the mouse liver through the molecule named programmed cell death-1 (PD-1), which is upregulated in the mouse spleen but downregulated in liver during infection with *T. gondii*, and is responsible for the low responsiveness to IL-7 [167]. Hence, reduced levels of IL-7 in aqueous humor in rOT could demonstrate the inability of the infected patients to cope with *T. gondii* infection. Though, the role of IL-7 in acute and recurrent OT is not distinctly determined. During acute infection with *T. gondii*, absence of both IL-7 and IL-15 in serum can cause an impaired $CD8^+$ T cell response and $CD8^+$ T cells of mice lacking IL-15 were not able to produce IFN-γ and lyse parasites, whereas depletion of IL-7 had no worsening effect [168]. In recurrent disease with *T. gondii*, $CD8^+$ T cell response is IL-15-dependent, resulting in failure of immune responses by $CD8^+$ T cells in both lymphoid tissue and liver (non-lymphoid tissue) when IL-15 is absent, with IL-7 only playing a minor role [169]. Contrary to these findings, a recent mouse experiment could emphasize the function of IL-7 during *T. gondii* infection by illustrating that the combination of IL-7 and IL-15 with DNA vaccination facilitated the humoral immune response against acute and chronic infection [170].

According to our results, IL-9 was decreased in aqueous humor in patients with rOT. Mouse experiments including infection with extracellular parasites such as *Trichuris muris*, *Trichuris spiralis*, *Nippostrongylus brasiliensis* and *Strongyloides stercoralis* have pointed out the function

of IL-9 in controlling mast cells' response, recruiting basophil as well as eosinophil leukocytes and supporting worm expulsion [171-173]. To date, IL-9 has not been known to have a distinct role in the immune response to intracellular parasites such as *T. gondii*. To our knowledge, IL-9 is produced by T_h17, T_h2 and T_h9 cells and Schmitt et al. (1994) found that its production is enhanced by IL-2 and inhibited by IFN-γ [174], suggesting that IL-9 has functions in OT similar to those of the above-mentioned infection with extracellular parasites, a reduction of its concentration in aqueous humor would lead to an impaired ability of the human organism to cope with *T. gondii* infection.

A study found that the cytokine IL-4 stimulates T_h9 cells to secrete IL-9 during human lymphatic filariasis [175]. This result corroborates our findings that IL-4 is elevated in pOT but not in rOT, with decreased IL-9 levels in rOT. By investigating the role of IL-9 in allergic inflammation in the lung, a murine model illustrated that IL-4 levels correlated with levels of IL-9 [176]. The same study also determined that IL-4 and IL-9 co-expression indicates that T cells are in transition between T_h9 and T_h2 cells. This result could also be the case in our patients group with pOT. Our results are not concordant with de-la-Torre et al. (2014), who detected a positive correlation between the number of recurrence and IL-5 and VEGF [127].

Further work measuring IL-7 and IL-9 concentrations during the patient's first consultation and follow-up visits may reveal further insights into the concentrations of these cytokines in the course of OT. In the case of patients with rOT, decreased immune mediator concentrations in the aqueous humor at first consultation may subsequently demonstrate a possible role for IL-7 and IL-9 as suitable markers for recurrence in OT.

Moreover, it would be essential to analyze correlations between genotypes of the *T. gondii* strains and clinical parameters like recurrences, macula involvement, synechiae, vitreous inflammatory level and strabismus in future studies.

5.1.3. The controversial role of IL-17 during *Toxoplasma gondii* infection

In our study, there was no elevated concentration of IL-17 in *primary* and *recurrent OT*. It has been stated that the immune response against *T. gondii* is driven mainly by T_h1 and T_h17 cells in humans [81]. Different models and studies determined the essential role of IL-17 for inflammation. Ye et al. (2001) found that IL–17 is a major cytokine-activating development and recruitment of neutrophil leukocytes in the immune response to murine infection with *Klebsiella pneumoniae*, and there were several *in vivo* studies on mice revealing that a depletion of

neutrophils shortened the time to death after *T. gondii* infection [82, 177, 178]. Also IL-17R-deficient mice were found to have a defect in recruitment of neutrophil leukocytes to the local side of infection with consequent increased parasite burden [123]. Chen et al. (2011) indicated that IL-17 activates the RPE cells and damages their barrier function supporting inflammatory cells entry to the tissue which led to the consideration of intravitreal administration of anti-IL-17 monoclonal antibodies to reduce intraocular inflammation [179]. Sauer et al. (2012) and Kelly et al. (2005) have shown that T_h17 cells are responsible for the inflammatory process in aqueous humor in pOT in mice and humans [126, 180].

