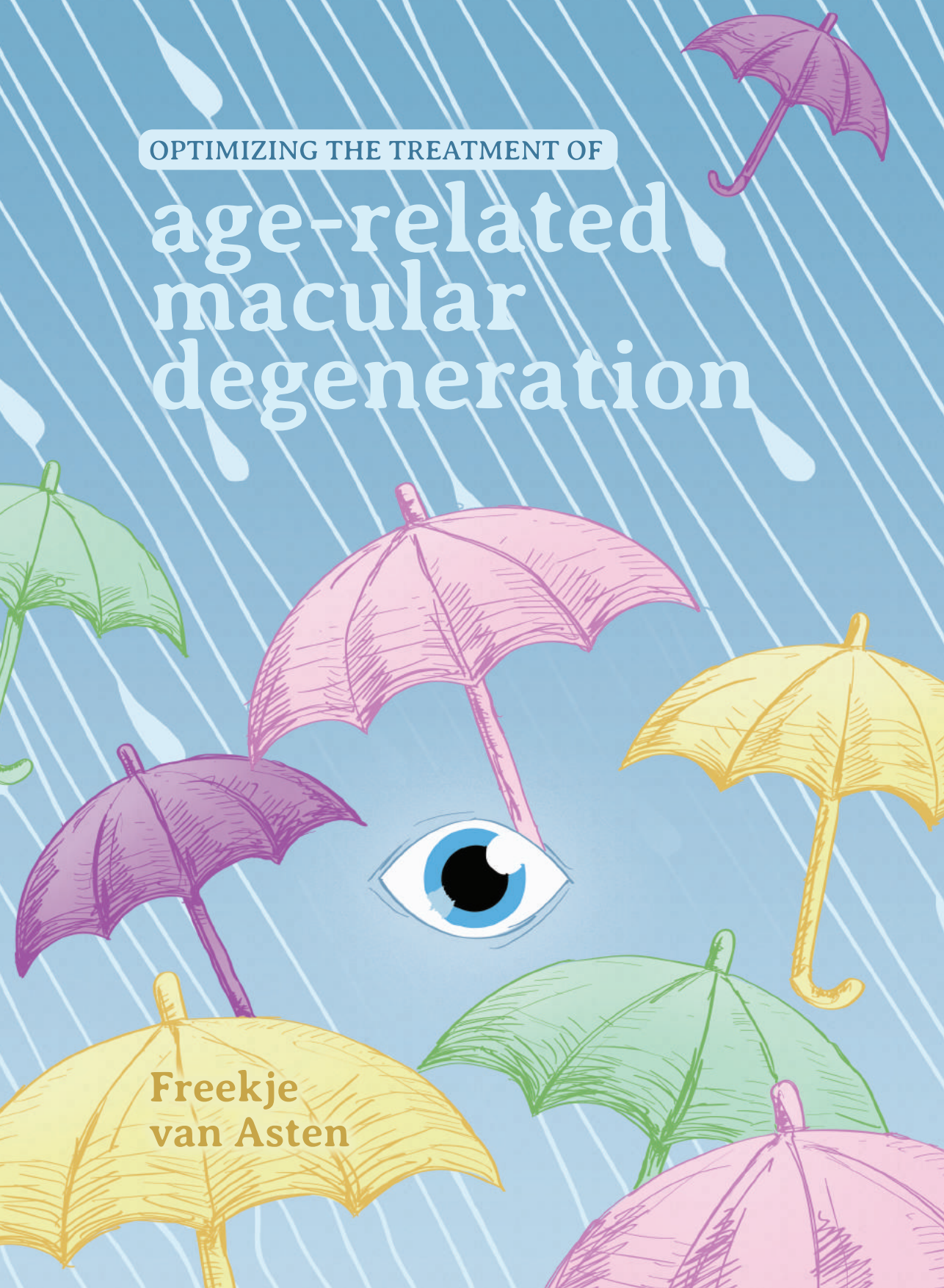
OPTIMIZING THE TREATMENT OF

age-related macular degeneration



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Chapter 1

General introduction

Age-related macular degeneration: a global health care burden

Age-related macular degeneration (AMD) is a widespread problem and a considerable health care burden. It is the most important cause of irreversible severe vision loss in the elderly Western population.[1] The global prevalence of AMD is estimated to be 9% and reaches up to 11% in those of European ancestry.[2] Prevalence increases with age, rising up to 20% in populations of over 80 years (figure 1).[3] The number of people affected is expected to rise and by the year 2020 there will be a staggering 200 million people with AMD worldwide. Approximately 12 million of these people will develop the debilitating neovascular advanced stage of disease, in many cases necessitating intravitreal anti-VEGF treatment.

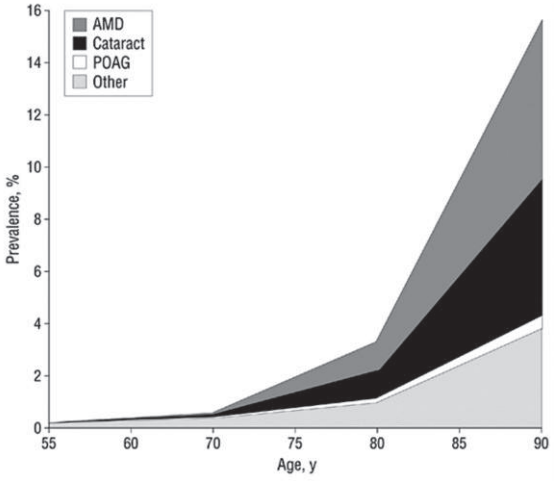

Figure 1. Total prevalence of poor vision as a function of age, specified by cause. AMD= age-related macular degeneration; POAG= primary open-angle glaucoma.[3]

The etiology is multifactorial, meaning there is not one specific cause, but there are multiple risk factors contributing to its development. Well-known environmental risk factors include smoking, family history of AMD, cataract surgery, obesity and dietary factors such as low antioxidant intake.[4-7] Genetic factors are an important component of the etiology as the proportion of disease variance due to genetic factors, or heritability, of AMD is estimated to be approximately 70%.[8] These genetic factors are usually so-called single nucleotide polymorphisms (SNPs). SNPs are small alterations in a person's DNA, or genetic variants, which may be commonly seen in the normal, unaffected population. However, some SNPs predispose the carrier to AMD. Genetic studies point

towards the involvement of the complement system in disease development, as several variants in complement genes (*CFH, C2/CFB, C3, CFHR1, CFHR3, CFI*) are strongly associated with AMD.[9-13] The role of the complement system was corroborated by presence of complement components in drusen, which are retinal deposits known as the hallmark lesions of AMD.[14] In addition, physiological measurements in blood serum of activated complement, demonstrated an increased level of complement activation in AMD patients versus controls.[15-17] The extensive application of genome-wide association studies (GWAS) in large AMD cohorts has led to the identification of several other genetic associations, indicating contributions to disease etiology of pathways involved in lipid metabolism, extracellular matrix remodeling and angiogenesis.[18]

The hallmark lesions of AMD are drusen: small deposits under the level of the retinal pigment epithelium (RPE) (figure 2). Drusen appear as yellowish spots in the retina and can vary in aspect and size from ≤63μm (hard drusen) to >63μm (soft drusen). The composition of drusen has been studied extensively. At least 40% of the druse consists of lipids that are potentially accounted for by the secretion of lipoproteins by RPE.[19] In addition, a number of druse proteins have been described, notably inflammatory factors including complement components as mentioned previously. Less than 10 small hard drusen may be physiological in healthy elderly people and are thought to reflect normal aging of the retina.[20]

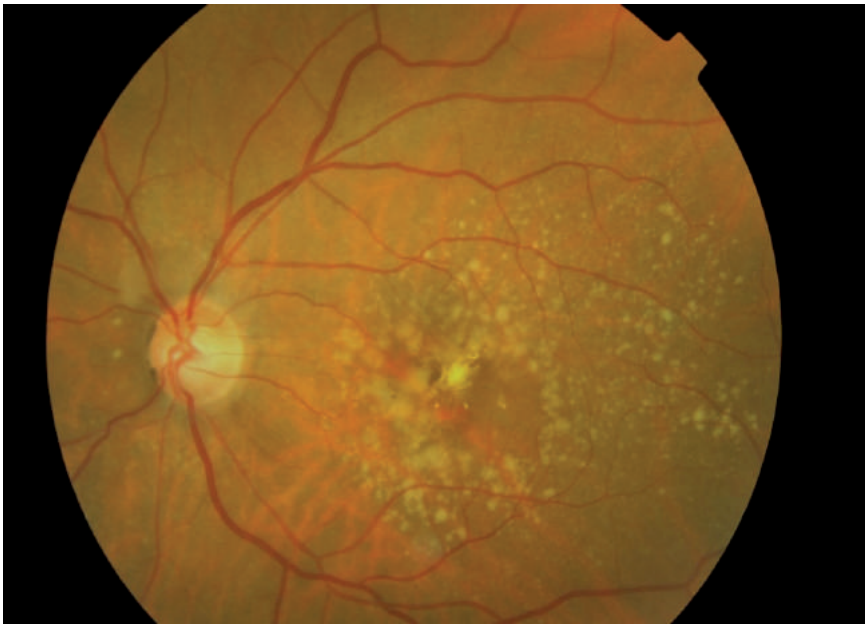

Figure 2. Fundus photograph of patient with intermediate age-related macular degeneration showing many small and large drusen.

The early and intermediate phases of disease typically show only drusen and pigmentary changes in the retina, vision loss is generally mild at this stage.[21, 22] However, approximately 25% of these people will progress to the advanced stage of AMD within 15 years and will experience severe vision loss.[23] Advanced AMD comprises two distinct phenotypic entities. Approximately half of patients that progress to advanced AMD will develop geographic atrophy, whereas the other half develops neovascular AMD.[2] In geographic atrophy the profound thinning of the outer retinal layers results in a complete loss of function, when the atrophy affects the center of the macula irreversible blindness is the result.[24] At the moment there is no proven therapy for geographic atrophy. The classification of AMD according to the Cologne Image Reading Center and Laboratory (CIRCL) protocol[25, 26] is summarized in table 1.

