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UNIVERSITY OF LÜBECK
DIRECTOR: PROF. DR. SALVATORE GRISANTI

PATHOGENESIS OF AGE RELATED MACULAR DEGENERATION (AMD)

Immunohistochemical evaluation of the macrophage activation pattern in human choroidal neovascular membranes

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Khaled Nassar
Born in “El Menoufia”
Egypt

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1. **Referee:** Prof. Dr. med. Salvatore Grisanti

2. **Referee:** Prof.Dr.med.Hartmut Merz

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1 INTRODUCTION

1.1 Introduction

The retina is the light-sensitive part of the eye. It is a thin film of tissue covering most of the inner wall of the eye. The macula is the central part of the retina which has the highest concentration of cone photoreceptors required to resolve fine detail. Age related macular degeneration (AMD) is a progressive late onset disease affecting the central macula, which is responsible for fine central vision needed for driving, reading, and recognizing people's faces. It is the leading cause of irreversible blindness in western countries. It is estimated that the number of people with AMD will double by the year 2020. Current treatment options for AMD are limited, mostly to the late neovascular stage of the disease, with no treatment currently available for the late atrophic form of disease.

Growing evidence suggests that inflammatory and immunologic events might play a role in AMD. Several human histological studies have suggested the participation of macrophages in choroidal neovascular membrane (CNV) formation. The macrophage is a highly adaptive and responsive cell which is able to recognize alterations in its micro-environment and maintain tissue homeostasis. Macrophages play a central role in a remarkable range of biological and pathological processes. The precise mechanisms of macrophage involvement in AMD are still not fully understood. Macrophage heterogeneity raises an important question regarding the beneficial and harmful roles of macrophages in AMD pathogenesis.

1.2 Scientific Background

1.2.1 Clinical definitions

1.2.1.1 *Age-related macular degeneration (AMD)*

AMD refers to spectrum of diseases associated with visual loss, retinal pigment epithelium (RPE) changes, drusen, geographical atrophy (GA), and CNV which usually occur in patients over the age of 50. A unified classification of AMD is not yet present; however, for simplicity it can be clinically and histologically classified into two major subtypes: dry and wet AMD. Dry AMD progresses more slowly and manifests with drusen, GA of RPE, and photoreceptor dysfunction and degeneration. Wet AMD on the other hand has a key feature of CNV, the growth of new blood vessels from the choroid into the region underlying the RPE or extending past the RPE into the subretinal space and the retina. This CNV can lead to leakage of blood into the subretinal space, which, along with RPE atrophy and photoreceptor degeneration, leads to vision loss (Bird et al., 1995; Age Related Eye Disease Study Research Group (AREDS) report Nr. 6, 2001).

1.2.1.2 *Drusen*

Cells of the RPE continuously ingest photoreceptor outer segments that are shed throughout life. The residue of intracellular digestion may eventually fill the cell. Drusen are extracellular deposits that lie between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane (Burns and Feeney-Burns, 1980). Drusen are either small hard drusen which are not associated with an increased risk for the development of CNV (Bressler et al., 1990) and are not age-related (Klein et al., 1992) or large soft drusen which their presence increases the risk for development of RPE abnormalities, GA, and CNV formation (Bressler et al., 1990; Klein et al., 1997).

1.2.1.3 *Atrophic macular degeneration*

The atrophic form of macular degeneration has been called GA because the areas of RPE atrophy tend to form well-demarcated borders that do not relate to specific anatomic structures (Blair, 1975). Atrophic macular degeneration leads to significant visual loss in almost all cases; patients describe a gradual and subtle blurring of vision that relates to the degree of foveal involvement. A minimum of 175 µm of the retina should be involved in order to classify a patient as having GA (Bird et al., 1995).

1.2.1.4 *Choroidal neovascularization (CNV)*

CNV refers to the growth of abnormal new vessels of choroidal origin beneath the sensory retina or RPE (Campochiaro and Glaser, 1986). CNV is an abnormality found in many diseases in which the integrity of the RPE, Bruch's membrane, and choriocapillaris has been compromised. The effect on vision from CNV derives from its tendency to leak fluid beneath and into the sensory retina, to bleed, and to create a fibrovascular disciform scar in the macular region (Schatz et al., 1979).

1.2.2 Epidemiology

AMD can be classified broadly into two categories: non-neovascular (dry) and neovascular (wet). Although non-neovascular AMD accounts for approximately 80% of all diagnosed cases, neovascular AMD is responsible for nearly 80% of significant visual disability associated with this disease. GA, the most severe non-neovascular manifestation of AMD, causes approximately 21% of the cases of legal blindness in North America (Leibowitz et al., 1980). The average age at onset of visual loss is about 75 years. After the age of 50, the incidence steadily increases, with more than one third of people in the ninth decade of life affected. The visual impact is significant; the Salisbury Eye Evaluation Study reported the prevalence of blindness, defined as visual acuity (VA) 20/200 or worse, associated with AMD as 0.38% in individuals aged 70–79 years and 1.15% in individuals aged 80–84 years (Muñoz et al., 2000).

In Germany forecasts show that the number of patients with advanced AMD will increase from 710,000 at present to over a million by 2020. During the same period, the number of patients with neovascular AMD will increase from 485,000 to 700,000. There are currently 50,000 new cases of neovascular AMD every year in Germany, and it has only just become possible to treat these with the new anti-vascular endothelial growth factor (VEGF) drugs. The new treatment options will cause additional annual costs of 1.1-2.9 billion euro for Germany alone (Knauer and Pfeiffer, 2006). No significant gender predilection has been identified for AMD. The Framingham Eye Study showed a slightly higher incidence of AMD in Caucasian women compared to men (Leibowitz et al., 1980). The Health and Nutrition Examination Survey (HANES) found no difference (Klein and Klein, 1982).

1.2.3 Diagnosis and ancillary testing

Clinical examination is usually sufficient to establish a diagnosis of AMD. Subtle macular abnormalities, especially subretinal fluid, are best detected by stereoscopic slit-lamp biomicroscopic examination using a contact lens. Fluorescein angiography is useful in any patient in whom CNV is suspected to determine the characteristics of the lesion and the patient's potential qualification for available therapeutic modalities. Determination of the presence of CNV and evaluation of the extent, location and composition of its components are critical in deciding whether treatment is indicated and if so, which therapeutic modality is appropriate. If a lesion is well demarcated, its location may be determined by the closest point to the centre of the foveal avascular zone (FAZ). Lesion location is classified angiographically as follows:

1. Extrafoveal (=200 µm and < 2500 µm from the centre of the FAZ).
2. Juxtafoveal (1–199 µm from the centre of the FAZ).
3. Subfoveal (under the centre of the FAZ).

Based on angiographic patterns of fluorescence, components of CNV lesions may be categorized as either classic or occult. Classic CNV is characterized by bright uniform early hyperfluorescence and exhibiting leakage in the late phase which obscures the boundaries. Occult CNV is recognized angiographically by one of two patterns: fibrovascular Pigment epithelium detachment (PED), or late leakage from an undetermined source (Chamberlin et al., 1989; [1]Macular Photocoagulation Study, 1991; [2]Macular Photocoagulation Study, 1991).

1.2.4 Natural history

The risk of visual loss in eyes that initially manifest drusen or RPE abnormalities varies, depending on the characteristics of the macula and the status of the fellow eye. In eyes of patients older than 65 years that have bilateral drusen but no significant visual loss initially, the risk of a new atrophic lesion or neovascular lesion that results in visual loss has been reported as 9% at 1 year, 16% at 2 years, and 24% at 3 years (Holz et al., 1994). Confluent drusen, focal hyperpigmentation of the RPE, and extrafoveal areas of chorioretinal atrophy are three clinical findings that increase the risk for the subsequent development of visual loss. In individuals who already have neovascular AMD in one eye, the risk of developing CNV in the fellow eye is estimated at about 7–10% per year. If the

fellow eye has no large drusen or focal RPE hyperpigmentation, the 5-year risk of developing CNV is only 10%. When both large drusen and RPE hyperpigmentation are present, however, the 5-year risk increases to approximately 60% (Bressler et al., 1990).

1.2.4.1 *Occult Choroidal Neovascularization*

Natural history studies of occult CNV demonstrate a poor visual outcome associated with these lesions. One retrospective study reviewed 84 eyes with occult CNV and showed a 63% rate of moderate visual loss over an average of 28 months; average visual acuity declined from 20/80 to 20/250 over this interval (Bressler et al., 1988). The verteporfin in photodynamic therapy (VIP) study followed 93 eyes with purely occult subfoveal CNV in a placebo control arm. At 12 months, 73% of these eyes experienced visual loss from baseline, with 32% manifesting severe visual loss. At 24 months, 79% experienced visual loss from baseline, with a 43% rate of severe visual loss (Verteporfin in Photodynamic Therapy report 2, 2001; Bressler, 2002).

1.2.4.2 *Classic Choroidal Neovascularization*

The natural history of subfoveal classic CNV can be discerned by evaluating the control arms of the Macular Photocoagulation Study (MPS) and the Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study. In the MPS, visual acuity in untreated eyes harbouring classic CNV decreased an average of 1.9 lines at 3 months and 4.4 lines at 24 months. Severe visual loss was noted in 11% of eyes at 3 months and 37% at 24 months. The TAP Study showed a similar trend (Photodynamic Therapy Study, 1999; Bressler, 2001).

1.2.5 Treatment

The management of either type of AMD continues to be a challenge for patients, ophthalmologists, and the healthcare system. Recently, the progress made in the comprehension of the basic pathological mechanisms in both types of AMD has led to novel developments in therapeutic strategies resulting in a widening of the available treatment options and improved prognostic perspectives (Augustin et al., 2009). Treatment options span a broad range of therapeutic approaches, including thermal laser photocoagulation ([1]Macular Photocoagulation Study, 1991), surgical approaches as excision, displacement, or transplantation

(De Juan and Machemer, 1988; Stone and Sternberg, 2002; Lüke et al., 2009), radiation therapy (Bellmann et al., 2003), transpupillary thermotherapy (Subramanian and Reichel, 2003), feeder-vessel laser (Shiraga et al., 1998), and new treatments targeting the CNV component and its pathogenic cascade, such as photodynamic therapy (PDT) with verteporfin (Harding, 2001), anti-inflammatory drugs: triamcinolone acetonide (Gopal and Sharma, 2007), anecortave acetate (D'Amico et al., 2003), squalamine (Higgins et al., 2000), and more recently anti-VEGF therapies: ranibizumab (Ferrara et al., 2006), macugen (Drolet et al., 2000), and bevacizumab (Rosenfeld et al., 2005). Other treatment modalities include gene therapy (Gehlbach et al., 2003). The next discussion will summarise the different treatments which were received by the recruited patients.

1.2.5.1 *Photodynamic Therapy (PDT)*

PDT using verteporfin entails a two-part process with the photosensitizer (verteporfin) injected intravenously first. This is followed by controlled exposure to blue laser light, at 689 nM, 600 mW/cm^2, 50 J/cm^2, for 83 seconds. Neovascularization is eradicated when the verteporfin, which accumulates in the choroidal vessels, is activated by the laser light and generates reactive oxygen species. These attach to localized endothelial cells, causing platelet binding and aggregation. This blocks further blood flow through the vessels, and in fairly quick order, atrophy of the neovascularization follows. Blood vessels that have been eradicated in this way do not grow back, although other vessels will still be formed within the subretinal space due to continued expression of VEGF (Augustin et al., 2009). The TAP study and VIP study showed that CNV patients, particularly those with a predominantly classic component, have a reduced risk of moderate visual loss at 12 and 24 months (Lim, 2002). More recently the VIP study has demonstrated some benefit for patients with pure occult CNV after 2 years of treatment with PDT (Verteporfin in Photodynamic Therapy Study report 2, 2001). In PDT, microvascular injury induces inflammation, hypoxia, and the expression of angiogenic and survival molecules including VEGF-A, which could lead to CNV persistence and recurrence. For that reason, it is likely that multiple rounds of AMD retreatment are necessary (Spaide et al., 2003). Immunohistological examination of surgically extracted membranes suggests that revascularization after PDT is caused by angiogenesis rather than recanalization ([2]Grisanti et al., 2004; Petermeier et al., 2006). Matrix metalloproteinases-9 (MMP-9) plays a role in

recurrence after PDT. PDT induced an early, temporary decrease in MMP-9 and endostatin expression. At longer intervals, MMP-9 increase is possibly associated with the angiogenic process responsible for recurrence after PDT (Tatar et al., 2006; [1]Tatar et al., 2007).

1.2.5.2 *Anti-VEGF Therapy*

A different approach to the treatment of ocular neovascularization is antiangiogenic therapy with targeted molecular therapy. One of the potential targets for antiangiogenic therapy is VEGF. VEGF not only makes a fundamental contribution to the neovascular processes but it also participates in the related physiological pathways (Grisanti and Tatar, 2008).

1.2.5.2.1 Ranibizumab (rhuFab) (Lucentis)

Ranibizumab (Lucentis, Genentech/Novartis, Inc, South San Francisco, California) is a humanized, recombinant monoclonal antibody fragment designed to recognise all five human isoforms of VEGF. In animal studies, it has been shown to penetrate through all retinal layers and inhibit VEGF-A, thereby decreasing vascular permeability and blocking angiogenesis (Krzystolik et al., 2002). In a 2-year study, ranibizumab provided greater clinical benefit than verteporfin PDT in patients with age-related macular degeneration with new-onset, predominantly classic CNV with lower rates of serious adverse reactions and complications (Brown et al., 2009).

1.2.5.2.2 Bevacizumab (Avastin®)

Bevacizumab (avastin®; Genentech, Inc, South San Francisco, California) is a full length humanized murine monoclonal antibody directed against human VEGF-A and thus a closely-related drug to ranibizumab. It was approved by the Food and Drug Administration (FDA) in 2004 for the intravenous treatment of metastatic colorectal cancer. Its potential for the treatment of CNV was first tested by Michels and his colleagues via intravenous infusion in a 12-week, open-label, uncontrolled study, (Michels et al., 2005). Striking effects were observed on both visual acuity, the optical coherence tomography (OCT) and the angiographic characteristics of the neovascular lesions. However, patients experienced a mean increase of 12 mmHg in systolic blood pressure. This systemic side effect, combined with the promising visual and anatomic results from the intravenous infusion of bevacizumab, led investigators to consider the intravitreal injection of

bevacizumab (Rosenfeld et al., 2005).

Bevacizumab significantly reduces VEGF-induced permeability and proliferation of cultured endothelial cells (Peters et al., 2007). Published case series of bevacizumab treatment for different neovascular ocular pathologies indicated positive anatomical and functional effects. A potential side-effect is the apparently increased incidence of RPE tears (5 to 10%), after large and hemorrhagic pigment epithelium detachment. It has been postulated that the fast resolution of fluid and/or contraction of the fibrous tissue may cause the rip (Grisanti and Ziemssen, 2007). Examination of surgically extracted human CNV that had prior treatment with bevacizumab showed a shift within the angiogenic balance in terms of decreased VEGF predominance over endostatin ([2]Tatar et al., 2009).

1.2.5.3 *Anti-inflammatory Therapy*

CNV usually contain histopathologic evidence of inflammation, especially macrophages. These cells secrete numerous proangiogenic factors, including VEGF, prostaglandins, MMP, and others (Grossniklaus et al., 2002). Intraocular injection of triamcinolone acetonide (TA) was effective in reducing the leakage from CNV (Rechtman et al., 2003). Combined treatment with intravitreal TA and PDT has shown improvement in visual acuity and less-frequent requirement for re-treatments in short-term follow-up (Spaide et al., 2003). In addition, it seems to exert angiogenesis inhibitory effects on CNV, which is achieved by enhancing endostatin expression rather than by suppressing VEGF expression ([1]Tatar et al., 2008).

1.2.6 The Pathogenesis of Choroidal Neovascularization:

CNV is the process leading to “wet” AMD. It is the result of abnormal choroidal vessels growing through Bruch’s membrane and proliferating beneath either the RPE or the neurosensory retina. These abnormal vessels then either leak and/or bleed. The result is a rapid impairment of retinal function resulting in visual distortion and loss of vision. Histologically, CNV starts as an ingrowth of capillaries through Bruch`s membrane and is typically associated with basal laminar or linear deposits (Green and Key, 1977). With time, these capillaries mature into arteries and veins, followed by fibrosis (Green and Enger, 1993). Gass initially subdivided CNV into two groups, based on where these vessels were

found within the retina. CNV type I referred to the vessels located in the sub-RPE space (between the RPE and Bruch`s membrane), whilst in CNV type II the vessels were located in the subsensory retinal space (between the neural retina and the underlying RPE). CNV type I was thought to be due to diseased RPE, where its attachment to the underlying Bruch`s membrane is loosened, thereby allowing vessels to infiltrate the sub-RPE space. This type of CNV was typically thought to occur in diseases such as AMD. CNV type II was attributed to conditions causing a breach in the RPE that allowed the ingrowth of vessels from the choroid into the sub-retinal space (Gass, 1994).

Clinically, the CNV is often seen as a grey-green membrane beneath the sensory retina. Serous detachment of the retina or subretinal hemorrhage is often associated, due to fluid leakage or hemorrhage from the CNV itself. In case of severe bleeding, large areas of subretinal hemorrhage may occur with associated sudden and catastrophic central visual loss. The blood may also extend into the vitreal cavity, resulting in a vitreal hemorrhage (Green and Enger, 1993).

Based on the pattern of leakage from the abnormal vascular complex as seen on fundus fluorescein angiography (FFA), CNV is classified into “classic” and “occult” varieties. In both types of CNV, visual loss results from leakage from the newly formed blood vessels or hemorrhage or both. With the advent of submacular surgery, when CNV is surgically excised from beneath the retina, correlation between the clinical and histological appearance of CNV in AMD has become possible. Several studies have now confirmed that well defined “classic” CNV on FFA correlates best with CNV type II, with the fibrovascular complex situated in the subretinal space; whilst ill-defined “occult” CNV on FFA corresponded best with CNV type I, where most fibrovascular tissue was located in the sub-RPE space (Grossniklaus and Gass, 1998; Lafaut et al., 2000). These studies also found that conditions resulting in a breach in the RPE were almost always associated with classic CNV type II, whilst CNV due to AMD could either be classic type II, occult type I, or a combination of both (Bressler et al., 1987; Grossniklaus et al., 1994; Grossniklaus and Gass, 1998; Grossniklaus and Green, 1998).

Histological analyses of CNV with cell markers have disclosed that the cellular components of these membranes include RPE, inflammatory cells, vascular endothelium, glial cells, myofibroblasts, and fibrocytes (Lopez et al.,

1991; Grossniklaus et al., 1992). Extracellular components have included several types of collagen (collagen types I, III, IV, and V), fibronectin, laminin, glycosaminoglycans (GAGs), and lipid (Grossniklaus et al., 1992). The formation of a disciform scar is always associated with poor central vision and is considered to be the end stage of CNV. Histologically, disciform scars predominantly develop from CNV, where it is thought that leakage and hemorrhage from the abnormal vascular complex lead to fibrous tissue proliferation and a fibrovascular scar with endothelium lined vascular channels (Lopez et al., 1991). The scar itself often has two components, one within layers of Bruch`s membrane and the other between the neural retina and RPE (Sarks, 1976; Green and Enger, 1993). Cells invading Bruch`s membrane may also alter it and release angiogenic factors. Macrophages increase in number (Killingsworth et al., 1990) and are thought by some to be the factor common to all diseases with neovascularization (BenEzra, 1978).