In a reinfection model of mice with OT, Sauer et al. (2013) determined that there is a shift from a T_h17 to a T_h1, T_h2 and T regulatory immune response [181]. However, cytokine ratios from our measurements (see Table 5) do not reveal a shift from T_h17 to T_h1 dominant immune response when comparing both *primary* and *recurrent OT*, as our study suggests a T_h1 dominant response in both *primary* and *recurrent OT*, with one cytokine quotient - TNF-α/IL-10 - shifting the immune response to a "more" T_h2 and "less" T_h1-dominated immune mediator pattern in rOT (Table 5). Our results also propose that an overbalance of T_h1 response can lead to a reduced number of recurrences (Figure 28).

Our findings for IL-17 corroborate the results of a Colombian study cohort with OT in a recent study by de-la-Torre et al. (2013) illustrating less IL-17 expression in aqueous humor [21]. Colombian patients in the same study showed a more severe clinical course of OT than in French patients. De-la-Torre et al. (2013) explained the different immune mediator patterns and different clinical characteristics with different *T. gondii* strains that were found in aqueous humor of Colombian and French OT patients. In contrast to de-la-Torre et al. (2013), another study with Brazilian patients detected an increased release of T_h17 cells and IL-17 from PBMC in patients with OT [182]. These findings seem contradictory. In a study in France by Guiton et al. (2010), it was found that IL-17 promotes inflammation rather than interfering with parasite control, showing an inflammatory process in the ileum of mice after oral infection with *T. gondii* as well as high levels of IL-17 release from splenocytes and lymph node cells in mice with severe neuropathology after infection with *T. gondii* [183]. London et al. (2011) pointed out that inflammation is a crucial process that mediates ocular lesion development in patients infected with *T. gondii* [184]. This results is not concordant with our study, as we did not find any correlation between immune mediator concentrations and size of active lesion (Table 9).

5.1.4. Increased levels of IL–6 in primary and recurrent ocular toxoplasmosis

In our study, IL-6 was elevated in *primary* and *recurrent OT*, showing that levels of this cytokine are similarly high in both groups. *In vitro* studies have demonstrated that neutrophil leukocytes have the ability to phagocytose and kill *T. gondii* tachyzoites [185, 186]. IL-6 has been identified as an activator of microbicidal functions in human neutrophil leukocytes [187]. However, IL-6 can also inhibit T_h1 cell differentiation as shown in a murine model [188].

Imbalanced production of IL-6 via the signal pathway through the gp130 receptor subunit can turn IL-6 from a protective to a pathological mediator in systemic *T. gondii* infection as well as in TE [189]. Antibodies against IL-6 reduce inflammation and development of brain cysts and enhance T_h1 cell response in mice [96, 116]. Another mouse experiment revealed that IL-6 could also inhibit the production of IL-12p40 in TE [109, 188]. Our results are not concordant with these findings, as IFN-γ is elevated in aqueous humor of patients with pOT and IL-12 is increased in aqueous humor of patients with rOT.

Moreover, our results with elevated IL-6 levels in aqueous humor in OT patients are consistent with studies on mice and humans with OT. Lyons et al. (2001) detected an elevated IL-6 expression in the vitreous humor, retina and choroid in mice with OT [121]. Further, de-la-Torre et al. (2013) could also show elevated IL-6 levels in the aqueous humor of Colombian OT patients [21].

Several studies on mice have shown that IL-6 promotes the differentiation of T_h17 cells [190, 191]. In this context, we can suggest that in our patients with pOT T_h17 cells' cytokine production is channelled to a secretion of IL-6 rather than IL-17.