Table 1. CIRCL protocol for AMD classification on fundus photography

Early AMD	≥10 small drusen (<63 µm) and pigmentary changes or the presence of 1–14 intermediate (63–124 µm) drusen within 6 mm of the fovea
Intermediate AMD	≥15 intermediate drusen or any large drusen (≥125 µm in diameter) within 6 mm of the fovea
Advanced: Geographic atrophy	Subfoveal atrophy of the RPE of at least 175µm in diameter within 1 mm of the fovea
Advanced: Neovascular AMD	Choroidal neovascularization secondary to AMD

Neovascular AMD and vascular endothelial growth factor

Neovascular AMD is the second form of advanced AMD and the most common cause for visual impairment. Vision loss from neovascular AMD is usually rapid and common complaints are blurred central vision, distortion of straight lines (metamorphopsia), and/or central visual field defects (scotoma).[27] In most patients vision loss starts unilateral and, initially, may go unnoticed by the patient due to the compensatory effect of the fellow eye. Often, however, the second eye will also develop neovascular complications, at a risk of 10% per year.[28, 29] Neovascular AMD is driven by increased levels of vascular endothelial growth factor (VEGF) that are typically released in hypoxic circumstances. The VEGF family consists of several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). VEGF-A is the predominantly involved factor in neovascular AMD pathogenesis. It is the primary regulator of angiogenesis and

exerts its action through activation of the VEGF receptor 2 (VEGFR2).[30, 31] Upregulation of the VEGF pathway in AMD causes newly formed, brittle vessels to grow from the choroid, through Bruch's membrane and into the retina (figure 3)[32]. This choroidal neovascularization (CNV) leaks fluid and plasma constituents into the retina leading to rapid and severe visual loss.[33]

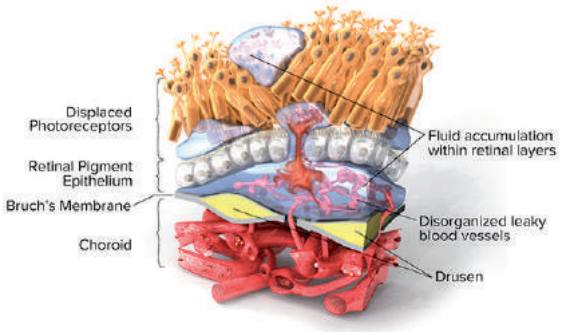

Figure 3. Invasion of choroidal neovascularization into the sub- and intraretinal space. Image from scienceofamd.org.[32]

Different CNV lesion types are recognized, each with their own phenotypic features. The CNV complex of the classic subtype lies between the RPE and the neurosensory retina[34]. Its appearance on fluorescein angiography is of a well-demarcated area of leakage, typically with a hypofluorescent rim surrounding the lesion (figure 4A).[35] The classic subtype is commonly referred to as "type 2 neovascularization". The most common is the occult lesion type (~50% of cases),[36] also known as "type 1 neovascularization", where the neovascular complex is located just beneath the retinal pigment epithelium (RPE). On fluorescein angiography, occult lesions are characterized as lesions with speckled hyperfluorescence with poorly demarcated borders (figure 4B).[35] The classic and the occult CNV subtypes regularly coincide in the same lesion, where the lesion is referred to as predominantly classic in case more than 50% of the lesion consists of classic CNV or as minimally classic when it is less. Less common is the retinal angiomatous proliferation (RAP) subtype, or "type 3 neovascularization", occurring in approximately 10-30% of cases.[36, 37] In RAP an anastomosis forms between a choroidal and a retinal vessel. It usually presents as small intraretinal hemorrhages on fundus photography, combined with a hyperfluorescent hotspot on fluorescein angiography (figure 4C). A pigment epithelium detachment develops in almost all patients during the later stages.[38, 39] The final CNV subtype is polypoidal choroidal vasculopathy (PCV), however there is ongoing discussion whether PCV should be treated as a subtype of AMD or an entirely different disease entity.[40] The characteristic lesion in PCV is an inner choroidal

network of dilated vessels which may be seen as red-orange polyp-like protrusions in the retina (figure 4E).[40] PCV is uncommon in Caucasians, but primarily affects pigmented individuals, especially Asians and African-Americans.[40] RAP and PCV may be easily missed with conventional techniques and usually require further diagnostic imaging, such as indocyanine green angiography (figure 4D,F).[41]

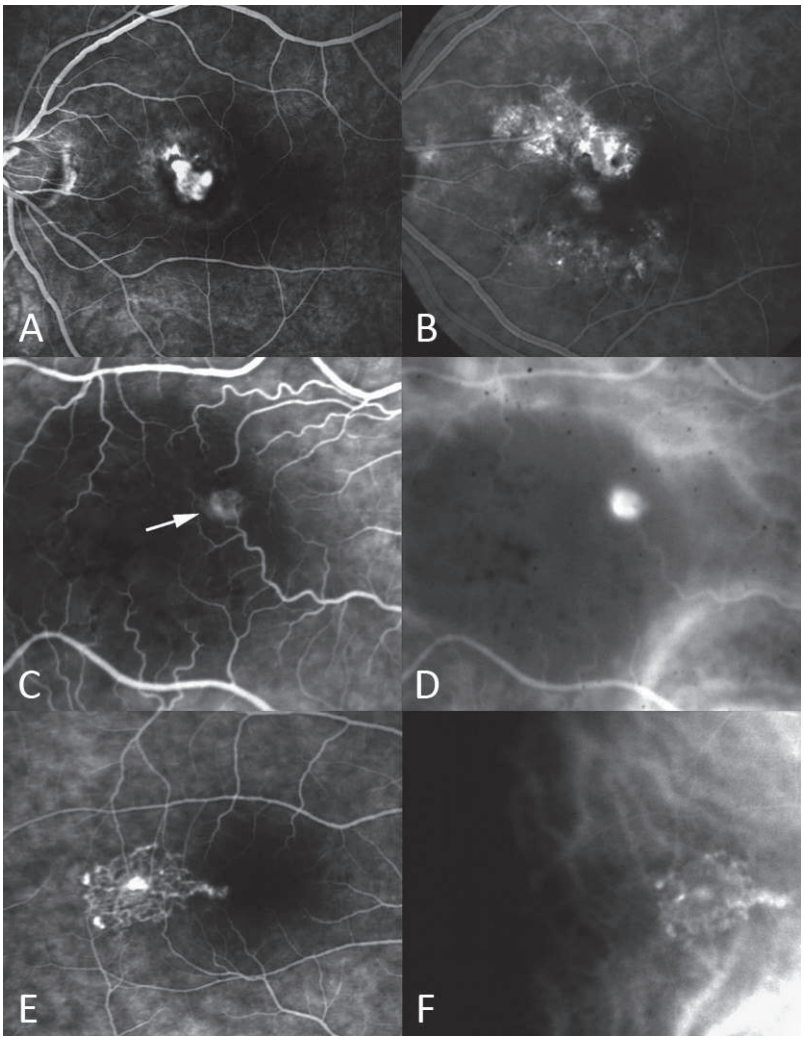

Figure 4. A. Fluorescein angiography (FA) of a classic neovascularization with a well-demarcated hyperfluorescent area of leakage. B. FA of occult neovascularization with speckled appearance. C. Retinal angiomatous proliferation (RAP) on FA, the lesion is indicated by the arrow. D. Indocyanine green angiography showing a hotspot at the site of the RAP lesion. E. Polypoidal choroidal vasculopathy (PCV) on FA with a clear network of polyp-like structures. F. Indocyanine green angiography confirming the presence of a polypoidal lesion in PCV. Images from Duane's Ophthalmology.[41]

Anti-VEGF treatment in AMD: a therapeutic revolution

Before the anti-VEGF era, photodynamic therapy (PDT) was the first-line AMD treatment. PDT was introduced in 1999 and was an important development in AMD treatment, being able to prevent severe vision loss in 61% of patients.[42] However, because of the availability of other highly effective drugs it is now seldom considered as a treatment option in AMD with the exception of PCV, where PDT may be administered in combination with anti-VEGF.[43]

Recognition of the pivotal role of VEGFs in the pathogenesis of neovascular AMD prompted the development of intraocular anti-VEGF drugs to suppress the neovascular lesions. This class of drugs represented a huge leap in the treatment for macular degeneration. The first anti-VEGF drug was pegaptanib sodium (Macugen), an aptamer (oligonucleotide strand) binding specifically to isoform VEGF 165.[44] In 2004 the first randomized controlled trial (RCT) of pegaptanib sodium versus sham injection was published showing how pegaptanib could prevent much of the visual loss associated with neovascular AMD. Despite this anti-VEGF treatment, patients were still losing approximately 6-10 letters on average

Neovascular AMD treatment was truly revolutionized when in 2006 the VEGF antibodies were discovered as a potential therapy. The first to be tried in clinical practice was bevacizumab (Avastin). Bevacizumab is a VEGF-A antibody originally developed as a systemically administered cancer drug for colon carcinoma, but in case reports[45, 46] it was shown that it could also successfully inhibit CNV in AMD through intravitreal injection. This finding gave rise to the development of an anti-VEGF drug made specifically for ocular administration. Thus ranibizumab (Lucentis) entered the playing field. Ranibizumab was developed with similar properties as bevacizumab (VEGF-A blocking), but consists only of the fragment antigen-binding (Fab) region without the fragment crystallizable (Fc) domain. The much smaller molecular size was thought to facilitate distribution across all retinal layers to the choroidal neovascular structure. In the two large RCTs MARINA and ANCHOR the superiority of ranbizumab over sham treatment was unmistakably proven.[47, 48] In contrast to pegaptanib, ranibizumab was able to improve vision, with patients gaining 8 to 10 letters on average over the course of 1 year. This led to the registration of ranibizumab as treatment for neovascular AMD. Although ranibizumab was registered for AMD and bevacizumab never entered the registration process, large price differences between the two (approximately a factor 20) meant ophthalmologists still used bevacizumab in clinical practice. A RCT was therefore designed to evaluate the difference in effectiveness. Published in 2011, the CATT study showed no discernible difference between the two drugs with respect to visual acuity change.[49] Subsequent studies confirmed this.[50-52] These comparative RCTs also did not find differences in adverse events. Both ranibizumab and bevacizumab can enter the systemic circulation, but the half-life of bevacizumab is much longer,[53] which had some

ophthalmologists worried that bevacizumab may increase the risk of stroke or myocardial infarction. A subsequent Cochrane review confirmed there were no differences in serious adverse events and specifically in arteriothrombotic events.[54] Hereafter, the favorable cost-effectiveness profile of bevacizumab prompted some national ophthalmological societies, such as the Dutch Ophthalmology Society, to adopt this drug as first choice therapy for neovascular AMD in their guidelines.[55] However, since it is not officially registered for AMD, other national guidelines still recommend not to use bevacizumab as a first choice treatment.[56]