Macrophages promote the growth of endothelial cells, pericytes, and fibroblasts. Furthermore, macrophage derived prostaglandins, especially prostaglandin E, may be a strong stimulus to neovascularization (Polverini et al., 1977). Activated macrophages produce enzymes such as collagenases and elastases, and may erode Bruch`s membrane by a combination of mechanical disruption, phagocytosis, and extracellular release of enzymes. With increasing age, this cellular response is not seen until Bruch`s membrane has membranous debris present beneath the RPE basement membrane and seems to occur preferentially beneath hard drusen (Killingsworth et al., 1990). Other cells may also play a role in Bruch`s membrane damage and the promotion of new vessels to invade (Heriot et al., 1984; Penfold et al., 1984). Endothelial cell processes have also been found to invade Bruch`s membrane normally (Guymer et al., 2004). The mechanism which initiates and modulates the normal rate of basement membrane dissolution and endothelial cell protrusion and the conversion of this phenomenon to neovascularization is unknown. Grisanti and colleagues studied the endoglin expression in surgically excised CNV. Endoglin expression was elevated in vascular endothelial cells contained within CNV. They suggested a persisting post-mitotic activation in an advanced stage of this neovascular tissue ([1]Grisanti et al., 2004). The angiogenesis process is likely to be determined by the relative concentrations of various growth factors and the nature of the collagen and inter-fiber matrix of Bruch`s membrane. Some have suggested that the

basement membrane may bind these factors, thereby modulating their immediate effect (Glaser, 1988). Ample evidence suggests the existence of many factors in Bruch`s membrane with the potential to modify cell behaviour (Sarks, 1976; Feeney-Burns and Ellersieck, 1985; Loffler and Lee, 1986; Van der Schaft et al., 1991). Angiogenic growth factors that may have a role in the formation of CNV secondary to AMD thus far include VEGF, basic fibroblast growth factor (bFGF), nitric oxide, and angiopoietins. Of these, the role of VEGF appears to be the most prominent. VEGF is a pro-angiogenic growth factor that is essential for normal embryonic tissue growth and is expressed by RPE cells in a paracrine fashion in the normal maintenance of the underlying choriocapillaris. Elevated intra-ocular levels of VEGF have also been implicated in the development of CNV secondary to AMD (Lopez et al., 1996; Kliffen et al., 1997). VEGF expression is increased by tissue hypoxia and oxidative stress and these factors have also been implicated in the pathogenesis of AMD. Indeed VEGF is a chemoattractant for vascular endothelial cells precursors, inducing their mobilization and promoting their differentiation (Asahara et al., 1999); VEGF induces CNV enlargement also by stimulating endothelial cells expression of MMPs, which degrade the extracellular matrix and facilitate neovascular tissue invasion (Lamoreaux et al., 1998). Lastly, VEGF represents a potent chemotactic signal for macrophages (Clauss et al., 1990; Barleon et al., 1996). However, the exact mechanism of VEGF over-expression in AMD remains to be fully elucidated. Nevertheless, treatments aimed at reducing intra-ocular levels of VEGF using various anti-VEGF monoclonal antibodies have been tested in multi-centre, controlled clinical trials with efficacy in the treatment of CNV in AMD (Bressler, 2009).

1.2.7 Aetiology

The cause and pathogenesis of AMD is still unknown. Several theories have been proposed and the most widely considered are discussed below. The general consensus is that AMD has a multi-factorial causation, where both genetic and environmental factors play a part.

1.2.7.1 *Genetics*

AMD can be considered as a genetically complex disorder of the photoreceptor- RPE - Bruch's membrane - choriocapillaris complex ([1]Ambati et al., 2003). A genetic influence on AMD pathology is well known from family and twin studies (De Jong et al., 1997; Gorin et al., 1999). First-degree relatives of patients

with AMD, compared with first-degree relatives in families without the disorder, are at increased risk of the condition (Seddon et al., 1997; Seddon et al., 2005), are affected at a younger age, and have an increased lifetime risk of late AMD (Klaver et al., 1998; Assink et al., 2005).

Variants in the complement factor H (CFH) gene at 1q31 have been shown by several independent studies to be associated with a significantly increased risk of AMD in the Caucasian population. These findings imply that the innate immune system might be implicated in AMD pathogenesis (Edwards et al., 2005). Variants within CFH and LOC387715 may contribute to the increased risk of advanced AMD largely or entirely through their impact on precursors, such as drusen and/or other RPE/Bruch's membrane changes (Schmitz-Valckenberg et al., 2006).

1.2.7.2 *Environmental Risk Factors*

The greatest risks for developing AMD are increasing age and a family history of AMD (Smith et al., 1996). The most consistently found modifiable risk factor, however, is smoking, which increases the incidence of AMD two to five times, or progression from early AMD to CNV in current smokers (Smith et al., 1996; Smith et al., 2001). Other studies have implicated hypertension, carotid-cardiovascular disease, and high cholesterol levels (Macular Photocoagulation Study, 1994; Vingerling et al., 1995; AREDS report Nr. 8, 2001). A positive relationship was found between early AMD and a high body mass index (BMI) (Klein et al., 2001). A number of studies have also linked increased cholesterol or total dietary fat intake to both early and late AMD when analyzed as the risk associated with the highest versus lowest quartiles of intake (Mares-Perlman et al., 1995; Hyman et al., 2000). Conversely, increased intake of long chain omega-3 polyunsaturated fatty acids and fish has been associated with reduced risk of early AMD (Seddon et al., 2001). Although AMD shares risk factors with atherosclerosis, the actual association between AMD and atherosclerosis is inconsistent. Some studies have demonstrated positive links between AMD and atherosclerosis (Vingerling et al., 1995), cardiovascular disease (CVD), and cerebrovascular disease (Vidaurri et al., 1984).

1.2.7.3 *Theories of AMD pathogenesis*

1.2.7.3.1 Vascular Theory

The observation that AMD and atherosclerosis share risk factors and

pathogenetic mechanisms, has led to the development of a hypothesis that is identified as a hemodynamic or vascular model of the pathogenesis of AMD. It holds that AMD is a vascular disorder characterised by impairment of choroidal perfusion of the RPE. This model, evolved over four decades, has now been updated to incorporate recently reported evidence that the changes affecting Bruch's membrane in old age and AMD involve lipoproteins processed by the RPE. The model proposes that these lipoproteins accumulate in drusen and in Bruch's membrane because the choriocapillaris do not clear them (Friedman, 2004).

The strength of this model is that it neatly explains the association between CVD and AMD, as both have been found to share similar risk factors (hypertension, smoking, obesity, and high dietary fat intake), with some studies showing an increased prevalence of AMD in those with CVD (Vingerling et al., 1995; AREDS report Nr. 8, 2001). Decreased choroidal blood flow and increased lipid content of Bruch`s membrane with increasing age have also been well documented (Friedman et al., 1995).

1.2.7.3.2 Oxidation Theory

Reactive oxygen species cause oxidative damage to cytoplasmic and nuclear elements of cells and cause changes to the extracellular matrix. The degree of oxidative damage is restricted by a range of potent antioxidants and the repair of damaged elements. However, some oxidative damage will occur and the accumulation of this damage throughout life is believed to be a major contributory factor in tissue aging. The retina is a typical example in which oxidative damage manifests in what we term "retinal aging" and includes loss of retinal cells, accumulation of lipofuscin within the RPE, drusen formation, and accumulation of degradative products in Bruch's membrane and changes in choroidal capillaries. Once these changes become excessive they are believed to contribute to the onset of AMD (Winkler et al., 1999).

1.2.7.3.3 Inflammation

Inflammation has been recently implicated in a number of degenerative diseases associated with aging, including atherosclerosis and Alzheimer disease (Parihar and Hemnani, 2004). The role of inflammation in AMD pathogenesis is a rapidly evolving area of research, particularly with discovery of CFH gene in 2005,

and has been reviewed extensively (Maller et al., 2006). A number of recent epidemiological studies have found an association between AMD and increased levels of blood inflammatory markers. For example, AMD has been linked to high leukocyte count (Klein et al., 1993), high plasma fibrinogen level (Smith et al., 1998), oxidized low density lipoprotein (LDL), and elevated C-reactive protein (CRP) (Seddon et al., 2004). With regard to CRP, elevated levels of this inflammatory marker have been found to be an independent risk factor for the development of CVD (Li and Chen, 2003) and recently it has been associated with increased risk of advanced AMD (Seddon et al., 2004). Inflammatory cells may be involved in the breakdown of Bruch's membrane, RPE atrophy, and CNV. Accumulation of inflammatory cells has also been demonstrated in choroidal vessels associated with drusen and disciform scars (Penfold et al., 2001). The recent finding implicating the CFH gene in AMD (Edwards et al., 2005) greatly strengthens the inflammatory theory of AMD, as single-nucleotide polymorphism (SNP) within this gene could result in a decreased regulation of the complement cascade and an uncontrolled inflammation. Another supporting genetic finding in favour of this inflammatory theory is the recent link between variants encoding Toll-like receptor 4 (TLR4) and an increased susceptibility to developing AMD (Zareparsi et al., 2005). As smoking is known to activate the complement pathway, the progression of AMD in those with impaired CFH may therefore be accelerated. However, what triggers the inflammatory pathway is still not known. The fact that the CFH gene is involved in the alternative complement pathway has led some to speculate that some microorganisms may act as a trigger and indeed some work has implicated Chlamydia in AMD. It is possible that a number of organisms could be the trigger that activates the alternate complement pathway which is then unable to terminate effectively in those with defective CFH, leading to chronic inflammation and disease (Kalayoglu et al., 2003).

1.2.8 Macrophages and immune competent cells in AMD

1.2.8.1 *Morphological evidence*

1.2.8.1.1 Macrophages, Bruch's membrane breaks and early AMD

Sarks noted that in eyes with early AMD, macrophages were found adjacent to breaks in Bruch's membrane and in association with subclinical CNV, which considered as the earliest evidence of neovascularization (Sarke, 1976). These observations were reinforced by similar follow-up findings (Van der Schaft

et al., 1993). Stromal and choroidal leukocytes, including macrophages, were found in increased numbers by Penfold and his colleagues once eyes have developed a continuous layer of basal laminar deposit (BLD). Mean macrophage counts were significantly higher in eyes with continuous BLD compared to normal aged eyes (Penfold et al., 1984; Penfold et al., 1985). Later reports found that macrophages appeared to be attracted to membranous debris deposition within Bruch's membrane in early AMD (Killingsworth et al., 1990). Macrophages were also found accompanying both "active" and "inactive" subclinical CNV (Sarks et al., 1997). These morphological observations suggest a link between macrophage infiltration and the earliest stages of the neovascular process, possibly by causing breaks in Bruch's membrane which allow the in-growth of newly formed vessels.

1.2.8.1.2 Macrophages in advanced AMD lesions

An ultrastructural study of membranes from eyes with neovascular AMD found leukocytes and macrophages within neovascular structures, at breaks in Bruch's membrane, and in contact with activated pericytes further reinforcing the view that macrophages induce new vessel growth. Macrophage association with the neovascular process appeared to persist in burnt-out disciform lesions (Penfold et al., 1987). A large histopathological survey of surgically excised subfoveal CNV lesions confirmed that macrophages were the most frequently found cell type after RPE in both neovascular membranes and disciform scars (Grossniklaus et al., 2005). Interestingly, giant cells appear to be the most common type of leukocyte found in eyes with GA (Penfold et al., 1985; Penfold et al., 1986), whereas the breaks in Bruch's membrane commonly found in eyes with disciform scars are absent. This observation further emphasises the association of Bruch's membrane breaks with the in-growth of neovascular tissue. Sarks proposed that macrophages and giant cells are attracted to Bruch's membrane when the phagocytic capacity of RPE is exceeded (Sarks et al., 1997).

1.2.8.2 *Macrophages and choroidal neovascularisation*

With the compelling morphological data that macrophages are intimately involved with AMD lesions, and particularly the suggestion that they may facilitate neovascularisation, investigators sought evidence of macrophage expression of angiogenic mediators. Oh and his colleagues found that macrophages in surgically excised neovascular membranes, identified by cluster of differentiation 68 (CD68)

labelling, expressed the proinflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNFα), while RPE cells admixed in the same lesion expressed VEGF. They suggested that the presence of activated, pro-inflammatory macrophages may induce VEGF production by RPE, promoting CNV development (Oh et al., 1999). Grossniklaus and his colleagues also examined surgically excised CNV membranes and they observed that CD68-positive macrophages expressed VEGF and tissue factor, while RPE cells expressed monocyte chemoattractant protein-1 (MCP-1) (Grossniklaus et al., 2002). These authors concluded that RPE might be important for macrophage recruitment and that recruited macrophages expressed two growth factors essential for neovascularisation.

Further evidence of the ability of macrophages to induce neovascularisation was provided by animal model studies. The laser photocoagulation animal model, most commonly used to approximate the CNV found in AMD, causes breaks in Bruch's membrane. Neovascular lesions typically form within one week of photocoagulation. Choroidal vascular endothelial cells migrated into the subretinal space via the laser-induced defects in Bruch's membrane in monkey eyes three days after photocoagulation and macrophage infiltration was observed three to seven days after photocoagulation (Ishibashi et al., 1997). In the same study, VEGF expression was first found in macrophages and later in RPE and Muller cells. Tsutsumi and colleagues explored this relationship further when they compared macrophage infiltration and extent of CNV after laser photocoagulation in a chemokine receptor 2 (CCR2) knockout mouse model (Tsutsumi et al., 2003). CCR2, the receptor for MCP-1, is normally expressed by macrophages and is essential for macrophage trafficking. Both the number of infiltrating macrophages and the extent of CNV were significantly less in the knockout mice compared to wild type mice. These observations suggested that the neovascularisation process depends much more on recruited and not resident macrophages. Further strengthening this observation, Sakurai and his colleagues found that depletion of circulating monocytes using intravenous clodronate reduced both CNV lesion volume and leakage (as measured by fluorescein angiography) (Sakurai et al., 2003). To demonstrate that the recruited macrophages were derived from circulating monocytes, Caicedo and his colleagues used fluorescently-labelled monocytes transplanted into the bone marrow of irradiated mice (Caicedo et al.,

2005). Care was taken to reduce the laser intensity and duration to limit its effect on Bruch's membrane and minimise retinal injury. Blood-derived monocytes in this study infiltrated the retina overlying CNV three days after laser photocoagulation. Activation of the overlying Muller cells occurred secondary to macrophage infiltration. Muller cell activation was abolished after depletion of circulating monocytes using clodronate.

In summary, the laser-induced model of CNV has produced a number of important observations. Firstly, it is likely that circulating monocytes, and not resident immune cells, are responsible for the inflammatory damage immediately after Bruch's membrane disruption. Indeed, infiltrating macrophages may activate local cells (Caicedo et al., 2005). Secondly, recruited macrophages express VEGF and the extent of neovascularisation and leakage depends on the extent of macrophage trafficking and infiltration. However, laser photocoagulation tends to destroy the outer retina, along with Bruch's membrane and choroid. So, laser induced model of CNV is an acute traumatic model which is very unlike the CNV caused by AMD. Not surprisingly, the first cells to infiltrate laser-induced CNV lesions are neutrophils, which are conspicuously absent in AMD lesions.

1.2.8.3 *Choroidal macrophage recruitment and turnover and AMD*

1.2.8.3.1 CCL2, CCR2 and Cx3CR1 Knockout mice and AMD like lesions

In 2003, Ambati and colleagues reported that knockout mice deficient in either MCP-1, also known as chemokine ligand 2 (CCL2) or its receptor CCR2 developed AMD-like lesions when left to age beyond 9 months. These lesions included RPE lipofuscin accumulation, RPE degeneration, photoreceptor fall out, and the development of drusen and CNV; features not seen in wild type mice even beyond 24 months of age ([2]Ambati et al., 2003).

Evidence of immunoglobulin G (IgG) and complement 3c (C3c) in the choroidal vessel walls of the knockout mice suggested an immune complex deposition. Complement 5 (C5), serum amyloid P protein, and advanced glycation end products were also immunolocalised on RPE or the choroids of knockout mice. In wild-type mice, an age-dependent increase in CCL2 expression by RPE cells, together with an age-dependent increase in choroidal macrophages, suggested that CCL2–CCR2 interaction was critical to normal choroidal macrophage recruitment. They also found that choroidal macrophages from wild

type mice were able to degrade C5 and IgG deposited on the choroids or RPE of knockout mice. Together, data from this report suggested that resident choroidal macrophages play a critical role in the elimination of opsonised debris in Bruch's membrane ([2]Ambati et al., 2003).

Macrophages expressing scavenger receptors for oxidised lipoproteins are found in human AMD eyes. Disruption of the normal recruitment or turnover of choroidal macrophages may lead to persistence of both the debris and the opsonising molecules C5 and IgG, resulting in lesions similar to those found in AMD. AMD-like lesions were also found in mice deficient in CCL-2 and Chemokine (C-X3-C motif) receptor 1 (CXC3R1), fractalkine, a chemokine receptor involved in leukocyte trafficking (Tuo et al., 2004; Tuo et al., 2007), further reinforcing this view. These reports highlight the differences between age-related pathological changes and those that result from laser disruption of Bruch's membrane in mouse models. While CNV is reduced in CCL-2 (MCP-1) knockout mice after laser photocoagulation, it develops spontaneously when the same mice are left to age beyond 9 months.

1.2.8.3.2 IL-10 knockout mice and laser-induced CNV

An interesting but counterintuitive finding in interleukin-10 (IL-10) knockout mice was recently reported by Apte and colleagues. IL-10 is a major immunosuppressive cytokine that programs macrophages along the non-inflammatory alternatively activated macrophages (M2) pathway. These authors reported that the knockout mice developed less extensive laser-induced CNV compared to wild type mice. Lack of IL-10 would be expected to permit macrophage programming along the pro-inflammatory pathway, resulting in more CNV. The authors explained their unexpected observations by proposing that it is the inhibition of normal macrophage recruitment (e.g. by local IL-10) that promotes CNV (Apte et al., 2006).

1.2.8.3.3 Polymorphisms in leukocyte recruitment and turnover genes

A number of genetic studies have supported the observation that normal macrophage recruitment and function are important in the maintenance of a healthy and debris-free Bruch's membrane. Goverdhan and colleagues examined polymorphisms in the human leukocyte antigen (HLA) gene and AMD risk. They found one at-risk allele and two protective alleles, as well as differential expression

of HLA system antigens in the choroid (Goverdhan et al., 2005). HLAs are important molecules for macrophage recognition of antigens. The HLA genes are the most polymorphic in the human genome, and the mechanism by which they confer susceptibility to AMD is not yet clear. These findings provide further evidence for the involvement of immune or inflammatory processes in AMD pathogenesis. A single gene polymorphism in the CX3CR1 gene was also found to be associated with AMD (Tuo et al., 2004).

1.2.8.3.4 Serum myeloid cells and AMD

There is some epidemiological evidence implicating circulating myeloid cells in AMD, suggesting a higher systemic inflammatory “set point”. A higher white cell count was found in patients with neovascular AMD and disciform scars compared to controls in a small case-control study in 1986 (Blumenkranz et al., 1986). High white cell count was also associated with neovascular AMD (Klein et al., 1993) and with the 10-year incidence of large (>125μm) drusen in the Beaver Dam Study (Klein et al., 2003). Finally, monocytes isolated from patients with neovascular AMD expressed more TNFα when stimulated with RPE blebs in vitro compared to controls (Cousins et al., 2004).

1.2.8.3.5 Choroidal dendritic cells and drusen biogenesis

Although dendritic cell networks are likely to be present in the normal human choroid, the functional role of choroidal dendritic cells is at present poorly characterised. Hageman and associates have proposed that sub-RPE debris can attract the processes of cells on the choroid side of Bruch’s membrane. Based on that, they proposed that these cells share immunophenotypic features with dendritic cells. These authors propose that dendritic cell processes are attracted to injured RPE or diffuse sub-RPE debris, becoming a focus for deposition of inflammatory proteins which eventually results in the formation of drusen.

Unfortunately they reported CD68 and HLA-DR immuno-reactivity in drusen, an immunophenotype more typical of macrophages. While it is an interesting pathogenic mechanism, there has so far been insufficient evidence to support it (Hageman et al., 2001).