5.1.5. Chemokines in primary and recurrent ocular toxoplasmosis

In our study, numerous chemokines were elevated, particularly in pOT. IP-10 is remarkably elevated in our study population as well, though there was no difference in its concentration in aqueous humor between *primary* and *recurrent OT*.

IP-10 reduced parasite burden and triggered nitrite oxide production in an *in vitro* and *in vivo* study on mice infected with *Leishmania amazonensis* and *Leishmania donovani* [192]. However, less IP-10 was produced by human polymorphonuclear leukocytes (PMN) infected with the Leishmania species *Leishmania donovani* and *Leishmania aethiopica* [193]. A study conducted on mice infected with *T. gondii* has shown that IP-10 played an important role in chronic infection through the maintenance of T cell populations and IFN-γ mRNA expression as well as the control

of parasite growth [194]. Our results suggest that IP-10 may have an importance in *primary* and *recurrent OT* in trafficking T cells into infected tissues in humans, as it was previously described in mice with systemic *T. gondii* infection [137, 138].

Our study revealed elevated concentrations of MIP-1α in the aqueous humor in both *primary* and *recurrent OT*. It should be noted that the role of MIP-1α in OT has not been understood yet. Previous studies on mice described participation of MIP-1α in the immune response to systemic *T. gondii* infection by means of neutrophil leukocyte, macrophage and monocyte infiltration into the organs targeted by other parasites (*Trypanosoma cruzi*) and bacteria (*Mycobacterium avium*) [195-197].

To date, the effect of MCP-1 on OT has not been described. Our results suggest that MCP-1 may contribute to the immune response in *primary* and *recurrent OT*. A study on human peripheral blood-derived macrophages infected with *Leishmania infantum* revealed that both MIP-1α and MCP-1 can enhance nitric oxide production and leishmanicidal ability [198]. Similar affects of MIP-1α and MCP-1 are possible in *primary* and *recurrent OT* in our study. A study on mice infected with the extracellular parasite *Trichuris spiralis* could show that MCP-1 supported worm expulsion out of the gut by induction of mucosa permeability [199].
A mouse model study with TE reported elevated RANTES, MIP-1α, MIP-1β and MCP-1 in the cerebrospinal fluid of mice, which corroborates to our findings for RANTES, MIP-1α and MCP-1 in aqueous humor of our patients [128]. MIP-1β was elevated neither in *primary* nor in *recurrent OT* in our study.
Moreover, we found elevated IL-8 levels in pOT. Studies have investigated the role of IL-8 in the process of coagulation in the blood, detecting an association with recurrent venous thrombosis [200, 201]. IL-1-dependent IL-8 secretion has been discussed before. A study has shown that activated protein C (APC)-mediated upregulation of IL-6 and IL-8 is independent of IL-1, and Protein S (a cofactor for APC) enhances APC to upregulate IL-6 and IL-8 production in human umbilical vein endothelial cells (HUVEC) [202, 203]. However, these results are not in agreement with a study on human colon adenocarcinoma cells and cervix epithelioid carcinoma cells infected with *T. gondii*, revealing that IL-8 secretion is IL-1 dependent [129].
IL-1-independent production of IL-8 could be possible in the pOT group in our study.

5.2. Do immune mediator levels in aqueous humor depend on the patient's age? Is the expression of immune mediators decreased in older age and increased in younger age?

Human age influences the immune response against outside pathogens. A study by Kohut et al. (2004) detected low levels of TNF-α and IL-12 in mouse blood [204]. Another animal study on beagles revealed a decrease in $CD8^+$ and $CD4^+$ cells with age [205]. McLachlan et al. (1995) found that levels of IL-1 and reactive oxygen species are decreased in human blood, leading to impaired responsiveness to tumor cells or intracellular microorganisms [206]. Contrary to these findings, a Dutch study detected that human serological levels of TNF-α and IL-6 increase with age and suggested that these two cytokines could be seen as predictors of mortality [207]. A study in Brazil found that an age of above 40 years is a risk factor for developing OT [7]. In addition to this finding, studies from the USA and Canada have had similar results with patients over 50 years old being predisposed to the development of ocular lesions in OT [208, 209].