The huge success of ranibizumab in neovascular AMD led to the development of new anti-VEGF drugs and aflibercept ("VEGF Trap") is the latest. It functions as a decoy for VEGF-A, VEGF-B and PIGF, containing the binding portions of VEGF receptor 1 and 2. The alternative design was thought to have a greater binding affinity to VEGF and showed more potency in vitro than ranibizumab or bevacizumab.[57] However, the pivotal RCT consisting of two cohorts from North-America and Europe, dubbed VIEW 1 and VIEW 2, was published in 2012 and showed that there was no difference in effect on visual acuity or retinal thickness between aflibercept and ranibizumab.[58] Aflibercept however did have the advantage that it could be administered less frequently, once every two months instead of monthly.

Since the effectiveness in large cohorts appears to be similar, it can be challenging for ophthalmologists to decide which should be the primary treatment in the individual patient; bevacizumab, ranibizumab, or aflibercept. At the moment, the world is divided as to which anti-VEGF drug to use. We do not know whether all three drugs can be expected to have the same effect in each individual and what factors would influence the differential response. Furthermore, the contribution of the drugs on benefits for society in terms of quality of life gain and cost reduction may influence this decision. In addition, once we have established the first choice drug after careful review of all the evidence, the next step is to determine a secondary drug for further treatment of non-responders, which will preferably be a drug with a different therapeutic action.

Anti-VEGF treatment: clinical practice

The first RCTs administered ranibizumab by intravitreal injection every month.[47, 48] In clinical practice monthly injections proved to be a logistic and financial challenge and many clinics could not keep up with the large flow of treatments in this big patient population. One of the first adaptations to the original monthly regimen was an attempt to reduce injection frequency by an as-needed strategy (pro re nata; PRN). The Pronto study showed that good visual acuity gains could also be achieved by treating only when patients had lost 5 letters of visual acuity in combination with new or persistent fluid on optical coherence tomography (OCT).[59] The Pronto study was able to achieve a 9 letter

gain in vision with a mean only of 5.6 injections over 1 year. In subsequent comparative studies it was shown that the PRN treatment strategy yielded similar visual gains as the monthly regimen.[49, 50] Thus, many clinics adopted this new PRN regimen. Other popular treatment regimens include the treat-and-extend regimen in which patients receive an injection every visit, but the time between visits is extended when there is no fluid on OCT or shortened when fluid has recurred.[51]

The visual outcomes in the pivotal RCTs were very encouraging. However, RCTs are highly controlled environments where only specific patients with mostly beneficial characteristics are allowed to participate. In clinical practice many patients will present themselves with AMD, who would not fit the criteria for inclusion in RCTs. Also, in clinical practice, we cannot maintain the highly controlled setting as in RCTs. The pivotal RCTs that we now base our treatment guidelines on, including those advocating PRN regimens, required patients to be monitored monthly. In clinical practice, due to practical and logistical issues, treatment may be delayed, follow-up may be inconsistent and there may be more divergence from protocol, such as terminating treatment at a patient's request while it would still be indicated. These issues are sometimes unavoidable, however, they may affect the value of the treatment in real life. We know from observational studies conducted in other European countries that effects in RCTs are often not reached in clinical practice.[60-65] Observational studies are needed to evaluate the actual effect of anti-VEGF drugs in clinical practices. This may have consequences for the general prognosis of a patient group and the amount of non-responders we may expect to encounter. Furthermore, evaluating our treatment effects can help us identify areas for improvement. With these questions in mind, we wondered what the effect of anti-VEGF therapy for AMD was in our own clinics in the Netherlands.

Non-response to anti-VEGF therapy

The visual prognosis of the average patient with neovascular AMD has considerably improved after the implementation of anti-VEGF therapy. Nevertheless, vison does not improve in all AMD patients treated with anti-VEGFs and in approximately 10% of patients the vision declines by 15 letters even in the pivotal RCTs.[47-50] Although this gives us a good indication of the effectiveness of the treatment and the amount of non-response, these numbers do not necessarily represent all people that should be considered non-responder. The amount and subset of non-responders varies according to the definition used. It may depend on what cut-off for visual acuity loss is considered relevant, or even using a different method to define non-response such as fluid resolution on OCT may yield a completely different subset of non-responders. There is currently no generally accepted definition of non-response, but roughly, non-response can be subdivided in two major subtypes: functional or anatomical. The functional type

refers to response in terms of visual acuity. Anatomical response is defined by retinal thickness on optical coherence tomography (OCT). Many studies have tried to find predictive factors for functional response, but anatomical response gains interest. The advantage of non-response as a functional measure is that it is directly related to quality of life and thus the most relevant outcome for the patient.[66] On the other hand, the anatomy on OCT is usually what determines the ophthalmologist's decision to retreat and it is considered a more objective measure of response with less confounding factors. Unfortunately, response on OCT and visual acuity are not very well correlated,[67] so although a convenient measure, OCT changes may not be very meaningful to an individual patient.

Although we do not know beforehand who will respond to therapy and who will not, there are certain characteristics that can help us predict who the non-responders are. These characteristics may be clinical, phenotypic, and genetic factors.[68, 69] Clinical and phenotypic factors consistently associated with poor functional treatment results are: increasing age and poor baseline visual acuity.[70, 71] Good baseline visual acuity has been associated with more risk of vision loss. This is most likely a results of the floor-ceiling effect where patients with bad baseline visual acuity are not able to lose any more vision, as opposed to patients with good baseline visual acuity. Type of CNV may also be predictive of non-response. In some studies patients with classic CNV were less likely to gain vision as opposed to those with occult CNV,[70] however this finding could not be replicated in other studies.[72, 73]

Given the importance of genetic predisposition in AMD, genetic markers have been of major interest in non-response prediction as well. To date many SNPs have been found to be associated with the development of AMD, notably risk variants in the *ARMS2* and complement factor H *(CFH)* genes. Several studies have tried to identify underlying genetic factors predictive of treatment response, but so far results have been inconsistent.[69] The *CFH* Y402H and the *ARMS2* A69S risk variants are both strongly involved in AMD etiology and were therefore the most likely candidates for response prediction. Some studies have reported that both risk variants are associated with poorer response to anti-VEGF therapy.[74-78] Conversely, several studies have not found any association of these risk factors with response or have even seen opposite effects.[79-81] To get a better view of the current evidence, recent meta-analyses have been performed for the *CFH* Y402H risk variant.[82-84] Although the included studies were heterogeneous in population and treatment outcome, they were able to show that the *CFH* Y402H risk variant was associated with worse response to anti-VEGF therapy, increasing the odds for poor response by approximately 1.6 when carrying two risk alleles compared to none.

The VEGF pathway is also highly interesting for non-response prediction as this pathway is of course the target of anti-VEGF therapy. Some SNPs in the *VEGF-A* gene have been tested, but only few studies have been able to show an association with

treatment response.[68, 69] Lack of association could be because relevant functional variants in the VEGF pathway are not present or have not yet been identified, or because studies were underpowered. In a large study by Smailhodzic et al. it was shown that although effect sizes of individual SNPs are small, an accumulation of risk alleles in *CFH* Y402H, *ARMS2* A69S and *VEGF-A* rs699947 can make a difference of as much as 10 letters between patients that carry few risk alleles and those that carry all risk alleles.[85] This indicates that for response prediction it may be more valuable to compose a risk score of multiple SNPs to strengthen the power of the analysis.

A number of genetic and non-genetic factors are thought to be associated with non-response, but it remains unclear what the relevance of these factors are in the prediction of non-response, considering that effect sizes, especially of genetic factors, are generally small. It is unknown how well a combined model of the known clinical and genetic factors may predict non-response and how much of variation in response is still unexplained.

So far, non-responders have been identified through trial and error. Patients are treated with the first line therapy, and in case of non-response two options remain: discontinue treatment or switch to a different anti-VEGF drug. Because withholding treatment ultimately means losing vision,[86] the patient and ophthalmologist will often opt for an alternative drug. The pharmacological mechanism of the three available anti-VEGF drugs is very similar and thus it is unclear what the value would be of switching to a different drug. Furthermore, although the mean effectiveness is comparable between agents, but we do not know whether individuals will respond differently to different drugs. In The Netherlands the first choice treatment is bevacizumab, because of its superior cost-effectiveness profile. The VEGF-blocking action of bevacizumab and ranibizumab is virtually the same, but the active portion of aflibercept is not a VEGF antibody but rather a VEGF decoy-receptor.[57] Also, switching from bevacizumab to ranibizumab has so far yielded little and contradicting results.[87-90] Therefore, when a patient does not respond to bevacizumab, switching to aflibercept would make the most sense. Considering the expense of aflibercept and the burden of intraocular treatment, it is however important to find out what the value is for the patient when switching to this drug.