1.2.8.4 *Heterogeneity of macrophages*

Macrophages are cells derived from bone marrow-derived monocytes that

have homed into tissues. Blood monocytes, which can engage in pinocytic and phagocytic activities, migrate in response to various chemotactic factors and express fragment crystallisable-gamma (Fc-ɣ) receptors and iC3b complement. These have been considered to be immature forms of tissue-resident macrophages but can be seen also as a population of circulating macrophages. Further differentiation takes place in tissues and the resulting macrophage populations are being referred to as resident macrophages (Ma et al., 2003). Leenen and his colleagues have reviewed a variety of immunological markers recognized by a selected panel of monoclonal antibodies that are characteristic of macrophage precursors and immature macrophages, mature macrophages, macrophage subsets, and interferon gamma (IF-γ)-stimulated macrophages (Leenen et al., 1994). Normal macrophages (resident macrophages) can be found in many tissues. Local populations of macrophages are maintained by proliferation of resident progenitor cells and influx of monocytes from blood. Inflammatory macrophages that are derived exclusively from monocytes have similar properties. Some types of macrophages identified, either by expression of distinct surface markers or by discrete secretory products, may be associated with distinct disease states. Tissue macrophages stand guard against foreign invaders and are able to instantly defend as well as sending signals for recruitment and presenting antigen to other immunological cells. Critical reason macrophages are so effective as a first line of defence is that they are distribute throughout the body in various organs, tissues, and fluids (Rutherford et al., 1993).

Macrophages, through their secretory products, control inflammation, changes in the composition of the extracellular matrix, tissue reorganization, angiogenesis, and other processes. Macrophages constitute an important link between the innate and adaptive immune systems through the presentation of antigens to T-cells and production of cytokines and chemokines. Tissue macrophages play a critical role in wound healing by producing chemoattractants which recruit and activate additional macrophages, growth factors which promote cellular proliferation, proteases, and extracellular matrix molecules, and factors restraining tissue growth once repair is completed. Macrophages have the capacity to secrete a plethora of cytokines, growth factors, and other mediators which can affect many other cell types (Douglas and Hassan, 1990).

Macrophages display great diversity of phenotype and function resulting

from their ability to adapt to the local environment ([1]Gordon et al., 1992). It is the exposure to particular tissues, cell types, and physiological states that leads tissue macrophages to vary maturationally, functionally, and metabolically as evidenced by their differential response to stimulation and their range of distinguishing markers (Gordon et al., 1986). For example, although phagocytosis is a hallmark of macrophage activity, skin-associated macrophages, Langerhans cells, are poorly phagocytic (Gordon, 1995). In addition, cytokine production, receptor expression, and perioxidatic activity are highly variable between macrophage subtypes. The diversity in macrophage phenotype and function has compounded the difficulty in the interpretation of the ever expanding volume of data concerning these cells. Since spatially distinct macrophages do not respond uniformly, assumptions about one population based on the evidence of another can be misleading. In truth, even macrophages within a single tissue do not behave similarly (Daems et al., 1976; [2]Gordon et al., 1992). Pathological conditions and inflammatory events influence macrophage response and activation state. For example, a Gram-negative bacterial infection may lead to recruitment of fully mature, fully activated cells ([2]Gordon et al., 1992; Kinnaert et al., 1996). On the other hand, it has been reported that tumour-associated macrophages are more immature and in some cases unresponsive (Dinapoli et al., 1996).

In broad terms, macrophages can be activated along a “classical” or “alternate” pathway. The term classically activated macrophages (sometimes abbreviated as caMPhi; also called M1 macrophages) refers to macrophages that have undergone cellular activation in response to lipopolysaccharides (LPS) or IFN-γ and more generally by Type 1 helper T cells (T_h1) cytokines which includes IFN-γ and TNF-α (Gordon, 2003). These macrophages express the enzyme inducible nitric oxide synthase (iNOS), which allows them to convert arginine to nitric oxide, producing nitrite, peroxynitrites, and superoxides. These powerful oxidants cause lipid peroxidation of affected cell membranes, leading to cell death.

The term alternatively activated macrophages (sometimes abbreviated as aaMPhi; called also M2 macrophages) refers to macrophages that have undergone cellular activation in response to IL4 and IL13 or glucocorticoids (Loke et al., 2002). Alternate activation of macrophages switches on a different metabolic program, which is transforming growth factor ß-1 (TGF-β1) dependent. Macrophages thus programmed to express the enzyme arginase, which converts

arginine to ornithine, promoting cell division and repair (Mills et al., 2000). M2 generated in vivo have a gene expression profile distinct from other macrophage populations. Both types of activated cells can co-exist and show differences in their capacity to secrete mediators and to express receptors (Mantovani et al., 2004).

Thus, these cells participate to different extents in pro-inflammatory and anti-inflammatory immune reactions. Particle-mediated cell activation of macrophages also differs from type 1 or type 2 activated cells in gene expression patterns (Gordon, 2003, Martinez et al., 2008). The term macrophage deactivation refers to a reversal of the activated state. This process requires the presence of a variety of mediators. The different types of macrophages constitute a heterogeneous population of cells that differ in their origin and differentiation stage and may have been subject to different tissue-specific micro-environmental influences. Frequently, any distinctions made to describe degrees of cell activation are more or less arbitrary. Depending in large part on the nature of the stimuli, macrophages may be primed only for selected functions and may not attain the full spectrum of functional capacities. Distinct subpopulations of macrophages or differently activated macrophages display various functions in immune reactions and this may be reflected by differences in the factors they produce, and the mediators they respond to, under different circumstances. Numerous studies have been carried out to identify genes expressed or repressed specifically in macrophages in response to a variety of stimuli (Mantovani et al., 2004).

2 THESIS AIMS

Over the past two decades, there has been significant progress in the pathophysiology and treatment of AMD. Recently, it has become clear that many chronic degenerative diseases associated with aging demonstrate important immune and inflammatory components. Although several human histological studies have suggested the participation of macrophages in CNV formation, the precise mechanisms are still not fully understood. It is still not possible to clearly demonstrate whether macrophages accumulate near CNV because they play a causative role in CNV or because they serve as an adaptive response against CNV-associated pathology.

Macrophages heterogeneity raises an important question regarding the role of macrophage in AMD, especially in light of conflicting theories regarding their beneficial and harmful roles in AMD pathogenesis. Further studies in the M1 and M2 subsets of macrophages as well as the differential role of these macrophages in AMD are necessary to parse out the various functions of macrophages in preventing and/or inducing AMD pathology.

This study aims at understanding the effect of the CNV related variables, patient variables and the treatment effect on the macrophage activation pattern through evaluation of M1 and M2 patterns of activation in surgically excised choroidal neovascular membrane specimens.

3 MATERIALS AND METHODS:

3.1 CNV specimens

This study is a retrospective review of interventional case series of 58 surgically excised CNVs derived from 58 eyes of 58 AMD patients. Before surgery, therapy options were discussed with patients. Each patient gave written informed consent after the nature of the treatment procedure and the risks and benefits of all treatment alternatives were discussed in details. The study followed the guidelines of the Declaration of Helsinki as revised in Tokyo and Venice. The 58 patients were divided into 9 subgroups, table 1. The groups represent different variables as regards to the angiographic classification (G1-3) and the different applied medical treatment modalities (G4-G8). CNV specimens of the first three groups with no prior treatment were used as the control group (G9) of CNV with prior treatment (G4-G8). A detailed description of the patient's characteristics is given in the attachment number 8.1.

Table 1. Study groups

Group	Description
Group 1	Patients received no treatment, classic CNV, (n=5).
Group 2	Patients received no treatment, occult CNV, (n=10).
Group 3	Patients received no treatment, hemorrhagic CNV, (n=10).
Group 4	Patients treated with verteporfin photodynamic therapy (PDT), (n=9).
Group 5	Patients treated with bevacizumab (Avastin®), (n=10).
Group 6	Patients treated with ranibizumab (Lucentis®), (n=4).
Group 7	Patients treated with triamcinolone acetonide (TA), (n=5).
Group 8	Patients treated with bevacizumab and PDT combination, (n=5).
Group 9	None treated CNV (G1-3, n=25) used as control group for CNV with prior treatment.

3.2 Histopathological Methods

Within minutes after surgery, excised CNVs were fixed in formalin 3.7% for 24-36 hours and embedded in paraffin. Serial sections of the embedded tissue specimens were then cut at 4 µm intervals. CNV were examined for the pattern of macrophage activation using three specific antibodies for macrophages (namely CD68, CCR7, and CD163 antibodies) staining for pan M, M1, M2 macrophages, respectively, table 2.

Tabel 2. Antibodies used to characterise macrophages and its phenotypes			
Antibody		**Macrophage**	
Name	***Abb.**	**Name**	**Abb.**
Cluster of differentiation 68	**CD68**	Pan macrophages.	**M**
Chemokine receptor 7	**CCR7**	Classically activated macrophages.	**M1**
Cluster of differentiation 163	**CD163**	Alternative activated macrophages	**M2**

***Abb. : Abbreviation**

The tissue sections were prepared for a two-steps indirect immunohistochemical staining by deparaffinization with xylene and rehydration through a graded ethanol series. Endogenous peroxidises were blocked through incubation in 0.3% H_2O_2 in 0.1% sodium azide for 10 minutes. A heat-mediated antigen retrieval technique that included a 30-min boil in 0.01 Molar citrate buffer, pH 6.0, was used. To prevent non-specific antibody binding, the slides were incubated for one hour with a blocking buffer of 10% normal goat serum (ab7481, Abcam plc, Cambridge, UK) at room temperature. The primary antibodies used were **CD68** without dilution (mAb Mouse, clone PG-M1, IS613, Dako GmbH, Hamburg, Germany), **CCR7** (mAb Rabbit, N-term, ab32527, Abcam plc, Cambridge, UK) antibody at 1:250 dilutions, diluted in blocking buffer and **CD163** (mAb Mouse, RM3/1, ab17051, Abcam plc, Cambridge, UK) at 1:100 dilutions, diluted in phosphate buffered saline (PBS). Anti-rabbit and mouse horseradish peroxidase (HRP) polymer secondary antibody was used as the secondary antibody (Goat, ab2891, Abcam plc, Cambridge, UK). Staining was concluded with 3-amino-9-ethylcarbazole (AEC) staining kit (AEC101, Sigma-Aldrich Gmbh, Munich, Germany) and Mayer's hematoxylin (CARL ROTH GmbH + Co. KG, Karlsruhe, Germany) followed by application of Fluoromount™ aqueous based mounting medium (Sigma-Aldrich Gmbh, Munich, Germany) then the slides were covered with cover slip. Cells with red-rose insoluble precipitates in the form of bands or dots were considered positive cells. Human tonsil was used as positive control. The blocking serum substituted the primary antibody for negative control. For every membrane two sections were stained for each antibody and one section was used as negative control. A detailed discussion of the used protocol is given in the attachment number 8.2 and 8.3.

3.3 Photography

Mosaic images were captured at 50x and 400x magnification using Leica DMI 6000 B microscope and Leica Application Suit LAS Software (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). The use of the automated mosaic image capture prevents the possibility of overestimation or underestimation of the counted positive cells caused by missed or overlapped manually captured images, figure (1).

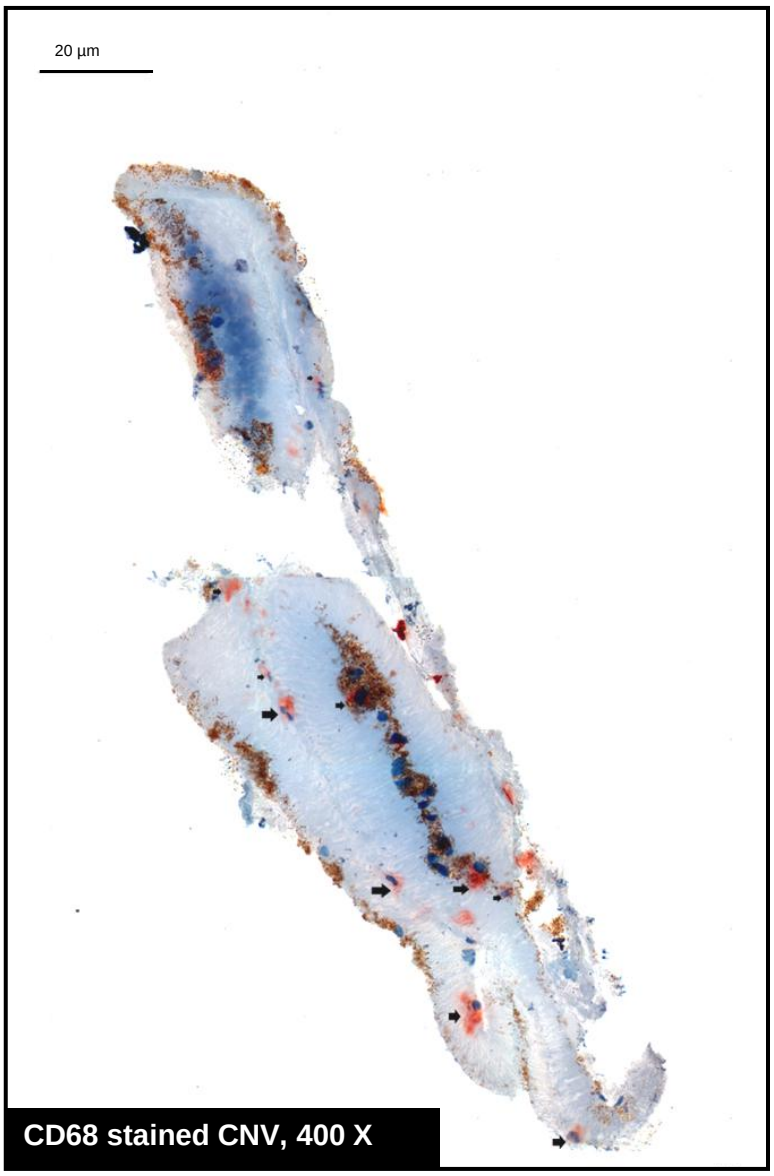


Fig. 1. A mosaic image formed of (21) images at 400X Magnification.

A predominantly classic CNV with prior PDT was stained for CD68 antibody. Cells with red–rose insoluble precipitates in the form of bands or dots were considered as positive cells (arrows), case number 28 in table 7.

3.4 Analysis

3.4.1 Calculation of the membrane surface area

The whole CNV surface area was calculated at 50 times magnification using Leica Application Suit LAS Software, analysis module, figure (2). The area of the CNV was expressed in mm^2.

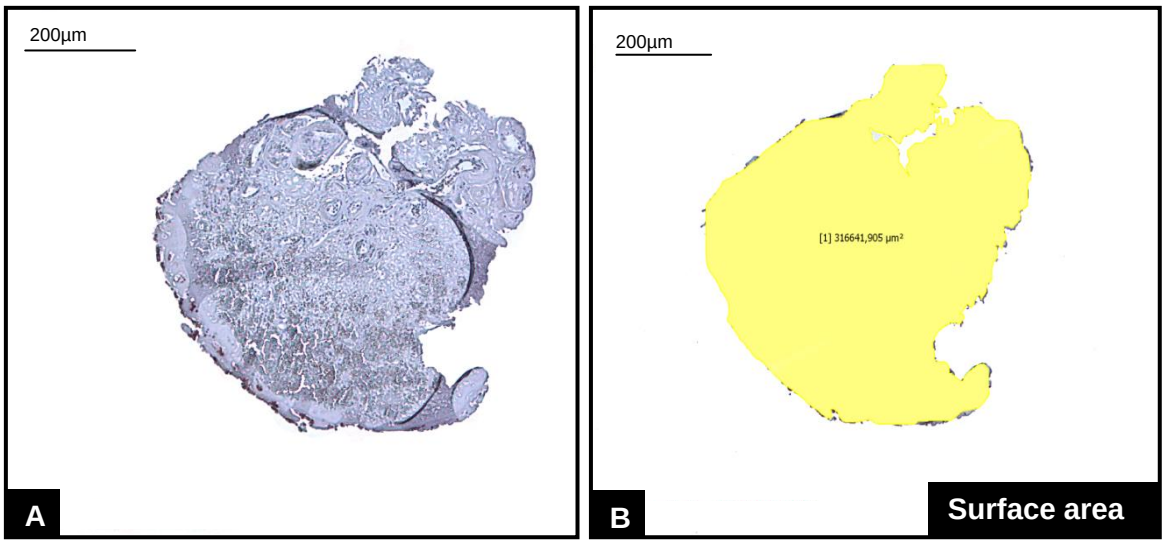


Fig. 2. Calculation of the membrane area at 50X magnification. A: Membrane image at 50X magnification. **B:** Surface area of the membrane superimposing the original photo, case number 13 in table 7.

3.4.2 Counting the immunopositive cells

The Immunopositive cells were counted for each specimen in the whole membrane using the 400 times magnification images. The cell counter and grid plug-in functions of free software ImageJ were used (Rasband, 1997). For each tested antibody, cell density was calculated in relation to the whole membrane surface area in mm^2. An M1/M2 ratio was then calculated for each specimen.

3.5 Statistics

Statistical analysis was performed with SPSS software for Windows version 16 (SPSS Inc, Chicago, Illinois, USA). Descriptive analysis for the study groups was reported as mean ± standard deviation (SD) for parametric results and the median and the interquartile range for non parametric results. Wilcoxon test was used to evaluate the difference between the CNV variables within the same group. Mann-Whitney test was used to evaluate the difference between the patient variables in between groups. P-values less than 0.05 were considered significant, tables (3-6).

4 RESULTS

4.1 Clinical characterization

This study retrospectively reviewed 58 eyes of 58 consecutive patients who had surgical extraction of CNV between 2004 and 2007. In all patients the cause of CNV development was AMD. The patient characteristics are described in attachment 8.1. The CNVs of the 58 patients, 32 females and 26 males, were divided into 9 groups as mentioned in the methodology, table 1. The mean age of the patients was 74.94 ± SD 7.16 years. In 34 patients the right eyes were operated on and in 24 patients the left eyes were operated on. The mean value of preoperative VA was 0.74 ± SD 0.82 LogMar notation. The angiographic diagnosis of the membranes was classic CNV in 15 membranes, occult CNV in 25 membranes and hemorrhagic CNV in 18 membranes. In 55 patients the membrane was located subfoveal, in 1 patient the membrane was located subfoveal and juxtafoveal, in 1 patient the membrane was located juxtafoveal and peripapillary and in 1 patient the membrane was located juxtafoveal and subfoveal. The membrane size was less than 2 optic disc diameter (DD) in 20.7% of the membranes, less than 3 DD in 48.3% and more than 3 DD in 31%. At the time of surgery, 43% patients had received no prior treatment. 15.5 % of patients had received PDT monotherapy, 17.2 % of patients had received intravitreal bevacizumab monotherapy. 6.9 % of patients had received intravitreal ranibizumab monotherapy once. 8.6 % of patients had received intravitreal TA monotherapy. 8.6% of patients had received combined bevacizumab and PDT treatment. The mean period between the medical treatment and the surgical extraction of the membrane in each group is shown in table (3).