In our study, we analyzed the correlation between age at first manifestation of pOT as well as rOT and immune mediator concentrations in aqueous humor, but no correlation could be detected. Moreover, looking at correlations between age at consultation and immune mediator concentrations in aqueous humor of pOT and rOT IL-7, IL-13 as well as VEGF and IL-10/IFN-γ show a positive correlation (Table 6). Subgroup-analysis revealed that in pOT there is a positive correlation between IL-10/IFN-γ and age at consultation indicating a T_h2 overbalance with less inflammation with age (Table 6a). In rOT, immune mediator concentrations of IL-9, MIP-1α, IL-1Ra, MIP-1β, RANTES, IL-15, IL-10/IFN-γ, IL-8, IFN-γ, IL-6, IL-12p70, Eotaxin, G-CSF, IL-7, IL-13, IP-10, MCP-1 and VEGF increase with age (Table 6b). Our results may suggest that the greater the number of recurrences of OT is, cytokine concentrations of IL-7, IL-9 and the TNF-α/IL-10 ratio decrease (Figure 28). Our findings suggest that IL-7, being responsible for T cell development, could be a factor for inducing maintenance of cytokine/chemokine production with age. However, IL-7 should be seen as only one out of many factors responsible for maintaining immune mediator levels in advanced age. Moreover, it is possible that in older patients, the regulatory function of IL-10 overbalances the T_h1-response. Interestingly, IL-10 was not elevated in pOT or rOT in our study. De-la-Torre et al. (2014) revealed a positive correlation between patients' age and concentration of IL-12, TNF-α, IL-7, IL-4, IL-17, IL-1β and IL-1Ra in aqueous humor in OT patients, without distinguishing between *primary* and *recurrent OT* [127].

5.3. Does younger age at first manifestation correlate with a higher number of recurrences in OT?

In our study, rOT is characterized by a low concentration of immune mediators in the aqueous humor. Table 8 shows that a negative correlation is present for all our tested immune mediators (except the IL-10/IFN-γ ratio), but is statistically significant for IL-7, IL-9 and the TNF-α/IL-10 ratio. Garweg et al. (2008) stated that patients under 21 years at the time of the first manifestation have a higher risk for recurrence [35]. This contrasts the results of Holland et al. (2008), who detected a peak age of recurrence at older than 40 years [34]. The mean age when the first manifestation occurs is 34 years for our study population, being comparable to the mean age of 25 and 30 years published by Friedmann et al. and Gilbert et al. [210, 211]. Garweg et al. (2008) found that patients in Europe appear to be at a mean age of 24 years at first ocular manifestation of Toxoplasmosis [35]. Moreover, Arantes et al. (2015) found that patients older than 40 years have a higher risk of developing a more severe retinochoroiditis [45].
In our study, the mean age at first manifestation for our rOT group is 25 ± 15 years which is comparable to Gilbert et al. (1999) and Friedmann et al. (1969). However, contrary to Holland et al. (2008), we found that patients younger than 40 years old have a higher recurrence rate than patients older than 40 years old (see chapter 4.6.2.1.) [34]. Our study's results are in agreement with Garweg et al. (2008), but not with Holland et al. (2008) [34, 35].

Previous studies have investigated the effect of aging on the immune system. Mouse experiments found that macrophages as well as monocytes secrete less cytokines with age [204, 212]. Another murine model study even found that TLR expression on macrophages is low in aged macrophages [213]. In humans, there was also found a decline of naïve T cells, a decrease in diversity of T cells, change of B cell compartment composition and a decrease of function of neutrophils, macrophages, NK cells as well as dendritic cells with aging [214]. According to this, one would conclude that young patients would be capable of initiating an appropriate immune response. But our results contradict this. Reasons for recurrences in young patients could be malnutrition, stress or an unknown genetic defect in the immune system (so called primary immune deficiency) which have been shown to lead to an impaired immune response and higher susceptibility to infections [215-219]. Our results state that with increasing age at first manifestation, immune mediator levels are elevated in aqueous humor in rOT (Table 7b) These findings contradict the statement that older individuals have a lower secretion of immune mediators. In pOT, however, levels of TNF-α

correlate with young age (Table 7a) so that younger individuals seem to have a greater inflammatory response.