Anti-VEGF treatment and non-response in diabetic macular edema

Following the success of anti-VEGF in AMD, the same strategy was applied in other retinal disorders that were characterized by fluid in the macula. Diabetic macular edema (DME) is such a retinal disorder with overlapping pathophysiological mechanisms.[91, 92] Loss of vision in diabetic retinopathy is most often caused by DME, both in type 1 and 2 diabetes. Approximately 7% of all diabetics are affected, making it the number one cause for vision loss in the working age population.[93, 94]

As in AMD, the VEGF pathway plays a crucial role in DME. In DME, fluid accumulation in the retina originates from the retinal capillaries which have become damaged by the hyperglycemic conditions. The increased permeability of the retinal capillaries is mediated through increased VEGF levels as well as inflammatory factors.[95] Although the pathophysiology is similar to AMD, the inflammatory pathways plays a much more prominent role in DME. Furthermore, in DME, the retinal capillaries are the origin of the fluid as opposed to the choroidal vessels in AMD.

Prior to 2012 when ranibizumab received approval for use in DME, laser photocoagulation was the primary treatment for center-involving DME. Laser photocoagulation mostly led to stabilization of vision and carried a substantial risk of scotoma formation.[96] RCTs comparing anti-VEGF therapy to laser photocoagulation in patients with central macular edema found that not only was anti-VEGF far superior, it also resulted in an average gain of ~10 letters of vision. Considering that these studies also could not find an added effect of anti-VEGF combined with laser, anti-VEGF quickly replaced laser as the primary choice of treatment.[97-102] The functional success was accompanied by anatomical improvement and the CRT of patients decreased by ~180 μm on average in the ranibizumab and aflibercept RCTs. For bevacizumab, the effect on retinal thickness was less pronounced with a mean decrease of approximately 110 μm. As in AMD, the question remained which drug should be the preferred primary treatment. In the protocol T study, a large head-to-head-to-head trial of the Diabetic Retinopathy Clinical Research Network (DRCRN) comparing all three anti-VEGF agents, aflibercept was shown to be superior to ranibizumab and bevacizumab in DME-patients with baseline Snellen visual acuity below 20/40 after 1 year.[103] After 2 years, aflibercept still had significantly better vision gains than bevacizumab, but not ranibizumab.[104] In patients with baseline visual acuity of 20/40 or more, there was no significant difference in functional effect. With regard to anatomical effect there was no obvious difference between aflibercept and ranibizumab, however as expected from previous RCTs, bevacizumab resulted in 50-70 μm less CRT reduction. Despite the difference in anatomical effect but with regard to the much lower costs of bevacizumab, the current advice is to administer aflibercept in patients with poor baseline vision (<20/40 Snellen) and bevacizumab in patients with moderate tot good baseline vision.[105]

Considering the involvement of an inflammatory component to DME pathophysiology, corticosteroids are also applied as a treatment option in DME. The most commonly used and studied steroid treatment is intravitreal triamcinolone acetonide (TAC), which may be administered up to every 16 weeks.[106] In the protocol I study comparing ranibizumab, laser and TAC, TAC was initially effective in reducing CRT but this effect seemed to wane over time.[97] Also improvement of visual acuity was limited to the pseudophakic eyes. Therefore, steroids are usually not a first choice treatment, since they have not been proven to be more effective than anti-VEGF and more importantly, steroids are associated with adverse events such as cataract formation and increased

intraocular pressure.[97, 107] However, steroids can be an appropriate second line treatment when anti-VEGF therapy fails, especially in pseudophakic eyes.[108-110] Possibly, the inflammatory component is more active in anti-VEGF non-responders, warranting an anti-inflammatory approach as opposed to an anti-VEGF approach.
Due to different pathophysiological mechanisms involved in DME, treatment effects of anti-VEGF may differ from AMD and study results can likely not be readily extrapolated from AMD to DME. Anti-VEGF therapy in clinical practice for DME needs to be evaluated separately from AMD to give us a good idea of whether our DME treatment is effective and how many patient do not respond to treatment. Management of non-responders may vary from AMD as well, as the specific involvement of the inflammatory pathway in DME may mean different optimal strategies for individual non-responders.

Other treatments: prevention of advanced AMD

In the previous sections we have seen that anti-VEGF therapy requires intensive treatment and monitoring, and importantly, often does not completely restore all vision. Prevention of disease progression to advanced AMD could be a potential strategy to reduce patient, physician, and societal burden. In the absence of a definite cure for AMD, much could be gained by slowing progression to the advanced stages by addressing modifiable AMD risk factors.
Currently, the only intervention proven to reduce the progression to advanced AMD is oral intake of high doses of antioxidants (vitamin C, vitamin E and lutein/zeaxanthin) combined with zinc supplements for intermediate or unilateral advanced AMD. The Age-Related Eye Disease Study (AREDS), a randomized controlled trial showed that these supplements were able to reduce AMD progression by 25% by 5 years.[111] The exact mechanism of the protective effects of antioxidants and zinc has not yet been elucidated. Improving our understanding of this mechanism could help us optimize preventive treatments, either by more optimal targeting of the involved pathways by other treatments or by targeting a specific subgroup of patients that benefit most of the preventive measure.

Aim of this thesis

In general, the aim of this thesis was to identify areas in the treatment of AMD that require optimization and to assess optimization strategies. Areas for optimization were sought on an individual level to support personalized healthcare decisions and on a general population level to support the development of high-quality treatment guidelines. Three sub-objectives were distinguished: 1) to predict non-response to

anti-VEGF treatment in patients with neovascular AMD, 2) to evaluate and identify new areas for improvement of both the injection procedure and anti-VEGF treatment in clinical practice, and 3) to evaluate the cost- effectiveness of anti-VEGF treatment for neovascular AMD. More specifically, chapter 2 and 4b this thesis aimed to evaluate effectiveness of anti-VEGF treatment in clinical practice for AMD and DME respectively. In chapter 3a-c, in support of personalized healthcare, we aimed to improve the prediction of non-response by modelling non-response and identifying new genetic predictors. In chapter 4a and b we assessed the effectiveness of second-line treatments for AMD and DME after first-line bevacizumab. In chapter 5 we compare and address the cost-effectiveness of the three anti-VEGF treatments for AMD. In chapter 6 we evaluate and identify new areas for improvement of the anti-VEGF injection procedure. Finally, in chapter 7 we aim to elucidate mechanisms behind AMD prevention with zinc supplements.

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Anti-VEGF treatment of AMD in clinical practice

Chapter 2

A prospective, observational, open-label, multicenter study to investigate the daily treatment practice of ranibizumab in patients with neovascular age-related macular degeneration.

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Purpose: The HELIOS (Health Economics with Lucentis in Observational Settings) study was designed on request of the Dutch Health Authority for an observational study to assess the effectiveness and safety of ranibizumab for neovascular age-related macular degeneration (wet AMD) in daily practice.

Methods: The HELIOS study was a two-year prospective, observational, open-label, multicenter study involving 14 sites. Patients with wet AMD were enrolled and observed for a period of 24 months. The data were collected at baseline and at the visits closest around the time points 3, 6, 12, 18 and 24 months after inclusion.

Results: Treatment with ranibizumab resulted in prevention of vision loss. The mean ETDRS score increased from 45.1 letters at baseline to 48.5 letters at 24 months. This was achieved with a mean of 7.8 injections over 24 months. Stabilization in visual acuity was also reflected by the scores on the quality of life EQ-5D questionnaire, which did not significantly change over the study period. The more subjective EQ-VAS questionnaire showed an overall improvement. The VFQ-25 questionnaire was also mostly stable over time. After 24 months, 32.2% of the patients gained ≥ 1 letter and 17.1% gained > 15 letters. Patients completing the loading phase were better responders, as demonstrated by increased long-term visual acuity. In addition, ranibizumab was well tolerated and had a safety profile commonly seen in routine clinical practice.

Conclusion: This study demonstrates that also in daily practice ranibizumab was effective in preventing vision loss over a period of 24 months. No new safety findings were identified.

Introduction

Neovascular age-related macular degeneration (AMD) is the primary cause of severe and irreversible vision loss in the Netherlands and frequently results in legal blindness with resulting considerable economic burden [1-3]. Its prevalence worldwide is increasing concomitantly with life expectancy, and epidemiological forecasts estimate that the number of affected patients over 65 years of age will rise up to two-fold by 2030. Well known risk factors for AMD include age, smoking and genetic predisposition [4, 5].

Several treatments are currently available for wet AMD patients. Ranibizumab (Lucentis®) is a monoclonal antibody Fab fragment, that inhibits neovascularisation and leakage from vessels by binding to vascular endothelial growth factor A (VEGF-A) [6, 7]. The efficacy of ranibizumab has been demonstrated in pivotal Phase III trials (MARINA, ANCHOR and PIER) in which patients with choroidal neovascularisation (CNV) associated with AMD receiving intravitreal injections of ranibizumab experienced a sustained improvement in mean visual acuity over time [7-9]. In addition, ranibizumab was well tolerated and considered safe [10, 11].

Although the efficacy data from the above mentioned phase III trials is convincing, we should be aware that the results observed in clinical trials do not always translate well to clinical practice. Observational studies located in several European countries have been conducted to evaluate the efficacy of ranibizumab for neovascular AMD in clinical practice in their respective countries [12-22]. Following marketing approval of ranibizumab for neovascular AMD, the Dutch Health Authority had requested an observational study to assess the effectiveness and safety of ranibizumab in daily clinical practice in the Netherlands. The HELIOS (Health Economics with Lucentis in Observational Setting) study was designed to fulfil this request. The objectives of this study were to describe effectiveness, safety, treatment patterns and patient reported quality of life (QoL) outcomes in patients with neovascular AMD being treated with ranibizumab in real life practice settings over 24 months.

Materials and methods

Study design

A two-year prospective, observational, open-label, multicenter study was conducted to assess the effectiveness, safety and associated practice patterns in patients with neovascular AMD being treated with ranibizumab. The HELIOS study involved 14 sites in the Netherlands and enrolled 243 patients. Approval from an Independent Ethics Committee for this study was not required, since no intervention was required in the treatment and/or behaviour of the patients, and patients were only observed. All included patients provided an oral informed consent, and treatment had to be compliant with the prescribing information [23]. This study adhered to the Tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO). Patients were eligible for this study if they were diagnosed with wet AMD and had a recent disease progression with or without prior therapy. Diagnosis of wet AMD was based on presence of macular drusen and retinal pigment epithelial changes and signs of subretinal neovascularisation involving the centre of the macula on fluorescein and/or indocyanin green angiography and/or optical coherence angiography (OCT). Disease progression was defined as recent vision loss of more than 5 ETDRS letters, macular haemorrhage or signs of increased subretinal or intraretinal leakage on OCT. Patients were excluded if they participated in a clinical trial, (i.e. used investigational drugs at the time of enrolment or within 30 days or 5 investigational half-lives, whichever is longer) or when they did not provide informed consent.