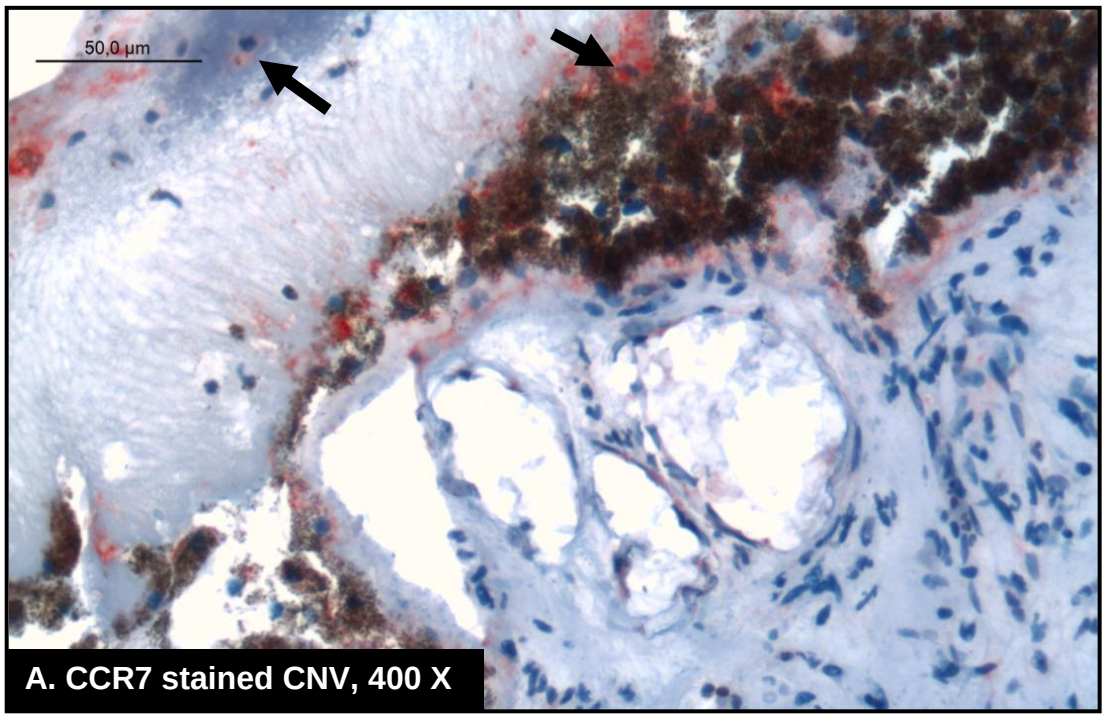
Table 3. The mean value of the duration (in days) between the date of the last given treatment and the surgical intervention

	N	Mean	Std. Deviation
Group 4 (G4)	9	93	51
Group 5 (G5)	10	49	64
Group 6 (G6)	4	18	17
Group 7 (G7)	5	15	22
Group 8 (PDT) (G8)	5	189	118
Group 8 (Avastin) (G8)	5	111	123

G4: Patients treated with PDT. **G5:** Patients treated with bevacizumab (Avastin®). **G6:** Patients treated with Ranibizumab (Lucentis®). **G7:** Patients treated with triamcinolone. **G8:** Patient treated with combined PDT and bevacizumab.

4.2 Immunohistopathologic findings

It was possible to detect macrophage cells in all examined membranes. Cells with red-rose insoluble precipitates in the form of bands or dots were considered as positive cells, figures (3-8). Macrophages were detected within the CNV stroma, RPE layer and close to the endothelial channels.



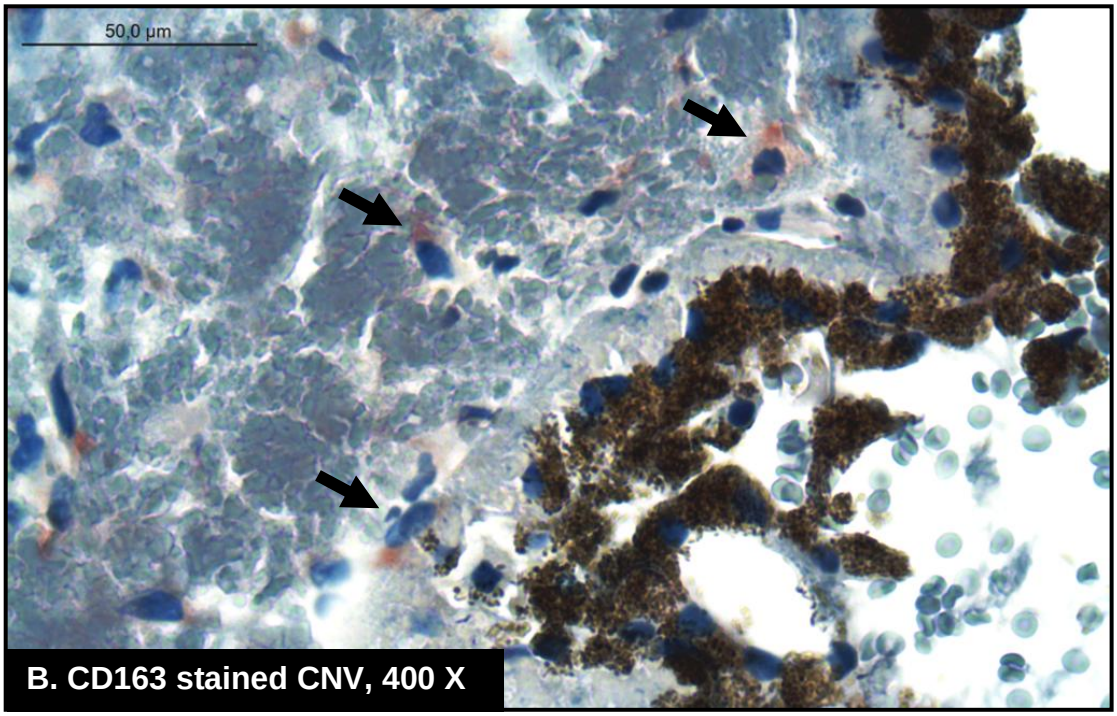


Fig. 3. Immunohistopathological staining results 1. Photomicrographs of a hemorrhagic CNV depicting CCR7 (M1) and CD163 (M2) positive (red-rose staining pattern) macrophages (black arrows), case number 21 in table 7.

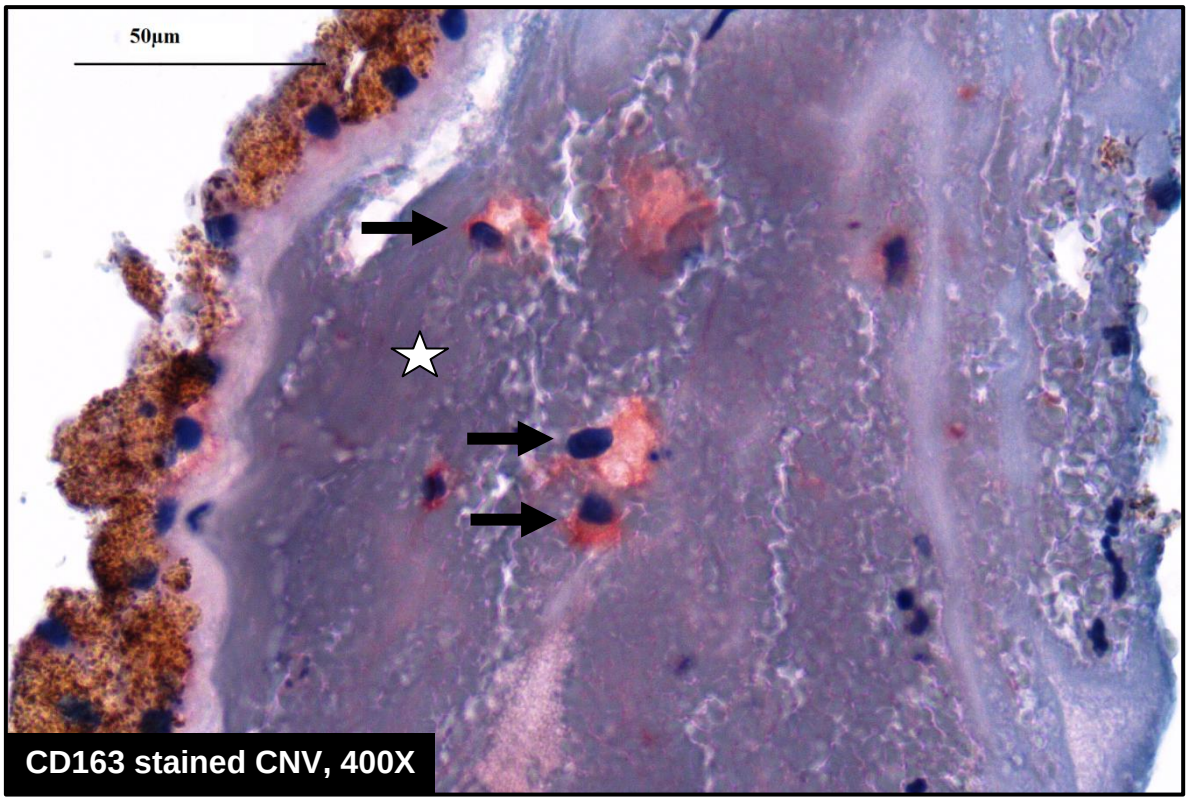


Fig. 4. Immunohistopathological staining results 2. CD163 stained membrane, positive macrophages (black arrows) within the CNV stroma; the white star indicates the hemorrhagic component, case number 25 in table 7.

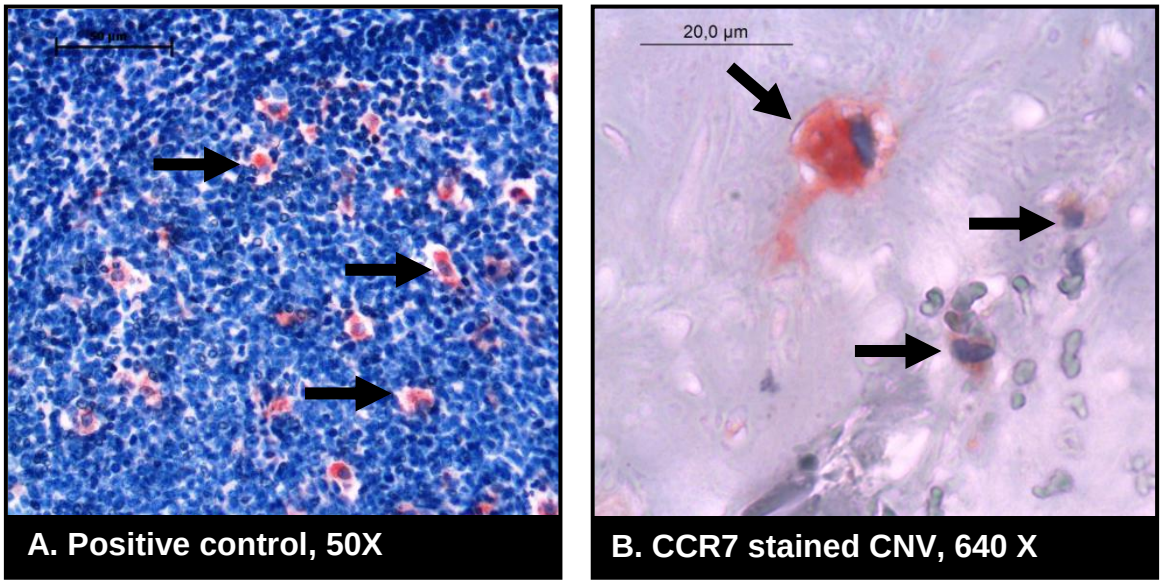
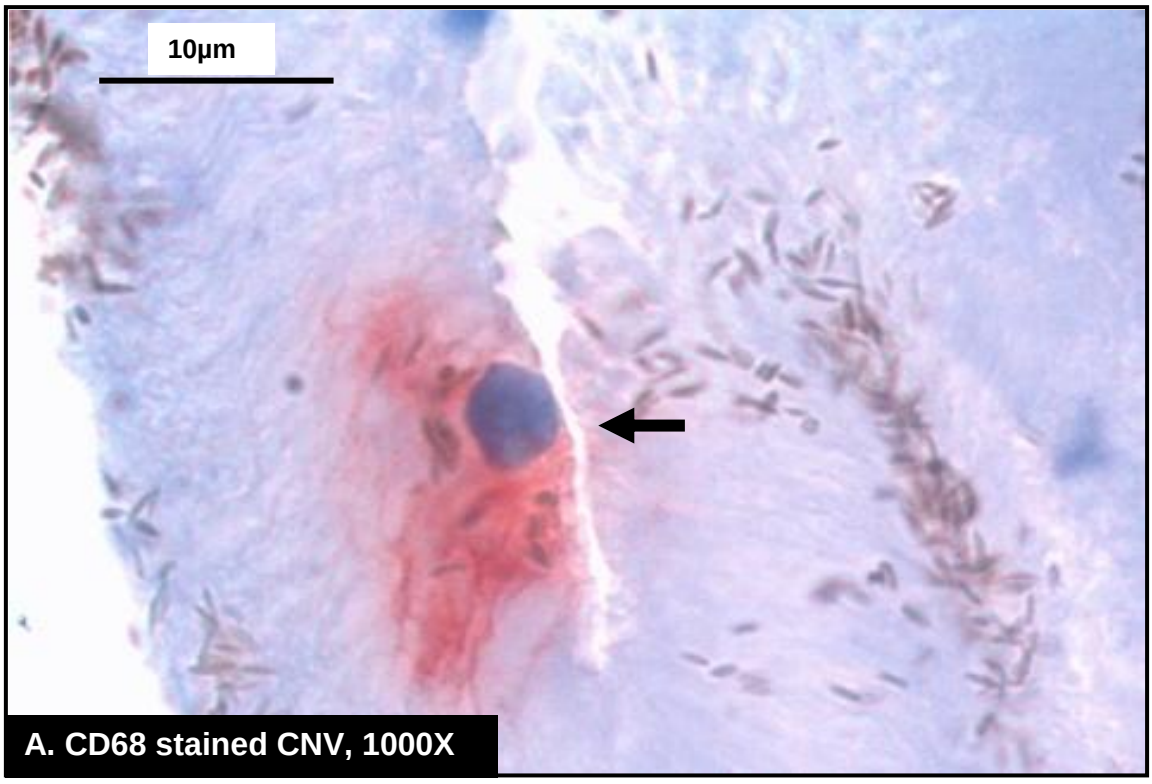


Fig. 5. Immunohistopathological staining results 3.

A. Positive control: human tonsil stained for CD163 antibody, 50x. **B.** Case number 14 in table 7, occult CNV without prior treatment depicting CCR7 positive macrophages (M1) (black arrows) within the CNV stroma.



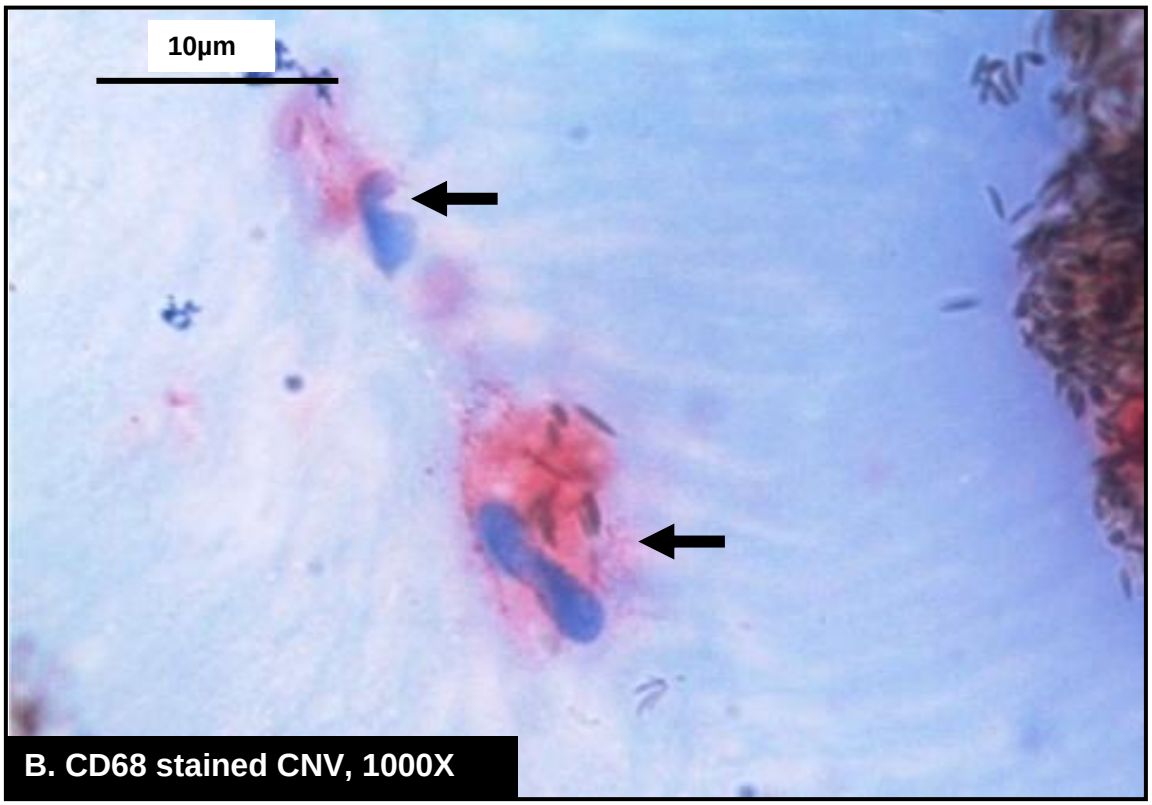


Fig. 6. Immunohistopathological staining results 4. Photomicrographs of a CNV after PDT monotherapy depicting CD68 pan macrophages positive cells (M), (black arrows), case number 28 in table 7.

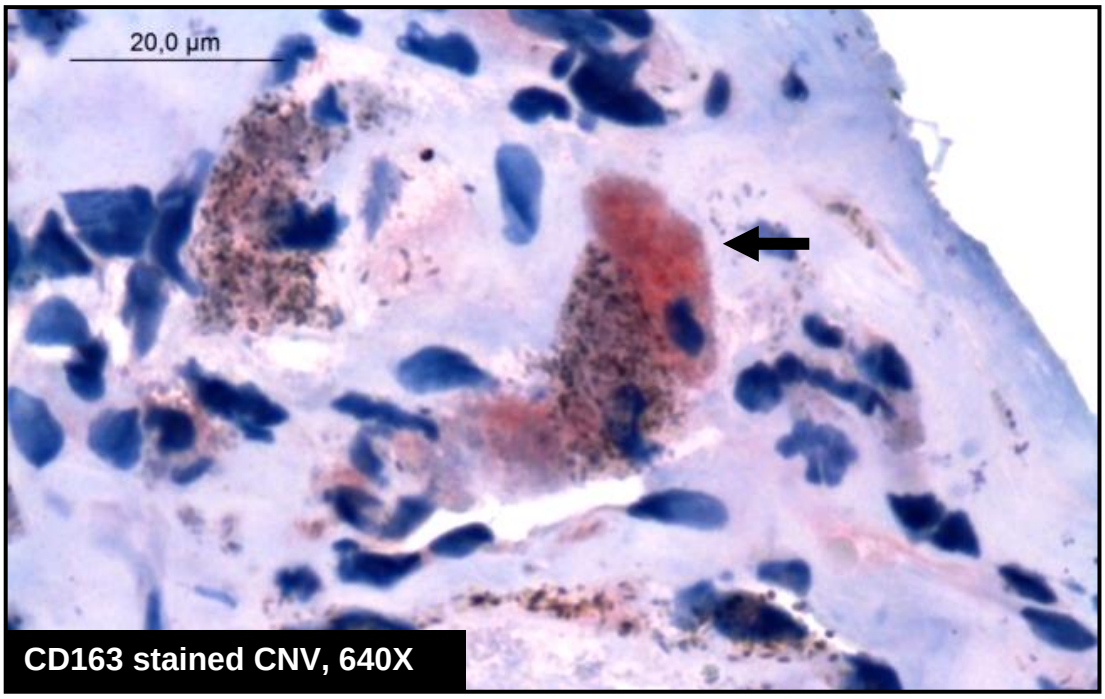


Fig. 7. Immunohistopathological staining results 5. Photomicrographs of a CNV after bevacizumab monotherapy depicting CD163 positive macrophages (black arrow), case number 39 in table 7.