5.4. Are concentrations of immune mediators elevated in samples with larger size of active lesion?

Our findings reveal that there is no correlation between immune mediator concentrations and size of active lesions (Table 9). These findings contradict to de-la-Torre et al. (2014), who showed positive correlations between the size of active lesions and concentrations of IFN-γ, TNF-α, IL-7, IL-4, IL-13, IP-10, IL-1β, IL-1Ra, MIP-1α, MIP-1β, RANTES as well as FGF basic in aqueous humor [127].

5.5. Is secondary chemoprophylaxis recommended to reduce the risk of recurrence of ocular toxoplasmosis?

Holland et al. (2004) and Reich et al. (2015) detected a high risk of recurrence in the first year after the most recent episode and a reduction of the risk of recurrence with the duration of the disease [220, 221]. Reich et al. (2015) also found that antibiotic treatment reduces the risk of recurrence of OT [221]. Silveira et al. (2002) conducted a prospective, randomized, open-label trial with administration of trimethoprim-sulfamethoxazole (dosage 160-800mg) every three days and a follow-up time of 20 months that showed a significant reduction of recurrences in the treatment groups [222]. However, in the study of Silveira et al. (2002) trimethoprim-sulfamethoxazole was discontinued after 20 months and in the 10-year-follow-up of this study Silveira et al. (2015) showed that there is the same risk of recurrence for OT due to discontinuation of the treatment [72, 222]. On the other hand, Felix et al. (2014) conducted a double-masked randomized placebo-controlled study with administration of trimethoprim-sulfamethoxazole (dosage 160-800mg) every two days for 45 days and a one-year-follow-up, which revealed no recurrences in the treatment group [73, 222]. A case series of 11 patients by Rothova et al. (1998) found that even though azithromycin or atovaquone are good therapy options for acute OT, they do not play a role in preventing recurrences in a one-year-follow-up after being applied for five weeks and then discontinued [223].

A two-year-follow-up study with administration of Clindamycin plus Dexamethasone as an intravitreal injection every two weeks with up to three injections in total showed the same

recurrence rate as with the therapy regime of pyrimethamine 25mg daily combined with sulfadiazine 500mg every six hours and folinic acid daily for a period of six weeks [224]. Here, advantages of intravitreal treatment with Clindamycin and Dexamethasone were reported with fewer follow-up visits and hematologic evaluations. Similar results were found by a study by Baharivand et al. (2013) [225].

Continuous long-term antibiotic treatment could lead to a reduction of recurrences in OT, however, a consistent scheme for the choice of antibiotic drug has not been found to date.

6. Conclusion

The measurement of immune mediator profiles in aqueous humor samples from patients infected with *T. gondii* could aid in characterizing and identifying patients at risk of a severe course of OT or developing recurrent OT. In our findings, concentrations of IL-7 and IL-9 in aqueous humor were decreased in patients with rOT at the time of aqueous humor sampling, which may suggest that those cytokines could serve as risk marker for recurrence. Thus, in accordance with several studies, an antibiotic chemoprophylaxis could be considered for these patients. Nevertheless, prospective studies need to be carried out to validate our results and to measure IL-7 and IL-9 over the course of the disease. This would provide more information as to whether IL-7 and IL-9 concentrations are decreased in the follow-up time as well and whether this correlation persists over time. Prospective studies confirming our results are also necessary in the context that we cannot rule out that whether our patients in the rOT group had low levels of IL-7 and IL-9 from the beginning of infection with *T. gondii*. With more confirming data, the overall duration of systemic therapy, secondary chemoprophylaxis and follow-up visits could consequently be adapted to improve treatment and monitoring of the patient. This way, patients with a severe course of OT with recurrences may possibly be identified at the first consultation and incureable damage to the retina may be prevented. Studies have shown that antibiotic chemoprophylaxis can impede recurrences if applied continuously. Depending on the antibiotic's side effects spectrum and the patient's health status, e.g. other illnesses, the appropriate antibiotic may be administered long-term without discontinuation. There is good evidence for the application of trimethoprim-sulfamethoxazole as a long-term therapy for preventing the recurrence of OT if applied continuously [72, 73, 222, 226].