Treatment and data collection

Treatment. All patients were treated with 0.5 mg ranibizumab (10 mg/ml) at the baseline visit. All patients should have been treated according to the prescribing information [23], which means starting with a loading phase of monthly injections for three consecutive months. The loading phase was followed by the maintenance phase, which includes that patients were monitored every month for disease progression. According to the prescribing information, reinjections are required when patients experience a loss of visual acuity of 5 letters or more, however, most physicians also retreated when there were clear signs of disease progression. All patients treated with ranibizumab at the baseline visit were followed in the study, even if the treatment had been interrupted.

Data collection. Patient data were collected at baseline and during routine visits approximately at 3, 6, 12, 18 and 24 months after enrolment, based on normal patient management procedures and upon discretion of the physician (there were no scheduled visits as per protocol and any visit could be recorded). At baseline, patient characteristics such as socio-demographic data, medical history, history of current disease, history of co-morbidities and ophthalmologic examinations were recorded, if performed. In

addition, information on concomitant AMD medication and prior AMD treatment was collected. At each visit, the treating physician recorded the patient's disease status, therapeutic decisions (including retreatment criteria for each additional ranibizumab injection) and assessed any adverse events.

Efficacy and safety assessments

Visual acuity outcomes. The primary objective of this study was to describe visual acuity outcomes in the real life setting in patients with wet AMD that were treated with ranibizumab for 24 months. These outcomes were described for the whole population, but also for the different lesion subtype groups (predominantly classic, occult, minimally classic), as well as for treatment-naive versus non-treatment naive patients.

Visual acuity was either measured as Early Treatment of Diabetic Retinopathy Study (ETDRS) scores starting at 2 m and converted to 1 m, or as Snellen fractions. All Snellen fractions were converted to approximate ETDRS scores, for analysis purpose, using the formula: approx ETDRS= 85+50xlog(Snellen fraction) [24]. The approximate ETDRS scores were rounded to the nearest letter.

Drug exposure and treatment patterns. To determine drug exposure and treatment patterns the number of injections per eye and the treatment intervals over the 24 month assessment period were analysed, separately for treatment-naive and previously treated patients, and for those who did or did not complete the 3 month loading phase.

Health related Quality of Life. The health related quality of life was assessed using the generic health assessment utility tool EuroQol (EQ-5D) as well as the visual-specific National Eye Institute Visual Function Questionnaire (NEI VFQ-25). The EQ-5D questionnaire comprises five levels: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. These five states were combined to produce a single index measure for quality of life. The EQ-VAS was used to record patient's self-rated health on a visual analogue scale, with 0 was labelled as "worst imaginable health state" and 100 as "best imaginable health state". The VFQ-25 consisted of 25 vision-targeted questions, representing 11 vision related constructs, plus one additional single-item general health rating question. The questionnaires were scored at baseline, and at visits around month 6, 12, and 24, as scheduled by the physician.

Safety Assessments. To evaluate safety and tolerability of treatment with ranibizumab all adverse and serious adverse ocular and non-ocular events (AE/SAE) were recorded.

Statistical Analysis

There was a moderate amount of missing data, which was due to the observational nature of the study, since physicians were not obliged to provide all data. All evaluations were considered explorative in nature. Descriptive statistics were used to report results.

Differences in absolute values of visual acuity measurements between visits were evaluated using repeated measures analysis of variance (ANOVA). Change from baseline in visual acuity or quality of life values were assessed with repeated measures analysis of covariance (ANCOVA) with baseline and lesion subtype as covariate. A Chi-squared test was used to assess differences in improvement categories between lesion subtypes. In case of significant results, post hoc tests were performed for further exploration. All statistical tests used were two-sided, with α=0.05 as level of significance.

Results

Patient disposition and demographics

In total, 243 patients with wet AMD were included into the study and started with ranibizumab treatment (the safety population, consisting of patients who received at least one ranibizumab injection). Of these 243 patients, 231 patients had a baseline and at least one post-baseline visual acuity measurement. This patient population was defined as the study population and used for all analyses except for the safety analysis. 153 patients of the study population completed the study. The reason for study discontinuation was loss to follow up (n=43), patient death (n=18), withdrawal of informed consent (n=5) or of unknown cause/other (n=12).

The patient characteristics were as expected for a wet AMD population and are described in table 1. Concomitant ocular conditions were observed in 20% of patients (table 1). Of these, 3.5% were treated by vitrectomy surgery of the primary involved eye. The other 16.5% consisted of other concomitant ocular conditions, such as glaucoma, cataract, pseudo-xanthoma elasticum, diabetic retinopathy, retinal vein occlusion, myopia, amblyopia, branch retinal vein occlusion, phaco emulsification and pseudophakia. Furthermore, patients in the study population reported on concomitant vascular conditions. Other relevant (but not specified) non-ocular conditions were reported for 19.5% of the patients in the study population (table 1).

The majority of the patients (61.9%) had an occult lesion subtype, 17.7% had a predominantly classic lesion, 16.9% of the patients had a minimally classic lesion and 3.5% had a lesion type which was characterized as other. The CNV location was subfoveal for most of the patients (80.5%), while 13.9% had the CNV lesion juxtafoveal and 5.6% extrafoveal.

A total of 192 patients (83.1%) included in the study were treatment-naive, while 39 patients (16.9%) were treated before with one or more therapies (11.7% with bevacizumab, 1.7% with ranibizumab, 2.2% with PDT, 2.2% with laser and 0.9% with other). Evaluation of concomitant therapies administered before and during the study revealed that only 26 patients received other treatment during the study, of which bevacizumab and 'other' were most frequently reported.

The baseline data for the different subgroups of lesion subtypes, and naive versus non-naive patients were comparable and only some small differences could be observed, but none were considered relevant for the outcome of the study.

Table 1. Key baseline demographics and disease characteristics of the study population

Baseline Characteristics	**Value**
Total population	n = 231
Mean age ± SD (years)	77.9 ± 8.2
Gender, n (%)	
male	95 (41.1%)
female	136 (58.9%)
Ethnicity, n (%)	
Caucasian	231 (100%)
AMD risk factors, n (%)	
current smoker	47 (20.3%)
former smoker	76 (32.9%)
family history of AMD	44 (19.0%)
Concomitant ocular conditions, n (%)	
history of vitrectomy surgery	8 (3.5%)
other	38 (16.5%)
Concomitant vascular conditions, n (%)	
coronary artery disease	44 (19.0%)
cerebrovascular artery disease	16 (6.9%)
peripheral vascular disease	50 (21.6%)
Other relevant non-ocular conditions, n (%)	45 (19.5%)
Mean visual acuity at baseline ± SD (letters)	45.1 ± 21.5
CNV lesion, n (%)	
occult	143 (61.9%)
predominantly classic	41 (17.7%)
minimally classic	39 (16.9%)
other	8 (3.5%)
CNV location, n (%)	
subfoveal	186 (80.5%)
juxtafoveal	32 (13.9%)
extrafoveal	13 (5.6%)
Treatment, n (%)	
no previous treatment	192 (83.1%)

Efficacy

Visual acuity

The mean ETDRS letter score for the study population at baseline was 45.1 ± 21.5 letters (n=231) which slightly increased during the study to a mean ETDRS score of 48.5 ± 25.1 letters (n=152) at 24 months, though not significantly (p=0.222) (table 2a). The treatment effects of ranibizumab, defined as change from baseline, were significantly different in the different lesion subtype groups (p=0.006). Also absolute visual acuity scores were significantly different between the lesion subtype groups (p<0.001) (table 2b). Patients in the occult subtype group had consistently higher visual acuity scores throughout the study period compared to the minimally classic and predominantly classic groups (figure 1).

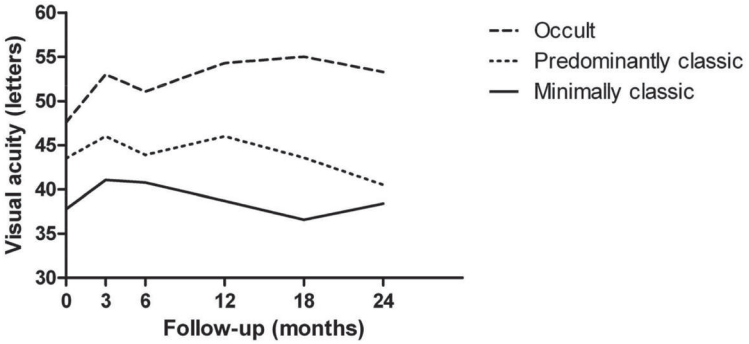

Figure 1. Course of mean visual acuity (EDTRS letters) over time for the different lesion subtype groups

Visual acuity was also compared in treatment-naive *versus* non treatment-naive patients. There seemed to be a slightly better response to ranibizumab in the treatment-naive patients (from 44.3 ± 21.6 letters at baseline to 49.3 ± 24.7 at 24 months) versus non-treatment naive patients (from 49.2 ± 20.8 letters at baseline to 45.0 ± 26.7 at 24 months), although not statistically significant (p=0.532) (figure 2). The injection pattern was similar for both patient groups, 7.8 ± 4.1 injections in the treatment naive group versus 8.1 ± 4.3 injections in the non-naive group after 24 months, demonstrating that the difference in visual acuity was not due to a larger amount of ranibizumab injections.