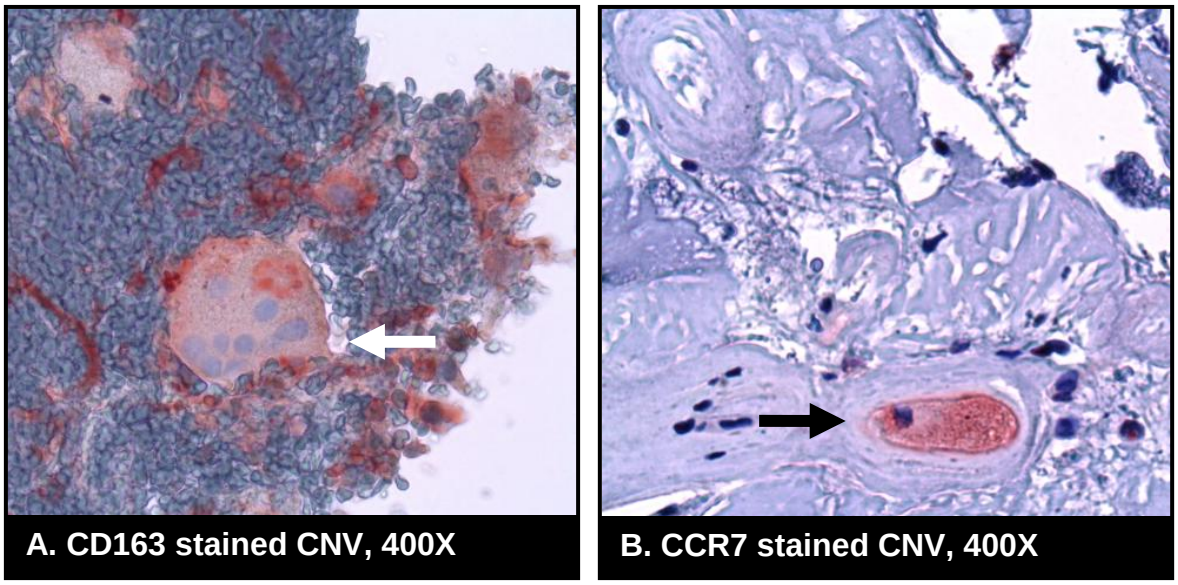


Fig. 8. Immunohistopathological staining results 6.

A: Case number 23 in table 7, Photomicrographs of a hemorrhagic CNV without prior therapy depicting CD163 macrophages positive (M2), (red-rose staining pattern). A multinucleated giant cell (white arrow) cell was detected, 400X. **B:** Case number 14 in table 7, An occult CNV showed suspected foam like cell (black arrow) positively stained to CCR7 antibody, 400X.

4.3 Macrophage activation pattern

Macrophage cell density was represented as the number of macrophage cells in relation to the surface area of the membrane in mm^2. The median value of cell density (cell/mm^2) for each macrophage phenotype and the M1/M2 cell density ratio in each group were calculated, tables (4-6) & figures (9-12).

In G1 "Classic CNV, n = 5", the median value of the M1 cell density (125 cell/mm^2 interquartile range 64 cell/mm^2 - 225 cell/mm^2) was slightly higher than the median value of its M2 cell density (112 cell/mm^2 interquartile range 62 cell/mm^2 - 210 cell/mm^2, p value 0.893). **In G2 "Occult CNV, n= 10"**, the median value of the M1 cell density (136 cell/mm^2 interquartile range 112 cell/mm^2 - 197 cell/mm^2) was slightly lower than the median value of its M2 cell density (156 cell/mm^2 interquartile range 98 cell/mm^2 - 224 cell/mm^2, p value 0.959). **In G3 "Hemorrahgic CNV, n= 10",** the median value of the M2 cell density (928 cell/mm^2 interquartile range 400 cell/mm^2 - 1263 cell/mm^2) was significantly higher than the median value of its M1 cell density (211 cell/mm^2 interquartile range 109 cell/mm^2 - 417 cell/mm^2, p value 0.005). As well, M2 cell density of hemorrhagic CNV (G3) (928 cell/mm^2 interquartile range 400 cell/mm^2 - 1263 cell/mm^2) was significantly higher than M2 cell density of classic CNV (112 cell/mm^2 interquartile range 62 cell/mm^2 - 210 cell/mm^2, p value) and occult CNV (156 cell/mm^2 interquartile range 98 cell/mm^2 - 224 cell/mm^2), p values, 0.014 and 0.003 respectively. A more global view for the pattern of macrophage activation was presented by G9, which represent all non treated CNV specimens (n=25). **In G9** a statistically significant higher M2 cell density (204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm^2) than its M1 cell density (159 cell/mm^2 interquartile range 105 cell/mm^2 - 264 cell/mm^2) was detected, p value 0.012.

To emphasize, in CNV with no prior treatment (G9), M2 cells were the prevalent macrophage cell type that was more pronounced in hemorrhagic CNV (G3). Hemorrhagic CNV had a statistically significant higher M2 cell density compared to its M1 cell density; classic CNV M2 cell density (G1) and occult CNV M2 cell density (G2).

CNV treated with PTD (G4, n= 9) was extracted at a relatively longer interval following treatment (Mean 93 days ± SD 51). Following PDT, the median value of M1 cell density was higher than its M2 cell density (143 cell/mm^2

interquartile range 115 cell/mm^2 - 236 cell/mm^2 versus 131 cell/mm^2 interquartile range 50 cell/mm^2 - 200 cell/mm^2, p value 0.26).

However the median value of M1 cell density was lower compared to control group (G9) (143 cell/mm^2 interquartile range 115 cell/mm^2 - 236 cell/mm^2 versus 159 cell/mm^2 interquartile range 105 cell/mm^2 - 204 cell/mm^2, p value 0.984). The median value of M2 cell density was significantly lower than that of the control group (131 cell/mm^2 interquartile range 50 cell/mm^2 - 200 cell/mm^2 versus 204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm, p value 0.044).

M1/M2 ratio of PDT treated CNV (1.75 interquartile range 1.03 – 3.37) was significantly higher than the control group (0.58 interquartile range 0.36 – 1.24), p value 0.01.

To emphasize, in PDT treated CNV (G4), activation of **M2** cells were significantly suppressed compared to the control. A significantly high **M1/M2 ratio** was present compared to the control group which was caused by the prevalence of M1 cells.

Following treatment with bevacizumab (Avastin®) "G5, n= 10", CNVs were extracted after therapy with mean value of duration of 49 days ± SD 64. The median value of the M2 cell density was lower than the median value of its M1 cell density (37 cell/mm^2 interquartile range 17 cell/mm^2 - 167 cell/mm^2 versus 81 cell/mm^2 interquartile range 51 cell/mm^2 - 94 cell/mm^2, p value 0.799).

However the median value of M, M1 and M2 cell densities was significantly lower compared to control group (G9). M cell density was significantly lower in bevacizumab treated CNV (G5) compared to control (G9) (182 cell/mm^2 interquartile range 66 cell/mm^2 - 318 cell/mm^2 versus 311 cell/mm^2 interquartile range 215 cell/mm^2 - 517 cell/mm^2, p value 0.026). The median value of M1 cell density was significantly lower than that of the control group (81 cell/mm^2 interquartile range 51 cell/mm^2 - 94 cell/mm^2 versus 159 cell/mm^2 interquartile range 105 cell/mm^2 - 264 cell/mm, p value 0.003). The median value of M2 cell density was significantly lower than that of the control group (37 cell/mm^2 interquartile range 18 cell/mm^2 - 167 cell/mm^2 versus 204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm, p value 0.003).

To emphasize, bevacizumab therapy (G5) suppresses macrophages infiltration to the CNV. Bevacizumab caused a statistically significant reduction of

M, **M1**, and **M2** macrophages (p value 0.026, 0.003, 0.003 respectively, Mann Whitney test) with more reduction of angiogenic M2 cells compared to the control group.

Following treatment with ranibizumab (Lucentis®) "G6, n=4", CNVs were extracted after therapy with mean value of duration of 18 days ± SD 17. The median value of the M1 cell density was higher than the median value of its M2 cell density (125 cell/mm^2 interquartile range 85 cell/mm^2 - 149 cell/mm^2 versus 29 cell/mm^2 interquartile range 19 cell/mm^2 - 51 cell/mm^2, p value 0.068).

M cell density was significantly lower in ranibizumab treated CNV (G6) compared to control G (9) (178 cell/mm^2 interquartile range 138 cell/mm^2 - 189 cell/mm^2 versus 311 cell/mm^2 interquartile range 215 cell/mm^2 - 517 cell/mm^2, p value 0.027). The median value of M1 cell density was lower than that of the control group (125 cell/mm^2 interquartile range 85 cell/mm^2 - 149 cell/mm^2 versus 159 cell/mm^2 interquartile range 105 cell/mm^2 - 264 cell/mm, p value 0.229). The median value of M2 cell density was significantly lower than that of the control group (29 cell/mm^2 interquartile range 19 cell/mm^2 - 51 cell/mm^2 versus 204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm, p value 0.003). There were no statistically significant differences in ranibizumab M, M1, M2 cell densities and M1/M2 ratios compared to bevacizumab treated group (G5). The median M1 /M2 ratio was significantly increased (3.87 interquartile range 1.97-8.67) compared to the control group (0.58 interquartile range 0.36-1.24), p value 0.007.

To emphasize, ranibizumab therapy (G6) caused a statistically significant reduction of the median M cell density and the median M2 density compared to the control group. The median M1 /M2 ratio was significantly increased compared to the control group. The M1 cells remain relatively unaffected compared to the control.

Following treatment with TA "G7, n=5", CNVs were extracted after therapy with mean value of duration of 15 days ± SD 22. The median value of the M1 cell density was significantly lower than the median value of its M2 cell density (33 cell/mm^2 interquartile range 21 cell/mm^2 - 52 cell/mm^2 versus 54 cell/mm^2 interquartile range 19 cell/mm^2 - 92 cell/mm^2, p value 0.043).

M cell density was significantly lower in TA treated CNV (G7) compared to control Group (G9) (163 cell/mm^2 interquartile range 97 cell/mm^2 - 187 cell/mm^2

versus 311 cell/mm^2 interquartile range 215 cell/mm^2 - 517 cell/mm^2, p value 0.013). The median value of M1 cell density was significantly lower than that of the control group (33 cell/mm^2 interquartile range 22 cell/mm^2 - 52 cell/mm^2 versus 159 cell/mm^2 interquartile range 105 cell/mm^2 - 264 cell/mm, p value 0.003). The median value of M2 cell density was significantly lower than that of the control group (54 cell/mm^2 interquartile range 19 cell/mm^2 - 54 cell/mm^2 versus 204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm, p value 0.006).

To emphasize, TA (G7) caused a significant reduction of pan M, M1, and M2 macrophages cell densities compared to the control group. The M2 cell density was significantly higher compared to M1 following triamcinolone treatment.

Following treatment with combined PDT and bevacizumab "G8, n=5", CNVs were extracted after therapy with mean value of duration of 189 days ± SD 118 for PDT and 111 days ± SD 123 for bevacizumab. The median value of the M1 cell density was lower than the median value of its M2 cell density (89 cell/mm^2 interquartile range 44 cell/mm^2 - 172 cell/mm^2 versus 148 cell/mm^2 interquartile range 106 cell/mm^2 285 cell/mm^2, p value 0.345).

M cell density was higher in combined PDT and bevacizumab treated CNV (G8) compared to control (G9) (424 cell/mm^2 interquartile range 384 cell/mm^2 - 615 cell/mm^2 versus 311 cell/mm^2 interquartile range 215 cell/mm^2 - 517 cell/mm^2, p value 0.140). The median value of M1 cell density was lower than that of the control group (89 cell/mm^2 interquartile range 44 cell/mm^2 - 172 cell/mm^2 versus 159 cell/mm^2 interquartile range 105 cell/mm^2 - 264 cell/mm, p value 0.080). The median value of M2 cell density was lower than that of the control group (148 cell/mm^2 interquartile range 106 cell/mm^2 - 285 cell/mm^2 versus 204 cell/mm^2 interquartile range 107 cell/mm^2 - 807 cell/mm, p value 0.42).

To emphasize, combined anti-VEGF and PDT (G8) showed no significant change in the M1/M2 ratio, pan M, M1 or M2 macrophages cell densities when compared to the control group.

Table 4. The pattern of macrophage activation represented as cell density (cell/mm^2)

		Pan macrophage(M)				Classically activated macrophages (M1)				Alternatively activated macrophages (M2)				M1/M2 ratio			
			Percentiles				Percentiles				Percentiles				Percentiles		
Groups	**n**	**M***	**25**	**50**	**75**	**M**	**25**	**50**	**75**	**M**	**25**	**50**	**75**	**M**	**25**	**50**	**75**
G1	**5**	266	197	266	422	125	64	125	225	112	62	112	210	0.69	0.48	0.69	3.40
G2	**10**	290	161	290	394	136	112	136	197	156	98	156	224	1.12	0.56	1.12	1.80
G3	**10**	517	234	517	946	211	109	211	417	928	400	928	1263	0.32	0.22	0.32	0.50
G4	**9**	366	87	366	500	143	115	143	236	131	50	131	200	1.75	1.03	1.75	3.37
G5	**10**	182	66	182	318	81	51	81	94	37	18	37	167	2.28	0.50	2.28	4.01
G6	**4**	178	138	178	189	125	85	125	149	29	19	29	51	3.87	1.97	3.87	8.67
G7	**5**	163	97	163	187	33	21	33	52	54	19	54	92	0.74	0.48	0.74	1.80
G8	**5**	424	384	424	615	89	44	89	172	148	106	148	285	0.44	0.21	0.44	2.10
G9	**25**	311	215	311	517	159	105	159	264	204	107	204	807	0.58	0.36	0.58	1.24

n: Number of CNV membranes. **G1:** No treatment, classic AMD CNV. **G2**: No treatment, occult CNV. **G3:** No treatment, hemorrhagic CNV. **G4:** Patients treated with PDT. **G5:** Patients treated with bevacizumab (Avastin®). **G6:** Patients treated with ranibizumab (Lucentis®). **G7**: Patients treated with triamcinolone. **G8**: Patient treated with combined PDT and bevacizumab. **G9**: No treatment control group. **M*:** Median value of the macrophage cell density.

Table 5. The significance of the difference between M1 and M2 cell density within the same group using the Wilcoxon test.	
Group	**Asymptomatic Significant (2-tailed)**
G1	0.893
G2	0.959
G3	0.005*
G4	0.260
G5	0.799
G6	0.068
G7	0.043*
G8	0.345
G9	0.012*
*Significant p value **G1**: No treatment, classic AMD CNV. **G2**: No treatment, occult CNV. **G3**: No treatment, hemorrhagic CNV. **G4**: Patients treated with PDT. **G5**: Patients treated with bevacizumab (Avastin®). **G6**: Patients treated with Ranibizumab (Lucentis®). **G7**: Patients treated with triamcinolone. **G8**: Patient treated with combined PDT and bevacizumab. **G9**: No treatment control group.	

Table 6. The significance of the difference in the macrophage activation between the study groups using the Mann-Whitney test, *p* values (*Asymp. Sig)* less than 0.05 were regarded as significant differences.				
	M	**M1**	**M2**	**Ratio**
G1&G2	0.903	0.624	0.540	0.800
G1&G3	0.221	0.270	0.014*	0.027*
G2&G3	0.112	0.290	0.003*	0.002*
G4&G9	0.424	0.984	0.044*	0.010*
G5&G9	0.026*	0.003*	0.003*	0.077
G5&G6	0.690	0.095	0.917	0.074
G6&G9	0.027*	0.229	0.003*	0.007*
G7&G9	0.013*	0.003*	0.006*	0.359
G8&G9	0.140	0.080	0.420	0.359
Significant p value **G1**: No treatment, classic AMD CNV. **G2**: No treatment, occult CNV. **G3**: No treatment, hemorrhagic CNV. **G4**: Patients treated with PDT. **G5**: Patients treated with bevacizumab (Avastin®). **G6**: Patients treated with Ranibizumab (Lucentis®). **G7**: Patients treated with triamcinolone. **G8**: Patient treated with combined PDT and bevacizumab. **G9**: No treatment control group.				

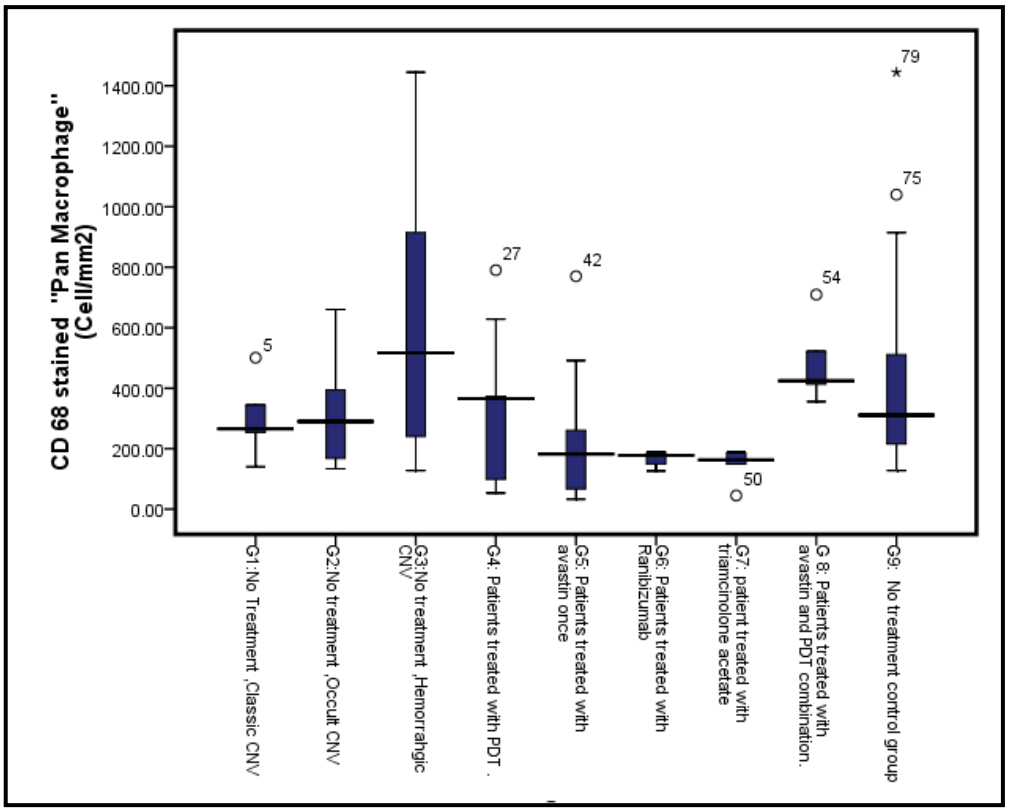


Fig. 9. The median values of the pan macrophage (M) cell density

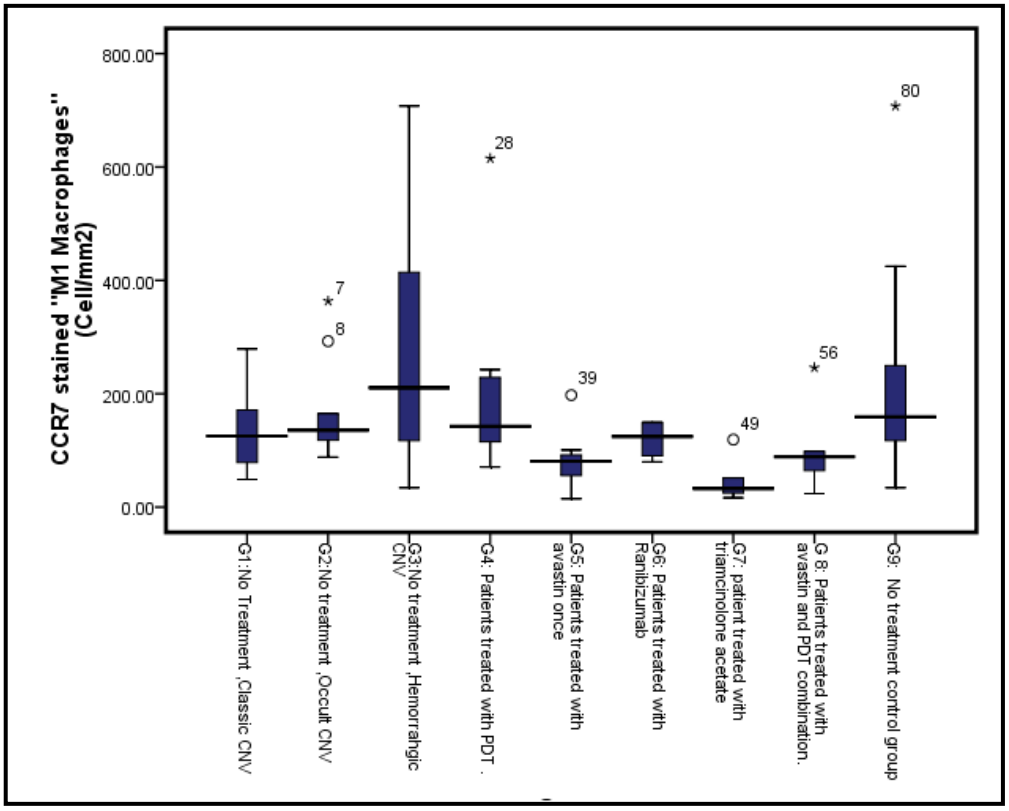


Fig. 10. The median values of the cell density of the classically activated macrophage (M1)