To conclude, discontinuation of antibiotic treatment even after long-term application leads to no reduction of recurrences of OT. Consequently, long-term treatment without discontinuation is necessary in this context. However, a discontinuation of long-term treatment can also result out of illnesses or incidences in the patient's life. Also, the choice of antibiotic drug for long-term treatment is not consistently clear at this point of time. Therefore, the identification of predictive factors can be of particular interest and cytokine profiles during a consistent time period with particular consideration of IL-7 and IL-9 could possibly give a hint of patients at risk of OT recurrences.

7. Limitations of the study

There are limitations of the study that have to be considered. First, clinical data was obtained from the medical records which were often not complete. In addition, the age distribution of the control group compared to the primary and recurrent OT group shows a large difference that is due to the nature of cataract patients, who were on average, over 60 years old. Moreover, we did not perform genotyping of the *T. gondii* strains that infected the eyes of the patients and therefore we could not outline strain-dependent influences on immune mediator concentrations in aqueous humor. In our study, we can see that immune mediators have a complex interaction scheme.

In conclusion, a statement about the previous and further course of the immune mediator concentration over time cannot be made, as our study design comprised one measurement of immune mediator concentrations in aqueous humor in each patient.

8. List of abbreviations

APC	activated protein C
CT	cerebral toxoplasmosis
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gp130	glycoprotein 130
HUVEC	human umbilical vein endothelial cells
IFN	interferon
IGIF	interferon-gamma-inducing factor
IL	interleukin
IP-10	interferon-γ-induced Protein 10
LOD	limit of detection
MCP -1	monocyte chemoattractant protein 1
MIP – 1	macrophage inflammatory protein 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
NKSF	natural killer cell factor
OT	ocular toxoplasmosis
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death-1
PDGF	platelet-derived growth Factor
PMN	polymorphonuclear leukocytes
RANTES	regulated on activation, normal T cell expressed and secreted
RPE	retinal pigment epithelium

SA-PE	streptavidin-phycoerythrin
TE	toxoplasma encephalitis
T. gondii	*Toxoplasma gondii*
T_h cells	T helper Cells
TNF	tumor necrosis factor
TLR	toll-like receptor
VEGF	vascular endothelial growth factor

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12. Affidavit and Declaration of any eventual publications

"I, Eve-Claudia Thieme, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Immune mediators in samples of aqueous humor in patients with primary and recurrent ocular toxoplasmosis". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

Eve-Claudia Thieme had the following share in the following publications:

Publication 1: Poster: Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U „The intraocular cytokinome is linked to clinical characteristics in human ocular toxoplasmosis". Conference of the ARVO (Association for Research in Vision and Ophthalmology) in Denver, USA, 03.-07.05.2015, Contribution in detail: involvement in the measurements in the laboratory (Bioplex Immunoassay), independent aquisition and organisation of retrospective data, involvement in the statistical analysis, involvement in drafting the poster, independent presentation of the poster

Publication 2: Talk: Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U „Zytokinprofil im Kammerwasserproben mit Erstmanifestation und Rezidiv einer okulären Toxoplasmose" on the winter meeting of BBAG (Berlin-Brandenburgische Augenärztliche Gesellschaft), Berlin, Germany, 5.-6.12.2014, Contribution in detail: involvement in the measurements in the laboratory (Bioplex Immunoassay), independent aquisition and organisation of retrospective data, involvement in the statistical analysis, involvement in drafting the talk structure and presentation slides, independent presentation of the talk