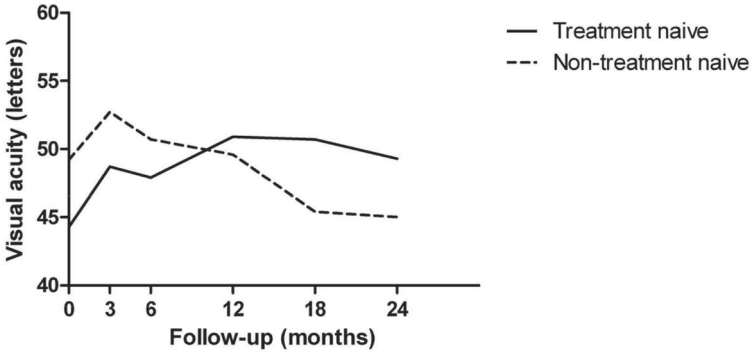

Figure 2. Course of mean visual acuity (ETDRS letters) over time for treatment naive vs. non-treatment naive patients

The visual acuity scores after 24 months were categorized in 4 BCVA outcome groups: significant improvement (gain of > 15 letters), improvement (gain of 15 - 0 letters), stabilization (loss of 0 - 15 letters) or deterioration (loss of > 15 letters). Of all patients, 79.6% demonstrated an improvement or a loss of less than 15 letters. As shown in table 3, 26 patients (17.1%) receiving ranibizumab showed a significant improvement in visual acuity, 49 patients (32.2%) showed an improvement, 46 patients (30.3%) showed a stabilization of visual acuity, and in 31 patients (20.4%) deterioration of visual acuity occurred.

A similar pattern was observed for the different lesion subtype groups. No statistical differences were observed when comparing the lesion subtype groups (p=0.914).

Table 2a. Mean visual acuity as ETDRS letter score, mean visual acuity change from baseline and mean number of injections over time for the study population

	Mean visual acuity EDTRS letter score ± SD		Mean visual acuity change from baseline EDTRS letters ± SD	Mean number of injections ± SD
Baseline	45.1 ± 21.5	(n=231)		1.0 ± 0.0
3 months	49.4 ± 22.7	(n=216)	4.6 ± 14.9	3.0 ± 0.7
6 months	48.4 ± 23.2	(n=203)	3.0 ± 18.1	4.1 ± 1.4
12 months	50.7 ± 24.0	(n=183)	4.4 ± 19.6	5.5 ± 2.3
18 months	49.7 ± 23.9	(n=168)	3.2 ± 22.1	6.7 ± 3.2
24 months	48.5 ± 25.1	(n=152)	1.0 ± 20.4	7.8 ± 4.2

Table 2b. Mean visual acuity as ETDRS letter score; mean VA change from baseline (ETDRS letters) over time for the different lesion types

	Lesion subtype groups								
	Predominantly classic			Minimally classic			Occult		
	Mean VA EDTRS letter score ± SD		Mean VA change from baseline (letters ± SD)	Mean VA EDTRS letter score ± SD		Mean VA change from baseline (letters ± SD)	Mean VA EDTRS letter score ± SD		Mean VA change from baseline (letters ± SD)
Baseline	43.5 ± 22.9	(n= 41)		37.8 ± 17.3	(n= 39)		47.6 ± 21.8	(n= 143)	
3 months	46.0 ± 26.2	(n= 39)	2.9 ± 11.9	41.1 ± 20.7	(n= 37)	4.2 ± 14.0	53.0 ± 21.7	(n= 135)	5.2 ± 16.0
6 months	43.9 ± 27.1	(n= 32)	3.2 ± 18.7	40.8 ± 20.5	(n= 33)	4.3 ± 13.8	51.1 ± 22.8	(n= 131)	2.6 ± 19.3
12 months	46.0 ± 27.3	(n= 30)	2.9 ± 19.4	38.7 ± 20.5	(n= 29)	3.2 ± 16.9	54.3 ± 23.5	(n= 118)	4.9 ± 20.7
18 months	43.6 ± 26.8	(n= 27)	0.2 ± 20.3	36.6 ± 21.8	(n= 27)	1.5 ± 18.0	55.0 ± 22.0	(n= 110)	5.0 ± 23.0
24 months	40.5 ± 27.4	(n= 26)	-2.5 ± 20.2	38.4 ± 22.4	(n= 26)	3.5 ± 19.3	53.3 ± 24.0	(n= 100)	1.3 ± 20.8

Table 3. Visual acuity scores after 24 months of ranibizumab treatment categorized in BCVA outcome scores

ETDRS category		Study population, n (%)
Significant improvement:	> 15 letters improvement	26 (17.1%)
Improvement:	1-15 letters improvement	49 (32.2%)
Stabilization:	0-15 letters loss	46 (30.3%)
Deterioration:	> 15 letters loss	31 (20.4%)

Drug exposure

Amount of ranibizumab injections

The total study population received a mean of 5.5 ± 2.3 injections during the first year (table 2a). The amount of injections in the second year were less than in the first year, since over the total 24 month period a mean of 7.8 ± 4.2 injections were administered. There were no differences observed in injection patterns for patients in the different lesion subtype groups.

Treatment patterns

In total, 184 patients (79.7%) completed the loading phase of one injection per month for 3 consecutive months, while 47 patients (20.3%) did not. Of the patients that did not complete the loading phase 28 (71.2%) were treatment naive, compared to 156 (81.2%) of the patients that completed the loading phase. This was not a statistically significant difference (p=0.194). The average treatment interval between the first and second injection was 31.9 ± 9.1 days and between the second and third injection was 34.3 ± 8.8 days for the patients that completed the loading phase. The average treatment interval between the first and third injection was 66.1 ± 12.6 days, which is in agreement with the three months duration of the loading phase.

The visual acuity in the patients completing the loading phase increased from 44.2 ± 21.6 at baseline to 51.0 ± 24.0 at 24 months follow-up. This increase in visual acuity was accompanied with an average of 7.4 ± 4.1 ranibizumab injections. This in contrast to patients who did not complete the loading phase, who had a decrease in mean visual acuity from 48.6 ± 20.9 letters at baseline to 37.6 ± 27.2 letters at 24 months follow-up, with an average of 5.6 ± 3.5 injections (p<0.001) (figure 3). Comparing the number of treatments between the two groups revealed a significant difference (p=0.005), suggesting that the total number of treatments is dependent on completion of the loading phase.

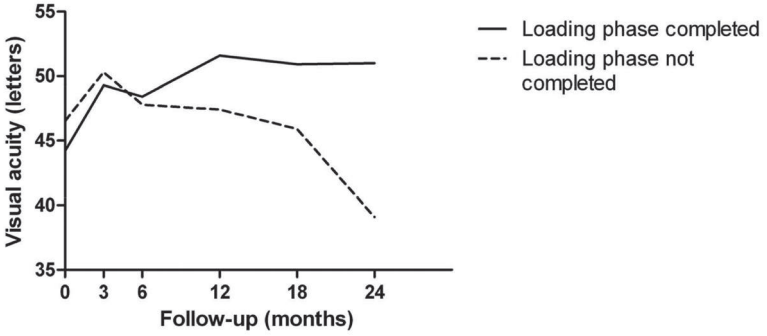

Figure 3. Course of mean visual acuity (ETDRS letters) over time for patients who completed the loading phase vs. patients who did not complete the loading phase

Health-Related Quality of Life and Visual Function Outcome

The EQ-5D questionnaire was used to assess quality of life and the EQ-VAS recorded the patient's self-rated health. Over the course of 24 months of ranibizumab treatment, there were no significant changes from baseline in the EQ-5D index scores, indicating stabilization of the disease (p=0.936) (table 4a). However, for the EQ-VAS score an increase was observed after 24 months of ranibizumab treatment (from 53.7 ± 32.6 at baseline to 70.9 ± 16.7 at 24 months; p<0.001), demonstrating an improvement in self-rated health as judged by the individual patient (table 4a). There were no differences in changes in quality of life between the lesion subtype groups (p=0.892).

wThe VFQ-25 questionnaire was used to assess the visual function outcome of patients being treated with ranibizumab. As demonstrated in table 4b, five of the eleven vision related subscale scores demonstrated a significant difference between visits for change from baseline between visits in visual function outcome during ranibizumab treatment: general vision (p = 0.023), ocular pain (p = 0.013), near activities (p = 0.043), distance activities (p = 0.014) and social functioning (p = 0.021). Results for the lesion subtype groups were similar, except for distance activities and peripheral vision, where the minimally classic lesion subtype showed slightly worse results over time (data not shown).

Table 4a. Health-related Quality of Life score of the EQ-5D and EQ-VAS questionnaires at baseline, 6, 12 and 24 months (study population)

	EQ-5d score, mean ± SD		EQ-VAS score, mean ± SD
Baseline	0.7880 ± 0.2193	(n=217)	53.7 ± 32.6
6 months	0.7800 ± 0.2532	(n=172)	60.6 ± 29.8
12 months	0.7804 ± 0.2319	(n=155)	59.6 ± 29.1
24 months	0.7864 ± 0.2127	(n=118)	70.9 ± 16.7

Table 4b. Visual function outcome score of the VFQ-25 questionnaire at baseline, 6, 12 and 24 months

	VFQ-25 questionnaire score mean ± SD				p-value*
	Baseline	6 months	12 months	24 months	
General health	48.71 ± 23.01 (n=214)	47.08 ± 22.66 (n=171)	44.84 ± 23.09 (n=155)	43.01 ± 22.38 (n=118)	ns
General vision	56.20 ± 17.95 (n=216)	59.77 ± 17.35 (n=171)	57.42 ± 16.39 (n=155)	55.42 ± 19.11 (n=118)	0.023
Ocular pain	86.41 ± 19.31 (n=217)	86.04 ± 18.83 (n=171)	82.82 ± 20.85 (n=155)	79.98 ± 19.76 (n=118)	0.013
Near activities	60.04 ± 29.37 (n=217)	61.14 ± 29.81 (n=171)	59.35 ± 28.23 (n=155)	56.53 ± 30.06 (n=118)	0.043
Distance activities	61.77 ± 30.62 (n=217)	62.55 ± 30.58 (n=171)	59.89 ± 30.10 (n=155)	56.78 ± 31.45 (n=118)	0.014
Vision specific:					
Social functioning	79.44 ± 28.36 (n=217)	80.19 ± 29.17 (n=171)	78.15 ± 28.40 (n=155)	75.21 ± 27.40 (n=118)	0.021
Mental health	67.96 ± 24.33 (n=217)	70.67 ± 23.67 (n=171)	68.27 ± 25.24 (n=155)	66.10 ± 25.47 (n=118)	ns
Role difficulties	60.37 ± 32.17 (n=217)	59.87 ± 33.13 (n=171)	56.69 ± 31.51 (n=155)	56.25 ± 31.64 (n=118)	ns
Dependency	77.62 ± 29.12 (n=216)	77.88 ± 29.63 (n=171)	77.74 ± 28.62 (n=155)	75.07 ± 31.38 (n=118)	ns
Driving	56.62 ± 39.88 (n=119)	58.00 ± 40.00 (n=99)	58.19 ± 40.20 (n=85)	57.23 ± 40.50 (n=64)	ns
Color vision	82.90 ± 28.06 (n=212)	84.76 ± 26.32 (n=169)	82.42 ± 27.65 (n=155)	83.19 ± 25.06 (n=116)	ns
Peripheral vision	76.28 ± 28.10 (n=215)	75.30 ± 31.10 (n=169)	71.94 ± 31.51 (n=155)	71.19 ± 29.35 (n=118)	ns

* p-values not corrected for multiple comparisons. ns= non-significant.