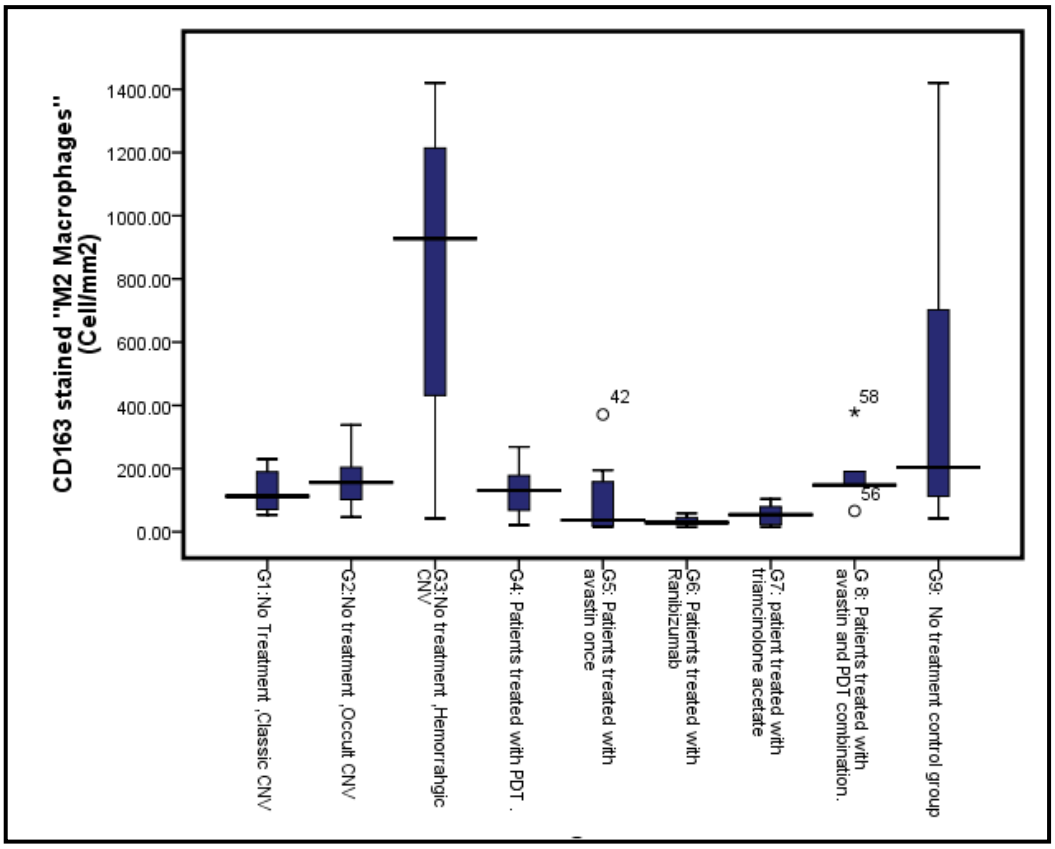


Fig. 11. The median values of the cell density of the alternatively activated macrophage (M2)

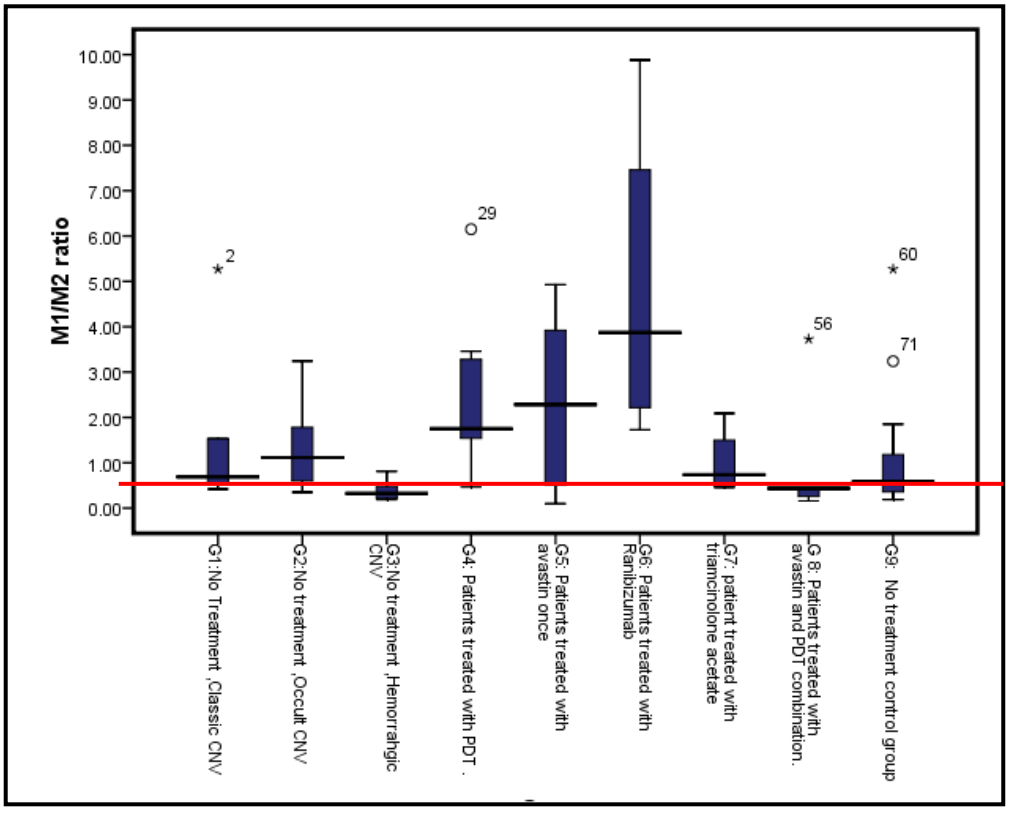


Fig. 12. The median values of the M1/M2 ratio, red line indicate equal cell density of both phenotypes (M1/M2 ratio).

5 DISCUSSION

Immune-mediated processes have long been associated with a variety of ocular diseases. Growing evidence suggests that inflammatory and immunologic events might play a role in AMD (McGonagle and Georgouli, 2008).

Since many years macrophages have been localized to sites of CNV in patients with AMD. Macrophages promote the growth of endothelial cells, pericytes, and fibroblasts. Furthermore, macrophage derived prostaglandins, especially prostaglandin E, may be a strong stimulus to neovascularization (Polverini et al., 1977). Activated macrophages may erode Bruch`s membrane by a combination of mechanical disruption, phagocytosis, and extracellular release of enzymes such as collagenases and elastases (Killingsworth et al., 1990). Oh and his colleagues suggested that the presence of activated, pro-inflammatory macrophages may induce VEGF production by RPE, promoting choroidal neovascularisation (Oh et al., 1999). Grossniklaus and his colleagues also examined surgically excised CNV membranes and observed that CD68-positive macrophages expressed VEGF and tissue factor, while RPE cells expressed monocyte chemo-attractant protein-1 (MCP-1) These authors concluded that RPE might be important for macrophage recruitment and that recruited macrophages expressed growth factors essential for neovascularisation (Grossniklaus et al., 2002). Experiments based on Laser photocoagulation model of CNV suggest that recruitment of macrophages is important for CNV development (Ishibashi et al., 1997; Tsutsumi et al., 2003). On the other hand, macrophage was suggested to be important for protection against CNV development. Duvall and Tso suggested a role of macrophages in clearance of drusen (Duvall and Tso, 1985). In 2003, Ambati and his colleagues introduced a new mouse model of AMD that highly simulate the AMD. They concluded that disruption of the normal recruitment or turnover of choroidal macrophages may lead to the persistence of debris and the opsonising molecules C5 and IgG, resulting in lesions similar to those found in AMD ([2]Ambati et al., 2003). Overall, these studies on AMD and animal models of AMD present conflicting evidence regarding the macrophages role**,** figure 13.

Indeed, macrophages display great diversity of phenotype and function resulting from their ability to adapt to the local environment (Gordon et al., 1986; [1]Gordon et al., 1992). Since spatially distinct macrophages do not respond uniformly, assumptions about one population based on the evidence of another

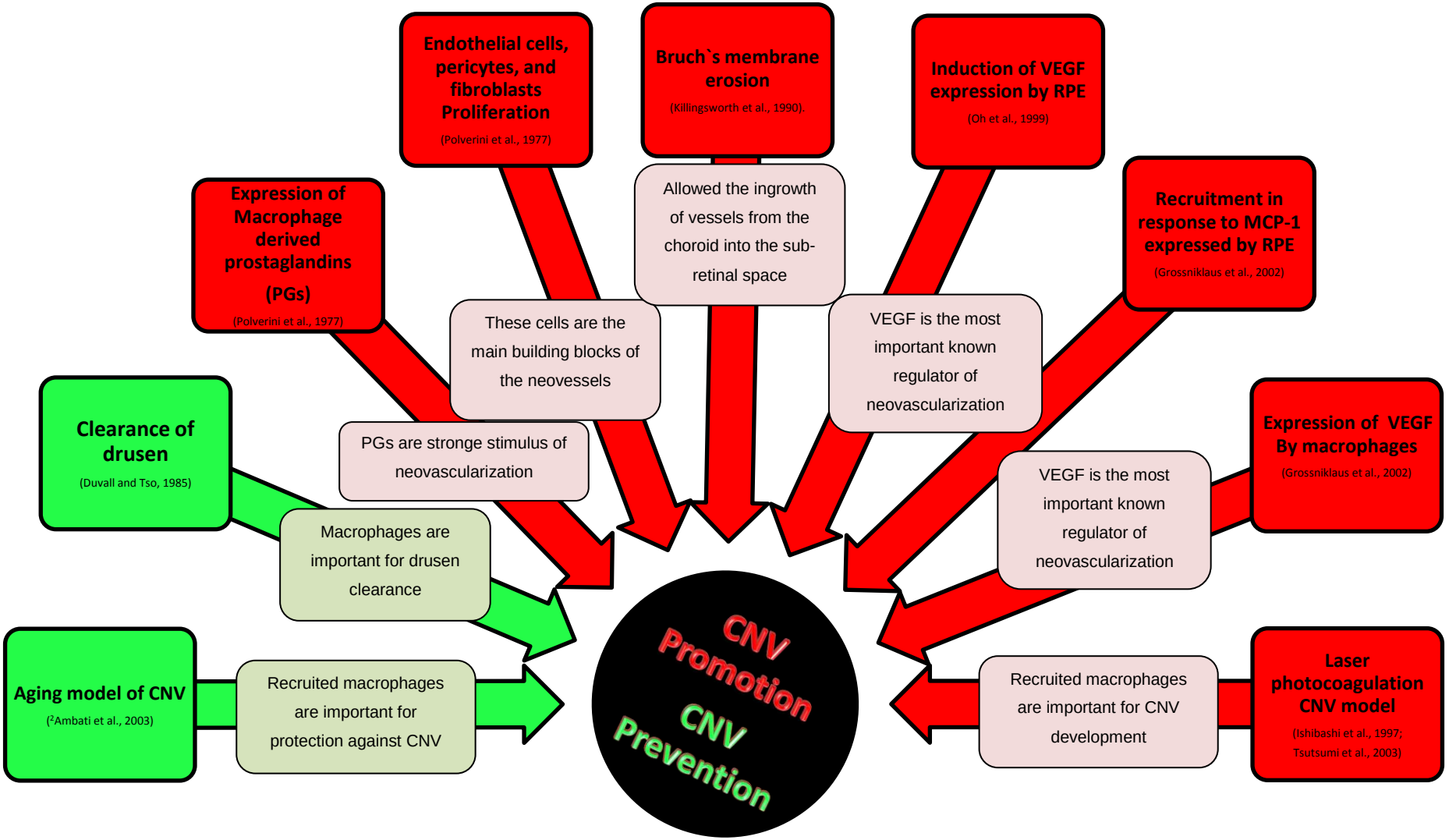


Fig. 13. Conflicting evidence regarding the role of macrophages in CNV development.

can be misleading (Daems et al., 1976; [2]Gordon et al., 1992). In order to understand their role in human CNV development it is necessary to identify and distinguish the phenotype of the involved cells.

Macrophages can be distinguished as classically activated (M1) or alternatively activated (M2) cells. These different types express specialized and polarized functional properties in response to cytokines and microbial products (Ma et al., 2003). M1 macrophages are involved in type I inflammation, tissue destruction, killing intracellular parasites and tumour resistance. They can produce powerful oxidants cause lipid peroxidation of affected cell membranes, leading to cell death (Gordon, 2003). M2 macrophages are programmed to express the enzyme arginase, which converts arginine to ornithine, promoting cell division, repair (Mills et al., 2000) and angiogenesis (Kodelja et al., 1997). Thus, macrophages participate to different extents in pro-inflammatory and anti-inflammatory immune reactions. However, both types of activated cells can co-exist and show differences in their capacity to secrete mediators and to express receptors (Mantovani et al., 2004), figure (14).

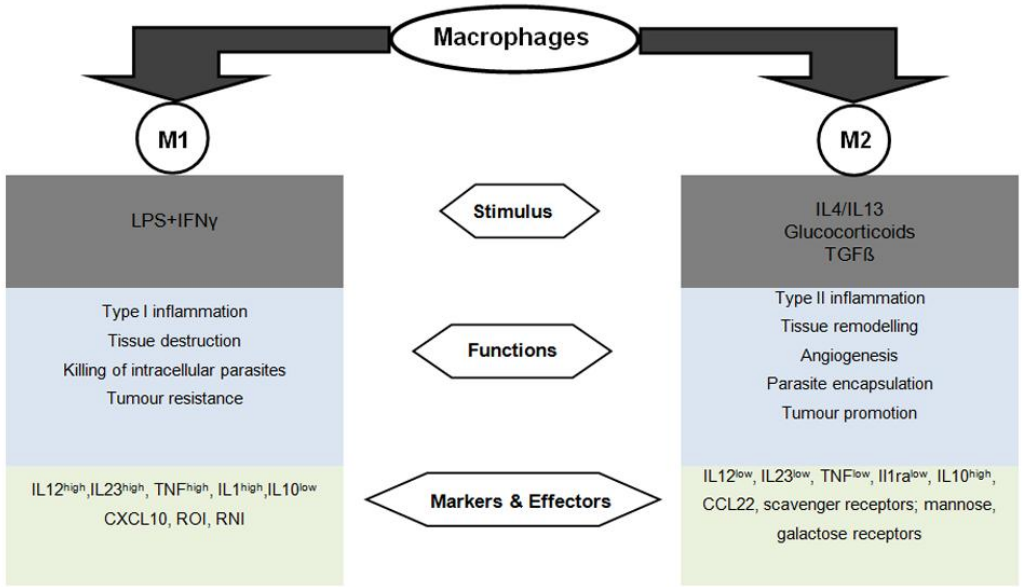


Fig. 14. Macrophage polarized activation.

A simplified view of immune and tissue-derived signals inducing classical (M1) and alternative (M2) macrophage polarized activation. The main functional effects on macrophage functions, molecular markers and effector molecules are schematically represented in both cases. "**Abbreviations: CXCL10**: Chemokine (C-X-C motif) ligand 10, **TNF-α:** Tumour necrosis factor alpha, **CCL22:** Chemokine ligand 22, **ROI:** Reactive oxygen intermediate, **RNI:** Reactive nitrogen intermediate."

The question addressed by the present study was to describe the pattern of macrophage activation in relation to the CNV variables. This present study demonstrates that the macrophages were activated within the CNV either as M1 or M2 subtype with a variable degree of activation of each type. The sum of the activated macrophages (M1+M2) was not always equal to the pan macrophage cell count. This in equality may be due to the presence of some macrophages in the deactivated form that stain for CD68 (Gordon, 2003).

The types of the CNV characteristics as well as the prior treatments have influence on the subtype of the macrophage activation. Although macrophage detection and/or its density in CNV were previously described (Lopez et al., 1991; Dastgheib and Green, 1994; Penfold et al., 2001; Schnurrbusch et al., 2001; Grossniklaus et al., 2002; Tsutsumi et al., 2003; [2]Ambati et al., 2003; Csaky et al., 2004; Grossniklaus et al., 2005; Suzuki et al., 2007; [2]Tatar et al., 2007; [1,2]Tatar et al., 2009), the author is unaware of previous reports that describe the pattern of macrophage activation in human CNV.

In this study we showed that M2 macrophages were significantly higher compared to M1 macrophages in non treated CNV (G9, n= 25), p value 0.012. The type of the CNV had an influence on the macrophage activation. Hemorrhagic CNV (G3, n=10) had a statistically significant higher M2 cell density compared to its M1 cell density; classic CNV M2 cell density, and occult CNV M2 cell density, p value = 0.005, 0.013, and 0.003 respectively. This high M2 density can have more than one explanation.

First explanation, it could be a cause of the hemorrhage within the CNV. It was reported that macrophages produce various angiogenic cytokines, including TNF-α, IL-1, bFGF, VEGF, and TGF-ß and thus play a key role in angiogenesis (Oh et al., 1999). Of the two major macrophages subtypes, M2 macrophages are well equipped to play an important role in protracted macrophages associated angiogenic processes (Kodelja et al., 1997). In addition, a high M2 cell density was present in other diseases which share with CNV some of its angiogenic hemorrhagic complications such as endometriosis (Bacci et al., 2009), atherosclerosis (Bouhlel et al., 2007, Slevin et al., 2009), and also growing and metastatic tumors (Lamagna et al., 2006; Lin et al., 2006). The increased susceptibility for hemorrhage can be related to the fact that the developed neovessels are immature and fragile which could contribute to the development of

an unstable haemorrhagic rupture-prone environment (Moulton, 2001; Slevin et al., 2009).

Second explanation is that the high density of M2 macrophages can be formed in response to hemorrhage. This can be supported by the fact that endocytosis of foreign and necrotic debris as well as scavenging components of damaged cells are well known functions of macrophages (Lamagna et al., 2006). CD163 is a scavenger receptor cysteine-rich (SRCR), a hemoglobin scavenger receptor exclusively expressed in the monocyte-macrophage system with a particularly high expression in M2 macrophages (Law et al., 1993; Kristiansen et al., 2001, Gordon, 2003). CD163 plays a major role in dampening the inflammatory response and in scavenging components of damaged cells (Moestrup and Moller, 2004; Borda et al., 2008). CD163-mediated endocytosis of haptoglobin-hemoglobin complexes formed upon red blood cell haemolysis leads to lysosomal degradation of the ligand protein and metabolism of heme by cytosolic heme oxygenase. CD163 regulates inflammation by at least two ways. Firstly, it is reported to directly induce intracellular signaling leading to secretion of anti-inflammatory cytokines. Secondly, and perhaps even more importantly, the CD163-mediated delivery of hemoglobin to the macrophage may fuel an anti-inflammatory response because heme metabolites have potent anti-inflammatory effects (Borde et al., 2008; Pilling et al., 2009).