Publication 3: Talk: Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U, „Aqueous humor cytokine profile of patients with primary or recurrent ocular toxoplasmosis“ on the conference EVER (European association of vision and eye research) Nice, France, 1.-4.10.2014, Contribution in detail: involvement in the measurements in the laboratory (Bioplex Immunoassay), independent aquisition and organisation of retrospective data, involvement in the statistical analysis, involvement in drafting the talk structure and presentation slides, independent presentation of the talk

Signature of the doctoral candidate	Signature, date and stamp of the supervising university teacher

13. Curriculum vitae and scientific publications

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Scientific publications:

Claudia Eve Thieme, Sibylle Winterhalter, Bianca Apitzsch und Nicole Stuebiger. Vogt-Koyanagi-Harada-Syndrome concurrent with Morbus Darier. J Rheum Dis Treat 2016, 2:036, Volume 2, Issue 2.

Moore NA, Harris A, Wentz S, Verticchio Vercellin AC, Parekh P, Gross J, Hussain RM, Thieme C, Siesky B. Baseline retrobulbar blood flow is associated with both functional and structural glaucomatous progression after 4 years. Br J Ophthalmol. 2016 Jun 13, 0:1-4.

Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U. The intraocular cytokinome is linked to clinical characteristics in human ocular toxoplasmosis. Poster presentation at the Conference of the Association of Research and Vision in Ophthalmology (ARVO) in Denver, USA 03.05.2015- 07.05.2015

Kagemann L, Thieme C, Ishikawa H, Wollstein G, Davis R, Loewen N, Conner I, Jonescu-Cuypers C, Schuman JS. Selective Laser Trabeculoplasty effect on trabecular meshwork. Poster presentation at the Conference of the Association of Research and Vision in Ophthalmology (ARVO) in Denver, USA 03.05.2015- 07.05.2015

Tobe L, Harris A., Verticchio Vercellin A, Eckert G, Thieme C, Hussain R, Geffen N, Wentz S, Wang J, Siesky BA. Lower retinal capillary blood flow predicts structural glaucoma progression after 5 years. Poster at the Conference of the Association of Research and Vision in Ophthalmology (ARVO) in Denver, USA 03.05.2015- 07.05.2015

Wang J, Harris A, Verticchio Vercelln A, Eckert G, Thieme C, Hussain R,
Gama W, Wentz, Siesky BA Lower baseline retinal capillar blood flow predicts functional glaucoma progression in obese patients with open-angle glaucoma. Poster at the Conference of the Association of Research and Vision in Ophthalmology (ARVO) in Denver, USA 03.05.2015-07.05.2015

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Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U. Zytokinprofil in Kammerwasserproben mit Erstmanifestation und Rezidiv einer okulären Toxoplasmose. Talk presentation at the winter meeting of the Association of Ophthalmologists in Berlin-Brandenburg (BBAG) in Berlin, Germany 05.12.2014-06.12.2014

Thieme C, Schlickeiser S, Dames C, Metzner S, Volk HD, Pleyer U**.** Aqueous humor cytokine profile of patients with primary or recurrent ocular toxoplasmosis“. Talk presentation at the conference of the European Association for Vision and Eye Research (EVER) in Nizza, France 01.10.2014- 04.10.2014

Thieme C, Winterhalter S, Apitzsch B, Joussen AM, Stübiger N. Case report: „Vogt-Koyanagi-Harada- Syndrom und M. Darier“. Poster presentation at the conference of the German Association of Ophthalmologists ((DOG), Leipzig, Germany 25.09.2014-28.09.2014

Schönfeld S, Metzner S, Winterhalter S, Thieme C, Pleyer U. Behandlungsergebnisse der Cytomegalie-assoziierten anterioren Uveitis. Poster presentation at the conference of the German Association of Ophthalmologists (DOG), Leipzig, Germany, 25.09.2014-28.09.2014

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Thieme C, Schönfeld S, Moser L, Joussen AM, Stübiger N. Optikusneuropathie nach Radiochemotherapie mit Temozolomid bei Gliobastoma multiforme. Poster presentation at the conference of the German Association of Ophthalmologists (DOG) Berlin, Germany 29.09.2015-02.10.2015

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