Safety

In total, 64 adverse events occurred in 45 patients (18.5%) of the safety population, of which 29 were serious adverse events (SAE) occurring in 27 patients (11.1%). The majority of adverse events had a mild to moderate intensity and were considered not related to the ranibizumab treatment. Furthermore, most observed events were ocular: 31 patients (12.8%) reported 40 ocular events.

The most commonly reported adverse events were detachment of retinal pigment epithelium (2.1%), eye pain (1.2%), conjunctival haemorrhage (0.8%), retinal pigment epithelial tear (0.8%), vitreous floaters (0.8%), visual impairment (1.2%) and cataract (0.8%).

The reported SAE's consisted of death (16), cardiac death (2), cardiac disorders of which one myocardial infarction (2), choroidal haemorrhage (1), iris bombé (1), retinal detachment (1), intraocular pressure increased (1), cardiac operation (1), cerebrovascular accident (1), gastric cancer (1), metastatic lung cancer (1) and lower limb fracture (1). The majority of the SAEs were considered by the physician as not suspected to have a relation with the study drug. Observations that were considered to be progression of the disease (e.g. pigment epithelial detachment) were not regarded as adverse events. The adverse events reported in this study were consistent with safety data from previous studies.

Discussion

Although the efficacy and safety of ranibizumab in licensed indications has been clearly demonstrated in the clinical trial program of ranibizumab [7-11], the efficacy and safety in routine clinical practice may differ. The present observational study was conducted to investigate this in more detail over a period of 24 months in 243 wet AMD patients, in clinical practice in the Netherlands.

Most patients (79.6%) demonstrated a stabilization or improvement of visual acuity in the primary involved eye after 24 months of treatment. This result was observed in the total study population as well as for the different lesion subtype groups. In comparison, in the MARINA and ANCHOR studies 94.5% and 96.4% of the patients showed stabilization or improvement.

The effect of ranibizumab was also investigated in treatment-naive and non treatment-naive patients. Non treatment-naive patients seemed to show stabilization of vision, while in treatment-naive patients an improvement in visual acuity score was observed after 24 months, suggesting that treatment-naive patients respond better to ranibizumab treatment, though this was not significant.

A mean of 5.5 ± 2.3 injections were administered in the total study population during the first year and 7.8 ± 4.2 injections over two years. The mean number of injections

per patient after one year were similar to that observed in the SUSTAIN study (5.6 injections) [25] and slightly higher than in the SAILOR study, which demonstrated a mean number of 4.9 injections (in cohort 1) [26]. There are not many data on an 'as-needed' (PRN) regimen for two years. The mean number of injections after two years was lower than in the ranibizumab as-needed arm of the 2-year CATT study (mean number of injections 12.6) and in the as-needed arm of the 2-year IVAN study (median of 13 injections) [27, 28]. The effectiveness of an as-needed regimen after two years has been shown by the CATT and IVAN studies. However, the mean gain in visual acuity after two years in this observational study was somewhat lower than seen in these large trials. After two years, patients in the CATT and IVAN study gained an average of 4-5 letters. This higher gain in letters could be explained by the larger number of injections in the 2-year CATT and IVAN studies combined with the monitoring which was much stricter that what is usual in daily practice. In addition, patients included in our study had relatively low baseline visual acuity. This could reflect a patient group with more advanced neovascular AMD and with lower capacity to improve due to more progressive photoreceptor damage.

The relatively low injection numbers together with the suboptimal functional outcomes at 24 months compared to the clinical trials, suggest that patient management could be improved.

Though substantial differences in efficacy could be seen compared to the clinical trials, the results from other observational studies on ranibizumab efficacy were in general similar to the current study [12-22]. Considering the treatment patterns, an apparent increase in long-term visual acuity was found in patients who completed the loading phase compared to a decrease in visual acuity in those who did not. The importance of the loading phase on final visual acuity in observational studies has been shown previously [21] and we could corroborate that finding. However, patients that completed the loading phase received a larger total amount of ranibizumab injections, which might also explain the better visual acuity outcome in the study of Gupta et al. as well as in ours. The Belgium HELIOS study [22], an observational study performed alongside but independent of the Dutch HELIOS study, had markedly less favourable visual acuity results of 1.6 letter gain after 1 year and a loss of 2.4 letters after 2 years, despite having a similar set-up and similar injection frequencies. A marked difference was that in the Belgium HELIOS study only about half of patients had a complete loading phase, compared to 80% in the Dutch HELIOS study, again showing the importance of completing the loading phase. Visual acuity gains in previous observational studies after 1 year ranged between 1 and 8 letters on ETDRS chart depending on which treatment regime was applied. Studies including monthly monitoring showed the better visual acuity results of 7 to 8 letter gains after 1 year [15, 17, 18]. In our study, monthly monitoring visits were not mandated and though part of the prescribing information this was left to the treating physician's discretion. Possibly, strictness of monitoring could explain the variation in outcomes between the

observational studies. Injection frequencies between observational studies were quite similar, ranging from 4 to 7 injection in 1 year.
The overall stabilization of visual acuity in our study was confirmed by the patient-reported stabilization of the disease as measured by the Quality of Life EQ-5D scores and by the NEI VFQ-25 scores. There was no clear effect of ranibizumab treatment on EQ-5D and VFQ-25 scores. Interestingly, the EQ-VAS indicated that ranibizumab treatment results in an increase in quality of life appreciation according to the patient. The difference observed between the EQ-5D index and EQ-VAS outcome might be explained by the fact that the EQ-5D index can be regarded as a deterministic, societal based measure, whereas the EQ-VAS is a single direct 'holistic' assessment from the patient's perspective. Therefore, the EQ-VAS leaves patients free to merge all relevant health state domains, and thus might be broader than the five EQ-5D domains. These data indicate that patients may benefit from ranibizumab treatment with regard to overall quality of life improvement (measured by the EQ-5D VAS score). Part of the effect might come from improvement in other (not-measured) health state domains.
Evaluation of the safety and tolerability demonstrated that the ranibizumab treatment during 24 months was associated with relatively few AEs in 45 (18.5%) patients and SAEs in 27 (11.1%) patients. The majority of SAEs were considered not to be related to the ranibizumab treatment. No new safety issues were identified in this study and the results are in line with safety data from previous studies [7-11, 25, 26]. The LUMINOUS study [29], a retrospective observational study of four European registries including HELIOS, showed that the safety profile was comparable between registries for most AEs. However, we cannot exclude that there is a possible underreporting of AEs due to the observational setting of the study.

In conclusion, this study demonstrates that ranibizumab treatment is effective in preventing vision loss over a period of 24 months. Completing the loading phase has a possible impact on long-term visual acuity outcome, thereby demonstrating the importance of monthly injections of ranibizumab for three consecutive months. This study was performed under the "old" label with a loading phase of three consecutive injections and then a PRN regimen. Ranibizumab now has a new label where treatment is given monthly and continued until maximum visual acuity is achieved. This might result in better treatment outcomes. Lastly, ranibizumab treatment was well tolerated and no new safety findings were identified. The suboptimal functional outcomes at 24 months, suggest that patient management could be improved.

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Prediction of treatment response

Chapter 3a

Predicting non-response to ranibizumab in patients with neovascular age-related macular degeneration.

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Purpose: To validate known and to determine new predictors of non-response to ranibizumab in patients with neovascular age-related macular degeneration (AMD) and to incorporate these factors into a prediction rule.

Methods: This multicenter, observational cohort study included 391 patients treated with ranibizumab for neovascular AMD. We performed genetic analysis for single nucleotide polymorphisms in AMD-associated genes and collected questionnaires regarding environmental factors and disease history. The primary outcome was non-response to treatment, which we defined as a loss of visual acuity of 30% of letters or more.

Results: Of the 391 patients, 47 were classified as non-responder. Independent predictors for non-response were: age, baseline visual acuity, diabetes mellitus and accumulation of risk alleles in the *CFH*, *ARMS2* and *VEGF-A* genes. The area under the receiver operating characteristic (ROC) curve was 0.77 (95% confidence interval: 0.70 - 0.84). We derived a clinical prediction rule, with possible total risk scores ranging from 0 to 19 points. The absolute risk of non-response varied from 3% to 52% between risk score groups.

Conclusion:. This is an important step towards a clinical prediction rule that can aid clinicians in identifying AMD patients with increased likelihood of non-response and consequently contribute to making shared treatment decisions.