A Third explanation can be a combination of the previously mentioned explanations. In this explanation the author postulates that local M2 macrophages can proliferate and accelerate the angiogenesis of such hemorrhage prone neovessels then once hemorrhage occurs; it starts to carry out the scavenger function. The presence of the M2 cells in non hemorrhagic CNV as presented in our study can support this explanation.

The above results are important for several reasons. *First*, they suggest that the alternative activation of macrophages could be a determinant factor for the susceptibility to hemorrhagic complications for CNV patients which are a major cause of poor visual prognosis in these patients (Hochman et al., 1997). *Second*, they suggest that it may be possible to avoid hemorrhagic complications of human CNV though local inhibition of alternatively activated macrophage. *Third*, since there is a continuous shedding of the extracellular domain of CD163 which leads to substantial amounts of soluble receptor in plasma (Borde et al., 2008; Pilling et

al., 2009), the author suggest that the M2 macrophages activity can be evaluated through an estimation of the plasma level of soluble CD163 receptor and in turn the susceptibility to haemorrhagic complications within CNV. *Fourth*, it brings attention to a possibility of involvement of peroxisome proliferator-activated receptor-gamma (PPAR-ɣ) in hemorrhagic CNV. There is growing evidence of the involvement of PPAR receptors in AMD pathogenesis (Herzlich et al., 2008). Bouhlel and associates reported that PPAR-γ activation primes primary human monocytes for M2 differentiation and M2 marker expression was positively correlated with that of PPAR-γ in human atherosclerotic lesions (Bouhlel et al., 2007). In addition, the similarity between the atherosclerosis and the development of AMD is well established (Friedman, 2004; Kamei et al., 2007).

Examination of membranes treated with photodynamic therapy (G4, n=9) revealed a statistically significant reduction of M2 cells compared to control, p value, 0.04 with a statistically significant high M1/M2 ratio. An increased M1/M2 ratio indicates prevalence of M1 in PDT treated membranes. PDT causes up-regulation of TNF-α (Schmidt-Erfurth and Hasan, 2000), and IFN-ɣ (Gollnick et al., 2006). Both cytokines are major stimuli for macrophage to be activated through the classic pathway (M1) (Mosser, 2003). Post PDT prevalent M1 macrophages could be due the effect of TNF-α and IFN-ɣ on local macrophages.

She and his colleagues suggested that PDT-induced inflammation in the CNV lesion might activate macrophages to produce iNOS, which results in excessive nitric oxide release and subsequently in photoreceptor apoptosis (She et al., 2007). The iNOS is one of the M1 macrophages products that causes to cell death (Gordon, 2003). Detection of M1 macrophage phenotype after long interval following PDT treatment (93 days ± SD 51), may indicate a need for long acting treatment strategies that target M1 macrophages following PDT.

M1 cells specifically express CCR7 (Martinez et al., 2006). That CCR7 has been identified as a key regulator of homeostatic T-cell trafficking to secondary lymphoid organs. However, recent studies suggest that this receptor also can be involved in the abnormal recruitment of T-cells from the circulation to sites of inflammation (Forster et al., 1999; Sallusto et al., 2000). Macrophages expressing CCR7 have been implicated in inflammatory skin disorders, (Ohl et al., 2004), were found within the inflamed atherosclerotic vessel wall (Waldo et al., 2008) and they are known to mediate inflammation-associated tumour progression (Mburu et

al., 2006). CCR7 expressing macrophages are exposed to increased levels of Chemokine ligand 19 (CCL19) and Chemokine ligand 21 (CCL21), potentially promoting inflammation, MMP activation, and thrombus formation (Serra et al., 2004; Burman et al., 2005). M1 cells could have a potential role in angiogenesis through MMP activation (Song et al., 2000; Ito et al., 2009).

Reduced M2 cells density following PDT could be explained in two ways. First, as we mention above, PDT causes up-regulation of TNF-α (Schmidt-Erfurth and Hasan, 2000) and IFN-ɣ (Gollnick et al., 2006), so macrophages are activated through classic pathway and no longer activated through the alternative pathway. Second, Although PDT cause up regulation of IL10 (a major stimulus of macrophage activation via alternative pathway) (Pazos and Nader, 2007), this stimulation is of a limited duration (72 and 120 h) post-PDT (Simkin et al., 2000). In our study the mean value of the duration between treatment and surgery was (93 days ± SD 51).

There are several activators of angiogenesis including VEGF, bFGF, TGF-ß, angiopoietin-1, and angiopoetin-2. However, it appears that VEGF signalling represents a critical rate-limiting step (Kaiser, 2006). The VEGF family currently has seven members: VEGF-A to VEGF-F and placental growth factor (Waisbourd et al., 2007). VEGF-A is the most important known regulator of angiogenesis (Kaiser, 2006). Different isoforms can be produced by alternative exon splicing of the VEGF-A, of these isoforms VEGF-165 is probably the major pathogenic species (Robinson and Stringer, 2001). Three VEGF receptors have been identified so far: VEGFR-1, VEGFR-2, and VEGFR-3 (Waisbourd et al., 2007).

Two anti-VEGF therapies are currently approved for intraocular use in patients with AMD and CNV: pegaptanib sodium (Macugen) and ranibizumab (Lucentis). Pegaptanib is a Ribonucleic acid (RNA) aptamer which specifically binds to and blocks the VEGF-165 isoform (Waisbourd et al., 2007). Ranibizumab is a recombinant humanized antibody fragment which inhibits all the biological active forms of VEGF (Raftery et al., 2007). Bevacizumab (Avastin®) is another anti-VGEF therapy which is a full length pan anti-VEGF humanized monoclonal antibody. It was approved for use in colon cancer but not for AMD (Steinbrook, 2006). Despite the lack of regulatory approval, it has been widely used off label for CNV and induces significant improvements in both visual acuity and retinal morphology (Spaide et al., 2006; Emerson et al., 2007).

This study found that bevacizumab (Avastin®) caused a statistically significant reduction of pan macrophages, p value 0.026; M1 macrophages, p value 0.003, and M2 macrophages, p value 0.003 with more reduction of M2 cells. The significant reduction of both types of macrophages can be a direct result of the reduction of the pan macrophage.

VEGF is a major chemo-attractant for inflammatory cells, including macrophages, neutrophils, dendritic cells, myeloid-derived suppressor cells, and T-cells (Ancelin et al., 2004; Huang et al., 2007; Dineen et al., 2008; Ko et al., 2009; Ozao-Choy et al., 2009; Shin et al., 2009). Hence a low macrophage infiltration following bevacizumab was previously reported. In tumour xenograft models, anti-VEGF therapy leads to a reduction in macrophage infiltration (Salnikov et al., 2006; Whitehurst et al., 2007; Dineen et al., 2008; Roland et al., 2009). The study carried out by Ronald and his colleagues showed that anti-VEGF therapy with bevacizumab inhibited the growth of an established breast tumour with reduced tumour microvessels density and limitation of the infiltration of tumour-associated macrophages (Roland et al., 2009). Salnikov and his colleagues also found that at 5 mg/kg dose, bevacizumab reduced the density of macrophages in xenograft carcinoma (Salnikov et al., 2006). Tog and his colleagues stated a significant correlation between tumour associated macrophages (TAM) and angiogenesis in renal cell carcinoma and they expected a reduction of TAM following anti-VEGF therapy (Toge et al., 2009). Tsutsui and his colleagues concluded that there is a close association between TAM infiltrations and both the VEGF expression and microvascular density. A prognostic implication of TAM infiltration is attributed to their involvement in tumour angiogenesis (Tsutsui et al., 2005).

Kiss and colleagues evaluated the effect of intravitreal bevacizumab on anterior chamber inflammatory activity. They found that no inflammatory response was detected clinically and by the laser flare meter after intravitreal bevacizumab administration in addition a slight reduction of the anterior chamber flare was present (Kiss et al., 2006).

Tatar and colleagues studied the effect of bevacizumab on the macrophage cell density in human CNV. They found a significantly higher macrophage cell density in bevacizumab treated CNV compared to control groups. The density of macrophages in the bevacizumab treated group ranged between (interquartile

range 321- 61821 cell/mm^2, median = 4661 cells/mm^2) (^{2}Tatar et al., 2008). This cell density is considerably high compared to our findings of pan macrophage cell density, (interquartile range 66-318 cell/mm^2, median = 182 cell/mm^2). The current study tried to improve the reliability of the cell counting through the use of mosaic software from Leica System which allowed the imaging of the whole membrane at high magnification levels without the overlapping of images which is likely to occur in membranes with large sizes. The captured images were then enrolled as a single stack to be manually counted with ImagJ software (Rasband, 1997). Through the use of this software we avoid the biases caused by the overlap or missing parts of the membrane that would occur with the use of manual photography. A simple example of a mosaic image (formed of 21 images) is given in figure (7).

This current study also evaluated the effect of combined photodynamic therapy and bevacizumab treatment on the macrophage activation. Results showed no significant change in the M1/M2 ratio, pan macrophages, M1 macrophages or M2 macrophages when compared to the control group. This can be explained by the anti-inflammatory effect of the bevacizumab could reduce the inflammation caused by the photodynamic therapy. These results are consistent with finding reported by Maier et al. They reported that PDT combined with injection of intravitreal bevacizumab tends to be more effective compared to PDT monotherapy by reducing the post-PDT increase of vascular growth and inflammatory factors (Maier et al., 2008).

Ranibizumab is a recombinant humanized antibody fragment which inhibits all the biological active forms of VEGF (Raftery et al., 2007). There was no significant difference in the macrophage activation pattern between ranibizumab and bevacizumab. We found that ranibizumab caused a statistically significant reduction of the median M cell density, p value 0.027 and the median M2 density, p value=0.003 compared to the control group. The median M1 /M2 ratio was significantly increased compared to the control group, p value=0.009. The M1 cells (pro-inflammatory macrophage) were not suppressed. Ranibizumab causes suppression of macrophage recruitment through its anti-VEGF action (Salnikov et al., 2006; Whitehurst et al., 2007; Dineen et al., 2008; Roland et al., 2009). Further reduction of macrophage activation can occur through inability of ranibizumab to

activate complement system, as it cannot bind complement because it lacks the Fc region (Gaudreault et al., 2005; Ferrara et al., 2006). Complement activation is essential for the activation of the macrophages via humeral pathway (Gordon 2003).

Although a lower incidence of inflammation was expected in patients treated with ranibizumab (Gaudreault et al., 2005; Ferrara et al., 2006), evidence of intraocular inflammation was still detectable (Brown et al., 2006; Rosenfeld et al., 2006; Kourlas and Abrams, 2007; Brown et al., 2009). Rosenfeld et al. reported an incidence of iridocyclitis (83%) and injection-site reactions (72%) following intravitreal injection of ranibizumab which did not increase with repeated injections, despite increasing the ranibizumab doses (Rosenfeld et al., 2006).

We suggest that this degree of inflammation can be explained by the presence of M1 (pro inflammatory macrophage). This suggestion is supported by the detection of IL-6, IL-1ß, and TNF-α cytokines following ranibizumab injection. Campochiaro et al found that IL-6, IL-1ß, and TNF-α were present in detectable amounts one month following ranibizumab injection in patients with vein occlusions and that their aqueous level was unaffected by the resolution of the macular oedema caused by the ranibizumab (Campochiaro et al., 2009). IL-6, IL-1ß, and TNF-α are the main proinflammatory cytokines produced by M1 cells (Gordon 2003).

Triamcinolone acetonide (TA) is a synthetic steroid of the glucocorticoid family with the hydrogen atom in the ninth position replaced with fluorine; its glucocorticoid potency is five times hydrocortisone (Gopal and Sharma, 2007). In this study we examined five occult CNV with TA prior to treatment. The CNV were extracted 15 ± 22 days after treatment. We found that there was a significant reduction of pan macrophages M, M1, and M2 macrophages cell density compared to control, p value 0.013, 0.003, and 0.006 respectively.

A low macrophage infiltration following TA therapy is well documented in previous studies. Singhal and colleagues found that TA attenuated macrophage/microglia accumulation associated retinal ganglion cell death and facilitates survival of Müller stem cell grafts (Singhal et al., 2010). Jahangier and his colleagues found that intra-articular treatment with glucocorticoids was associated with a considerable reduction of macrophage infiltration in the synovium which resulted in decreased arthritis activity at 6 months follow up

(Jahangier et al., 2006). Young and his colleagues found that intra-articular glucocorticoids may reduce CD68 positive macrophage infiltration into the synovial lining layer, 1 month following treatment (Young et al., 2001). In vitro, TA has been shown to downregulate the expression of TNF-α, VEGF, IL1, and MMP and subsequently influences neovascularization (Kim et al., 2007). Down regulation of TNF-α can be the cause of low M1 cell density because it is one of the major signals required for classic activation of macrophages (M1) (Gordon, 2003). The reduction of the M1 and M2 types can be secondary to the overall reduction of macrophages caused by TA. In our study we found that M2 cell density was significantly higher compared to M1 following TA treatment, p value 0.043. This is explained by the role of glucocorticoids in alternative activation of macrophages. Monocytes can be a source of M2 alternatively activated macrophages in response to IL-4, IL-10, IL-13, TGF-β1, or glucocorticoids (Gordon, 2003).

Tatar et al. evaluated the early effects of TA (5 ± SD 2.34 days) on macrophage infiltration to CNV. They found that TA treated CNV had a macrophage density similar to the control CNV ([1]Tatar et al., 2009). In our study we evaluated the delayed effects of TA (15 ± SD 22 days) on macrophage infiltration. We found that TA caused a reduction of macrophages infiltration to the CNV with a selective shift towards activation via an alternative pathway (M2). This discrepancy can be due to the difference in the point of time in which the CNV were examined. The mean value of the duration between the treatment and the extraction was 5± SD 2.34 days in Tatar's study, and 15 ± SD 22 days in this study. We argue that the extra time between treatment and extraction in this study has allowed the therapeutic action of TA to become evident, hence the significant reduction of macrophage cell density (Jonas et al., 2004; Jonas et al., 2005).

In addition to the description of the macrophage activation profile in AMD related CNV; we were able to detect foam-like cells and giant cells within the membranes. Foam cells were more prevalent in M1 macrophages. Macrophage foam cell formation in the arterial intima is the hallmark of early atherosclerosis (Ross, 1999). Ocular foam cells can be formed as macrophages take up the oxidized phospholipids caused by oxidative stress (Suzuki et al., 2007) or due to the activation of TLR secondary to pathogen exposure (Cao et al., 2007). This finding can support the vascular theory of CNV development.

There were some limitations in the present study. Firstly, the determination

of M1 versus M2 phenotype was based upon a limited number of cell surface markers. Secondly, it is unknown whether macrophages that were not committed to the M1 or M2 phenotype were recruited to the site of CNV and then stimulated to differentiate locally or whether phenotype-committed macrophages were selectively recruited to sites of neovascularization depending upon the antigens that were present. However, this study was able to introduce an evidence of the presence of macrophage heterogenicity in human CNV and highlight the effect of the CNV type and the prior treatment on the activation pattern of macrophage.

In conclusion, Macrophages play major and different roles in CNV development. The identification and understanding of different activation patterns may be important for distinct therapeutic strategies.

6 SUMMARY

Inflammatory processes play an integral and causative role in the pathogenesis of AMD. Evidence suggests that macrophages are involved in CNV pathogenesis; however, conflicting functions were present. Macrophages express specialized activation patterns which are referred as classically activated macrophages (M1) and alternatively activated macrophages (M2). **In this study** we retrospectively examined 58 surgically excised CNV for the pattern of macrophage activation using immunohistochemical technique. CD68, CCR7, and CD163 antibodies were used to stain for M, M1 and M2 macrophages, respectively. Macrophage activation pattern was examined in CNV specimens belonging to patients with and without prior treatment

In CNV with no prior treatment, M2 cells were the prevalent macrophage cell type. Hemorrhagic CNV had a higher M2 cell density compared to its M1; classic CNV M2 and occult CNV M2 cell densities. High M2 cells density can be responsible for CNV growth via secretion of angiogenesis cytokines. The increased M2 cells density of hemorrhagic CNV can be an indirect cause of hemorrhage, a response to hemorrhage or both.

Photodynamic therapy suppressed M2 cells activation with a significant high M1/M2 ratio caused by the prevalence of M1 cells compared to the control. It resulted in a state of inflammation caused by the relatively high M1 cells (pro-inflammatory) and low M2 cells (anti-inflammatory, angiogenic).

Bevacizumab anti-VEGF therapy suppressed infiltration of all macrophages subtypes probably by blocking the chemo-attractant effect of VEGF. It promotes an anti-inflammatory and anti-angiogenic effects. Combined Bevacizumab anti-VEGF and PDT attenuate the inflammatory state caused by PDT. **Ranibizumab anti-VEGF therapy** suppressed the overall macrophage infiltration, However M1 cells were more prevalent.This suppression can be explained by its ability to block VEGF and inability to cause activation of monocytes into macrophages via humoral pathway as it cannot activate the complement system. **Triamcinolone acetate therapy** caused a reduction of macrophages' infiltration with selective shift towards the M2 activation.

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8 ATTACHMENT

8.1 Patient characteristics

TABLE 7. Clinical Characteristics of the Patients Involved in The Study

Patient	Eye/ Gender	Age (years)	CNV type	Location	Size	VA	Treatment	Time to surgery /days
1	L/F	76	P. Classic	Subfoveal	<2DD	0.3	-	-
2	L/F	79	M. Classic	Subfoveal	<3DD	1	-	-
3	L/M	81	M. Classic	Subfoveal	<3DD	0.9	-	-
4	R/F	71	M. Classic, Hemorrahgic	Subfoveal	<3DD	0.8	-	-
5	R/F	76	M. Classic	Subfoveal	≥3DD	2	-	-
6	R/F	78	Occult	Subfoveal	≥3DD	0.3	-	-
7	R/F	77	Occult	Subfoveal	≥3DD	1	-	-
8	R/M	79	Occult	Subfoveal	≥3DD	0.4	-	-
9	L/M	80	Occult	Subfoveal	≥3DD	0.8	-	-
10	R/F	80	Occult	Subfoveal	≥3DD	2	-	-
11	R/F	79	Occult	Subfoveal	<3DD	0.5	-	-
12	L/F	76	Occult	Subfoveal	<3DD	1.5	-	-
13	R/M	73	Occult	Subfoveal	<3DD	0.6	-	-
14	R/M	74	Occult	Subfoveal	<3DD	0.2	-	-
15	L/F	72	Occult	Subfoveal	<3DD	0.05	-	-
16	L/M	81	Hemorrhagic	Subfoveal	<3DD	2	-	-
17	R/M	63	Hemorrhagic	Subfoveal	<2DD	0.05	-	-
18	L/M	86	Hemorrhagic	Subfoveal	<3DD	2	-	-
19	L/F	79	Hemorrhagic	Subfoveal	<2DD	2	-	-
20	R/F	78	Hemorrhagic	Subfoveal	<3DD	0.01	-	-
21	L/F	64	Hemorrhagic	Subfoveal	≥3DD	0.4	-	-
22	R/M	70	Hemorrhagic	Subfoveal	≥3DD	0.2	-	-
23	L/F	66	Hemorrhagic	Subfoveal	≥3DD	2	-	-
24	L/F	66	Hemorrhagic	Subfoveal	≥3DD	0.1	-	-
25	R/M	75	Hemorrhagic	Subfoveal	≥3DD	0.05	-	-
26	R/F	63	P.Classic	Subfoveal	<2DD	0.06	1PDT	90 days
27	R/M	72	Hemorrhagic	Subfoveal	<2DD	0.8	1PDT	42 days
28	R/F	72	Hemorrhagic	Subfoveal	<3DD	1.32	1PDT	26 days
29	R/F	76	Occult	Subfoveal	≥3DD	0.2	1PDT	84 days
30	L/M	52	Hemorrhagic	Subfoveal	<3DD	0.2	2PDT	154 days

31	L/F	79	P. Classic	Subfoveal	<2DD	0.2	2PDT	47 days
32	R/M	58	Occult	Subfoveal	<3DD	0.1	2PDT	172 days
33	R/M	75	M. Classic	Subfoveal	<3DD	0.1	2PDT	90 days
34	R/F	76	P. Classic	Subfoveal	<3DD	0.1	2PDT	135 days
35	R/F	85	P. Classic	Extrafoveal, Peripapillary	≥3DD	0.26	1Avastin	121 days
36	L/M	69	Occult	Subfoveal	≥3DD	0.76	1Avastin	4 days
37	L/F	73	Occult	Subfoveal	<3DD	0.76	1Avastin	2 days
38	L/M	81	Occult	Subfoveal	≥3DD	0.26	1Avastin	42 days
39	L/F	77	Occult	Subfoveal	<3DD	0.74	1Avastin	32 days
40	R/M	75	P. Classic	Extrafoveal, Juxtafoveal	<3DD	0.74	2 Avastin	4 days
41	L/F	83	Hemorrhagic	Juxtafoveal	<3DD	0.26	2 Avastin	1 days
42	R/F	91	Hemorrhagic	Subfoveal	≥3DD	0.74	2 Avastin	200 days
43	L/M	74	P. Classic	Subfoveal	<3DD	0.76	2 Avastin	41 days
44	R/F	73	P. Classic	Subfoveal	<3DD	0.74	2 Avastin	38 days
45	R/M	79	P. Classic	Subfoveal	<3DD	2.0	Ranibizumab	5.0
46	R/F	70	Occult	Subfoveal	<3DD	0.3	Ranibizumab	7.0
47	R/F	81	Hemorrhagic	Subfoveal	≥3DD	3.0	Ranibizumab	20.0
48	R/F	70	Occult	Subfoveal	<33DD	0.12	Ranibizumab	42.0
49	F/R	78	Occult	Subfoveal	<2DD	0.1	Triamcinolone acetonide	3.0
50	F/R	70	Occult	Subfoveal	≥3DD	0.3	Triamcinolone acetonide	7.0
51	F/L	80	Occult	Subfoveal	<3DD	0.2	Triamcinolone acetonide	8.0
52	F/L	75	Occult	Subfoveal	3DD	0.4	Triamcinolone acetonide	55.0
53	M/R	73	Occult	Subfoveal	3DD	0.05	Triamcinolone acetonide	3.0
54	L/M	78	Occult, Hemorrhagic	Subfoveal	<2DD	0.25	1PDT+5 avastin	245/48 days
55	R/F	72	Occult	Subfoveal	<3DD	0.25	1PDT+1 avastin	68/40 days
56	L/M	79	P. Classic	Subfoveal	<2DD	0.1	1PDT+1 avastin	63/60 days
57	R/M	70	Occult	Subfoveal	<2DD	0.25	1PDT+1 avastin	240/76 days
58	R/F	92	Hemorrhagic	Subfoveal	≥3DD	2	1PDT+ 2 avastin	330/330 days

P. classic: Predominantly classic; M. classic: Minimally classic; L= left; R= right; F= female; M= male; CNV= Choroidal neovascular membrane; PDT= Photodynamic therapy; DD= size of an optic disc diameter. VA: Visual acuity expressed in Log MAR notation (logarithm of the minimum angle of resolution).

8.2 Materials and stock solutions preparation protocol

8.2.1 Positive control:

Human tonsils were used as positive control for the three antibodies.

8.2.2 Washing buffer:

Tris Buffered Saline (TBS) washing buffer: 1XTBS/0.1% Tween-20 was the washing buffer throughout the experiments.

8.2.2.1 *To prepare stock solution of 10X TBS:*

- Add 24.2 g Trizma base and 80 g sodium chloride to 1L of dH2O. Adjust pH to 7.6.

8.2.2.2 *Working solution:*

- 1XTBST/0.1% Tween-20: add 100ml 10XTBS to 900 ml dH2O. Add 1 ml Tween-20 and mix well.

8.2.3 Antigen Retrieval Solution: 0.1M Sodium Citrate Buffer, pH 6.0

8.2.3.1 *To prepare stock solutions:*

- Solution A. 0.1 M citric acid solutions: dissolve 21.0 g of citric acid, monohydrate (C6H8O7.H2O) in 100 ml of dH2O.
- Solution B. 0.1 M sodium citrate solutions: dissolve 29.4 g trisodium citrate dihydrate (C6H5Na3O7.2H2O) in 100 ml of dH2O.

8.2.3.2 *Working solution:*

- Add 9 ml of Stock solution A and 41 ml of stock solution B to 450 ml of dH2O. Adjust pH to 6.0.

8.2.4 Blocking buffer:

PBS (Dulbecco's Phosphate Buffered Salts, 1X, catalogue #21-031-CV from Mediatech, Inc.) + 10% goat serum

8.2.5 Primary antibody:

8.2.5.1 *CD 163, Abcam, CD163 antibody [RM3/1] (ab17051)*

- Original concentration 0.1 mg/ml.
- The primary antibody was diluted with PBS.
- Prepare dilutes of 1:50, 1:100, and 1:200.
- Test the prepared dilution for optimal dilution that produces the best staining.

- Best staining results obtained with dilution of 1:100.

8.2.5.2 *CD68, Dako, FLEX Monoclonal Mouse Anti-Human CD68 Clone PG-M1*

- Ready-to-use monoclonal mouse antibody provided in liquid form in a buffer containing stabilizing protein and 0.015 mol/L sodium azide.

8.2.5.3 *CCR7, (N-term) rabbit monoclonal [Y59] antibody*

- Recommended dilution 1:250, 1:100
- The antibody was diluted with blocking buffer.
- Best staining results obtained with dilution of 1:250.

8.2.5.4 *Secondary antibody: Abcam, anti rabbit and mouse HRP polymer secondary antibody (ab2891).*

- The secondary antibody used is ready to use prediluted, so no need for further dilution.

8.2.6 AEC chromagen:

➢ AEC chromagen was prepared as instructed in the manufacture manual.

8.2.7 Phosphate Buffered Saline – PBS

1. 8 g NaCl
2. 0.2 g KCl
3. 1.44 g Na2HPO4
4. 0.24 g KH2PO4
5. 800 ml dH2O
6. pH - 7.4
7. dH_2O up to 1 litter.

8.3 Staining Protocol

8.3.1 Specimen Cutting

8.3.1.1 *Materials required*

1. CNV specimens embedded in paraffin blocks.
2. 9 microscope glass slides.
3. Water bath.
4. Human tonsil was used as positive control.

8.3.1.2 *Procedure*

1. Serial cutting of the embedded tissues at 0.4µm thickness.
2. The cuts are transferred carefully to water bath where they are brought to the glass slides.
3. Then the slides are left to dry at 58°C over night or at 65°C for 1 hour.

8.3.2 Deparafinization

8.3.2.1 *Materials required*

1. Xylene.
2. Ethanol (100%, 95%, 80%, 70%).

8.3.2.2 *Procedure*

1. Put the slides into a rack for IHC.
2. Dip the rack into 4 consecutive stain jars containing xylene to remove paraffin (10 minutes).
3. Dip the rack into ethanol to remove xylene :
    a. 100% ethanol 5 min.
    b. 95% ethanol 5 min.
    c. 80% ethanol 5 min.
    d. 70% ethanol 5 min.
4. Prepare a container which is large enough to afford 2 racks. Put the rack at one side of the container and, then, make water flow from the other side. Don’t make the tissue side face the water flow directly. Rinse the rack with tap water to remove ethanol for 5 minutes.

8.3.3 Washing solution

8.3.3.1 *Materials required*

1. TBST washing buffer: 1XTBS/0.1% Tween-20

8.3.3.2 *Procedure*

1. Slides are immersed for washing for 5 min.

8.3.4 Blocking of endogenous peroxidise

8.3.4.1 *Materials required*

1. 0.3%H_2O_2 in 0.1% sodium azide.

8.3.4.2 *Procedure*

1. Dip the rack in 0.3%H_2O_2 in 0.1% sodium azide for 10 minutes.
2. Rinse the rack with tap water for 15 minutes.

8.3.5 Antigen retrieval

8.3.5.1 *Materials required*

1. Sodium Citrate Buffer, pH 6.0.
2. dH_2O.

8.3.5.2 *Procedure*

1. Place covered staining dish into the rice cooker. Add 120 mL dH_2O and press “cook”.
2. Place a staining dish containing Antigen Retrieval Solution (Sodium Citrate Buffer, pH 6.0) in the rice cocker and turn it on for 30 minutes.
3. Immerse slides into staining dish containing Antigen Retrieval Solution (Sodium Citrate Buffer, pH 6.0).
4. When “cook” is turned to “warm” (about 20–30 min), unplug the cooker and remove the staining dish to the bench top.
5. Allow to cool down, without cover, for 20 min.

8.3.6 Serum blocking

8.3.6.1 *Materials required*

1. Blocking solution: Goat Serum.

8.3.6.2 *Procedure*

1. Wash slides with TBST for 5 min on a shaker.
2. Block slides with the blocking solution for 1 hour, (don’t wash).

8.3.7 Primary antibody

8.3.7.1 *Materials required*

1. Chamber with a lid.
2. Marking pen.

3. Tissue for moistening.
4. Primary antibody.

8.3.7.2 *Procedure*

1. Dilute primary antibody if indicated (see under 8.1.61, 8.1.6.2 and 8.1.6.3).
2. Apply primary antibody to each section and incubate overnight in the humidified chamber (4 ºC). Insure complete cover of the tissue (a volume between 30µl:50µl is sufficient).
3. Wash slides three times with TBST (3 min each on a shaker).

8.3.8 Secondary antibody

8.3.8.1 *Materials required*

1. Secondary antibody.

8.3.8.2 *Procedure*

1. Apply to each section **secondary HRP-conjugated secondary anti-rabbit antibody** diluted in the blocking solution per manufacturer's recommendation; incubate for 1 hour at room temperature.
2. Wash slides three times with TBST (3 min each on a shaker).

8.3.9 Chromagen

8.3.9.1 *Materials required*

1. AEC Chromagen.
2. Demineralised H_2O.

8.3.9.2 *Procedure*

1. Add freshly prepared **AEC** substrate to the sections.
2. Incubate tissue sections with the substrate at room temperature until suitable staining develops (generally 2–5 min).
3. Rinse sections with demineralised water (2min X 3).

8.3.10 Counter Staining

8.3.10.1 *Materials required*

1. Mayer's Haematoxylin.

8.3.10.2 *Procedure*

1. Add haematoxylin to the sections.
2. Incubate tissue sections for 3 min.
3. Rinse sections with water for 3 min.
4. No further dehydration step is required.

8.3.11 Mounting

8.3.11.1 *Materials required*

1. Fluoromount™ aqueous based mounting medium.
2. Cover slips.

8.3.11.2 *Procedure*

1. Let the slid to adequately dry off.
2. Mount cover slips on slides using aqueous based mounting medium.

8.4 Abbreviations

aaMphi	Alternatively activated macrophages.
AEC	3-Amino-9-Ethyl Carbazole.
AMD	Age related macular degeneration.
AREDS	Age-related eye disease study research group.
bFGF	Basic fibroblast growth factor, also known, FGF2 or FGF-β.
BLD	Basal laminar deposit.
BMI	Body mass index.
caMphi	Classically activated macrophages.
C3c	Complement 3c.
C5	Complement 5.
CCL2	Chemokine ligand 2.
CCL19	Chemokine ligand 19.
CCL21	Chemokine ligand 21.
CCR2	Chemokine receptor 2.
CCR7	Chemokine receptor 7.
CD68	Cluster of differentiation 68.
CD163	Cluster of differentiation 163.
CFH	Complement factor H.
CNV	Choroidal neovascularization, choroidal neovascular membrane.
CRP	C-reactive protein.
CVD	Cardiovascular diseases.
CX3CR1	Chemokine (C-X3-C motif) receptor 1.
DD	Optic disc diameter.
FAZ	Foveal avascular zone.
Fc	Fragment crystallisable region.
FFA	Fundus fluorescein angiography.
GA	Geographic atrophy.
GAGs	Glycosaminoglycans.
HANES	The health and nutrition examination survey.
HLA	Human leukocyte antigen system.
HLA-DR	Major histocompatibility complex, MHC class II.
HRP	Horseradish peroxidase
iC3b	iC3b is the proteolyticly inactive product of the complement cleavage fragment C3b
IFN-ɣ	Interferon gamma.
IgG	Immunoglobulin G.

IL	Interleukin.
iNOS	Inducible nitric oxide synthase.
LDL	Low Density Lipoprotein.
LOC	Specific location of a gene or DNA sequence on a chromosome.
LogMar Notation	Expression of visual acuity as logarithm of the minimum angle of resolution.
LPS	Lipopolysaccharides.
M	Macrophages stained for CD68.
M1	Classically activated macrophage (macrophages stained for CCR7).
M2	Alternatively activated macrophages (macrophages stained for CD163).
M1/M2 ratio	Expression ratio of M1/M2.
MCP-1	Monocyte chemoattractant protein-1.
MMP	Matrix metalloproteinase.
MPS	The macular photocoagulation study.
OCT	Optical coherence tomography.
PBS	Phosphate buffered saline
PDT	Photodynamic therapy.
PED	Pigment epithelium detachment.
PPAR	Peroxisome proliferator-activated receptor.
RNA	Ribonucleic acid.
RPE	Retinal pigment epithelium.
SD	Standard deviation.
SNP	Single-nucleotide polymorphism.
SPSS	Statistical package for the social sciences.
SRCR	Scavenger receptor cysteine-rich.
TA	Triamcinolone acetonide.
TAM	Tumour associated macrophages.
TAP	The Treatment of Age-Related Macular Degeneration with Photodynamic Therapy Study.
TGF-ß	Transforming growth factor beta.
T_h1	T helper cell 1.
TLR	Toll-like receptors.
TNF-α	Tumour necrosis factor alpha.
VA	Visual acuity.
VEGF	Vascular endothelial growth factor.
VEGFR	Vascular endothelial growth factor receptor.
VIP	The verteporfin in photodynamic therapy study.

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10 CURRICULUM VITAE

10.1 PERSONAL DATA

Name	Khaled Nassar
Birth date	16.10.1975
Nationality	Egyptian
Marital status	Married with one son.
Work address:	Schleswig-Holstein University
	Campus Lübeck, Clinic Eye Hospital
	Department of ophthalmology
	Ratzeburger Allee 160
	Telephone:0049 (0)451-5004010
	Fax: 0049 (0)451-5004952
Private address:	Alfstrasse18
	23552 Lübeck
	Deutschland
	Telephone: 0049(0)4518831116
	Cell phone: 0049(0)1744030067
e-mail:	khaledmohnassar@yahoo.com

10.2 EDUCATION

	Date	
Primary school, Monufia, Egypt	**1981**	**1986**
Prep school, Monufia, Egypt	**1986**	**1989**
High school, Monufia, Egypt	**1989**	**1993**

10.3 CERTIFICATIONS

1.	**Bachelor of Medicine, Bachelor of Surgery (M.B., B.Ch.)** Cairo University, Egypt.	1. Oct **1993**	1. Oct **1999**
2.	**Master degree of Ophthalmology (MSc)** Cairo University, Egypt.	1. Mar **2000**	1. Nov **2003**
3.	**Doctorate exam of Ophthalmology (MD)** Cairo University, Egypt.		1. May **2007**

10.4 POST GRADUATE TRAINING AND CONFERENCES

The Wuerttemberg Ophthalmological Association Meeting University Hospital Tübingen Centre for Ophthalmology, Tübingen, Germany	22. Feb **2008**	23. Feb **2008**
Pars-plana-Vitrectomy and Cataract-Surgery Wet-Lab: Hamburg University, Hamburg, Germany	19. July **2008**	
Borsteler Autumn seminar for Pathology und Biomedicine Research Center Borstel, Borstel, Germany	12. Sep **2008**	13. Sep **2008**
Lübeck Ophthalmology meeting Lübeck University, Lübeck, Germany	1. Nov **2008**	
Meeting of the Vasculitis Interdiscipline Centres Lübeck University, Lübeck, Germany	16. Jan **2009**	
12th VItreoretinal Symposium, Frankfurt-Marburg 2009, Johann Wolfgang Goethe University Frankfurt/Main, Germany	27. Aug **2009**	29. Aug **2009**
Lübeck Ophthalmology meeting Lübeck University, Lübeck, Germany	28. Nov **2009**	28. Nov **2009**
8th International Symposium on Ocular Pharmacology and Therapeutics. Rom, Italy.	3. Dec **2009**	6. Dec **2009**
ARVO 2010 Annual meeting: The future of Eye and vision research.	2. May	6. May

Fort Lauderdale, Florida, USA	**2010**	**2010**
World Ophthalmology Congress 2010 (WOC® 2010) Berlin, Germany	5. June **2010**	9. June **2010**
23rd International Congress of German Ophthalmic Surgeons Hamburg, Germany	21 Oct 2010	24 Oct 2010

10.5 POSTGRADUATE WORK

1.	**Research Fellow** Department of Ophthalmology University of Lübeck, Germany	1. May **2008**	18. Aug **2011**
2.	**Research Fellow** Tübingen University Department of Ophthalmology, Vitreoretinal surgery Laboratory research Department.	1. Dec **2007**	30. April **2008**
4.	**Assistant Lecturer of Ophthalmology,** Department of Ophthalmology Fayoum University, Egypt	10. Jan **2005**	30. Nov **2007**
5.	**Ophthalmology Resident** Cairo University, Egypt	17. May **2001**	16. May **2004**
6.	**Transitional year** Cairo University, Egypt	1. Mar **2000**	28. Feb **2001**

11 PUBLICATIONS LIST

11.1 Original work

1. Lüke J, **Nassar K**, Lüke M, Tura A, Merz H, Giannis A, Grisanti S. The Effect of Adjuvant Dimethylenastron, a Mitotic Kinesin Eg5 Inhibitor, in Experimental Glaucoma Filtration Surgery. Curr Eye Res. 2010 [Epub ahead of print]. **IF** 1.519.
2. **Nassar K**, El-Far E, Tatar O, Lüke J, Lüke M, Tura A, Grisanti S. Macrophage Activation Pattern in Human Choroidal Neovascular Membranes. ARVO 2010 Annual Meeting, USA, Florida, 2010, Presentation abstract, 401/D1061.
3. Lüke M, Lüke J, Tura A, **Nassar K**, Grisanti S. The effects of pegaptanib sodium on retinal function in isolated perfused vertebrate retina. Curr Eye Res. 2010; 35:248-254. **IF** 1.519.
4. Beutel J, Wegner J, Wegner R, Ziemssen F, **Nassar K**, Rohrbach M, Hilgers, Lüke M, Grisanti S. Possible implications of MCAM in metastasis of primary uveal melanoma patients. Curr Eye Res, Curr Eye Res. 2009; 34:1004-1009. **IF** 1.519.
5. **Nassar K**, Lüke J, Lüke M, Kamal M, Abd El-Nabi E, Soliman M, Rohrbach M, Grisanti S. The novel use of decorin in prevention of proliferative vitreoretinopathy. 8th ISOPT, Rome, 2009, Abstract: page 139.
6. Lüke J, **Nassar K**, Lüke M, Tura A, Giannis A, Grisanti S. The effect of adjuvant Dimethylenastron, a mitotic kinesin Eg5 inhibitor, in experimental glaucoma filtrating surgery. 8th ISOPT, Rome, 2009, Abstract: page 120.

11.2 Books contribution

1. **Nassar K:** Illustrator of **Laser in Ophthalmology** "**Principles Of Ophthalmic surgery**". In: Taha A (Editor in Chief), 1. edition, EL BLAGH, Cairo (2007)