Introduction

Age-related macular degeneration (AMD) remains the leading cause of severe visual impairment in the Western world [1]. Neovascular AMD accounts for approximately 10% of all patients with AMD, but is responsible for the majority of AMD-related severe vision loss [2, 3]. In this type of AMD, choroidal blood vessels invade the central retina and subretinal space, leaking serous fluid, lipids and blood leading to fibrous scarring. Untreated, choroidal neovascularizations (CNV) will result in a loss of 1-3 Early Treatment Diabetic Retinopathy Study (ETDRS) lines (5-15 letters) at 3 months and 3-4 lines at 1 year [4]. In recent years, intraocular administered drugs (ranibizumab and bevacizumab) aimed at blocking vascular endothelial growth factor A (VEGF-A) have become the mainstay of treatment for neovascular AMD. Although these agents have improved visual prognosis considerably [5, 6], about 10% of the patients do not respond to this therapy and still experience substantial visual loss, namely a loss of 15 letters or more, which is comparable to the natural course of the disease [6, 7]. Treatment can become quite burdensome particularly for the elderly patients, as patients have to visit the clinic at monthly intervals. By reliably identifying non-responders early in the treatment process, the burden from otherwise ineffective and costly intraocular injections may be reduced. Several studies have suggested that genetic factors influence the response to anti-VEGF therapy in AMD. To date no single nucleotide polymorphism (SNP) has been consistently associated with treatment response. Both the Y402H risk allele in the *CFH* gene and the *ARMS2* A69S have been linked to a reduced treatment effect, subsequent reports, however, could not corroborate this effect [8-16]. Recently, Smailhodzic and co-workers demonstrated that accumulation of high-risk alleles in the *CFH*, *ARMS2* and *VEGF-A* (rs699947) genes were associated with poor response rates to ranibizumab treatment and a younger age of onset of neovascular disease [17]. Besides underlying genetic factors, several reports have shown that non-responders tend to be older, demonstrate larger neovascular lesions and present with a higher visual acuity at baseline [18-21].
In this study, we assessed the combined effect of known and newly determined response-related factors in neovascular AMD. The objective of this study was to develop a clinical prediction model to help identify non-responders for ranibizumab in patients with neovascular AMD.

Materials and Methods

Population
We evaluated 391 eyes of 391 unrelated Caucasian patients aged 50 years or older with active CNV secondary to AMD. All study participants were enrolled between June 2008 and June 2010 in the European Genetic Database (EUGENDA), a multicenter

database for the clinical and molecular analysis of AMD. All patients were treated at the Departments of Ophthalmology of the Radboud University Nijmegen Medical Center, the Netherlands; the University of Cologne, Germany; or the McGill University Health Center, Montreal, Canada. This observational cohort study was performed in accordance with the tenets of the Declaration of Helsinki (1983 revision) and the Medical Research Involving Human Subjects Act (WMO). The approval of the local ethics committee was obtained for all three centers and written informed consent was acquired from all participants.

The diagnosis active neovascular AMD was established by retinal specialists based on signs of leakage on fluorescein angiography (FA)[8] (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany; or Imagenet, Topcon Corporation, Tokyo, Japan), combined with presence of fluid or blood during ophthalmic examination or on spectral-domain optical coherence tomography (OCT) (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany). The lesion type of the CNV was determined by FA, and was classified into 4 categories: occult with no classic component, minimally classic, predominantly classic and retinal angiomatous proliferation (RAP) [6]. Prior to retinal imaging, pupillary dilatation was achieved with topical 1.0% tropicamide and 2.5% phenylephrine. Patients with retinal disorders other than AMD, including manifest diabetic retinopathy, were excluded.
Each participant underwent best-corrected visual acuity (BCVA) assessments prior to and after treatment with three monthly 0.5 mg ranibizumab injections. For 314 (80.3 %) patients the Snellen visual acuity was collected retrospectively at a later time point after treatment, whereas 77 (19.7%) patients were followed prospectively during treatment using ETDRS visual acuity. We did not adopt any exclusion criteria based on visual acuity, because we aimed to collect a patient cohort that represents clinical practice. This way, any predictive factors found in this study can be applied in the broadest possible way.
After the three loading injections, patients received on demand re-injections whenever signs for CNV activity were detected by clinical examination, funduscopy or FA. OCT was used when available.
Patients were excluded from this study if they had received prior treatments for active subfoveal CNV secondary to AMD. If both eyes were eligible for inclusion, the eye that received treatment first was chosen as the study eye. If both eyes started treatment simultaneously, the study eye was chosen at random.

Predictive factors
Questionnaires were used to collect information regarding disease history and environmental factors, which included smoking habits, body mass index and disease history. Venous blood for genotyping was drawn before onset of treatment. Genotyping

of SNPs in the *CFH* (Y402H; rs1061170), *ARMS2* (A69S; rs10490924), *VEGF-A* (rs699947 and rs833069), *KDR* (rs2071559 and rs7671745), *LPR5* (rs3736228), and *FZD4* (rs10898563) genes was performed with TaqMan probes and primers, using assays developed by Applied Biosystems, and an ABI 7900HT system (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) as described previously [17]. Total CNV lesion size was measured on FA by dividing the total CNV lesion area by the optic disk area (DA). Total CNV lesion size was defined as the area of CNV, blocked fluorescence from hemorrhage or from other causes, pigment epithelial detachment and fibrosis. Measurements were performed using the open source image processing tool ImageJ (version 1.47f; available at http://rsb.info.nih.gov/ij) [22]. The multiple imputation function in SPSS using all available factors was applied to impute missing values when needed to maintain statistical power. The outcome variable was available for all patients.

Outcome

The main outcome measure in this study is non-response. We based non-response on a functional outcome, namely the change in visual acuity. We chose not to use an anatomical response as seen on OCT because, from a patient's perspective, vision is the only relevant outcome. An definition often used to define non-response is the loss of at least 15 ETDRS letters. This approach, however, tends to underestimate the number of non-responder in patients with a low visual acuity, as the amount of vision that can be lost is limited (floor effect). In contrast, patients with a high baseline visual acuity have greater potential to lose vision and the possibility of improvement is limited (ceiling effect). To avoid this floor-ceiling effect, we defined non-response as a loss of 30% of letters or more from baseline, which roughly corresponds with a loss of three EDTRS lines from the mean baseline visual acuity in this cohort. In table 1 we show the cut-off points for being non-responder with respect to baseline visual acuity. When baseline visual acuity was 20/640 or lower, a loss of 0 letters or more was considered non-response as visual acuity from that point was deemed too low to expect any more vision loss and changes in visual acuity would become too small to measure accurately. Because patients were treated according to an on demand schedule, follow-up and total number of injections varied among patients. To achieve a comparable and standardized evaluation of the response to ranibizumab, we analyzed the data to identify non-responders after the first three consecutive monthly ranibizumab injections. In the majority of patients the maximum change in visual acuity will have been reached after the first three monthly injections with ranibizumab, making it a good indication of long-term response [19]. Using this method, we calculated the cut-off point for non-response for each individual patient and classified each patient accordingly.

Table 1. Cut-off point for non-response with respect to baseline visual acuity

Baseline visual acuity (Snellen)	**Baseline visual acuity** (letters)	**Cutoff point for non-response** (letters)	**Cutoff point for non-response** (Snellen)
20/20	85	59	20/65
20/25	80	56	20/76
20/32	75	52	20/89
20/40	70	49	20/105
20/50	65	45	20/123
20/63	60	42	20/145
20/80	55	38	20/170
20/100	50	35	20/200
20/125	45	31	20/235
20/160	40	28	20/276
20/200	35	24	20/324
20/250	30	21	20/381
20/320	25	17	20/448
20/400	20	14	20/526
20/500	15	10	20/618
20/640	10	10	20/640
20/800	5	5	20/800
20/1000	0	0	20/1000

For each individual the cutoff point for non-response was determined by calculating the visual acuity after a loss of 30% of letters from baseline. Patients with a visual acuity after treatment equal to or lower than the calculated value (last column), were considered non-responder. Patients with 20/640 or lower were considered non-responder when their vision remained stable, as more vision loss in this group was not to be expected.

Statistical analyses

Snellen and EDTRS visual acuity was converted to the logarithm of minimal angle of resolution (logMAR) visual acuity for the purpose of statistical analysis. Change in visual acuity was calculated as the difference between the vision at baseline and after three months of follow-up.

The association of the environmental and genetic factors with the main-outcome measure was assessed with univariable logistic regression analyses. Variables were dichotomized or categorized when possible. Visual acuity prior to treatment was categorized into groups by taking the quartiles of the range of baseline visual acuity in this cohort (approximately 0.00-2.00 logMAR). Thus, the following four groups were established: > 0.50 logMAR

(20/63), between 0.50 and 1.00 logMAR (20/63-20/200), between 1.00 and 1.50 logMAR (20/200-20/640) and ≤ 1.50 logMAR (20/640). The various genotypes were assessed separately as well as cumulatively in association with non-response, taking into account any additive effects that may exist between risk alleles as described before [17].
Factors that were possibly associated with non-response (overall $P < 0.20$) were included in multivariable logistic regression analyses. The model was reduced by stepwise elimination of factors with a P-value of > 0.15 until four variables remained [23, 24]. The model's ability to discriminate between non-responders and responders was estimated as the area under the curve (AUC) for the receiver operating characteristic (ROC) curve. The Hosmer-Lemeshow goodness-of-fit test was used to estimate the reliability of the model. The model was validated internally using bootstrapping techniques, yielding a shrinkage factor for the regression coefficients and the ROC AUC [25, 26].
To obtain a prediction rule that is easily applicable in clinical practice, the adjusted regression coefficients of the model were divided by the lowest coefficient and rounded to the nearest integer. Scores for each individual patient were obtained by assigning points for each variable and adding the results. Patients were grouped according to risk score and the absolute risk of non-response for each risk score group was calculated. All statistical analyses were performed using SPSS, version 20.0 (SPSS, Inc., Chicago, IL).

Results

Of the 391 patients that met the eligibility criteria, 47 (12.0%) could be classified as non-responder and 344 (88.0%) as responder. Mean change in baseline visual acuity was plus 7.6 ± 10.7 letters for responders and minus 21.2 ± 13.1 letters for non-responders ($P < 0.001$). Demographic and disease characteristics of responders and non-responders are summarized in Table 2 and genotype characteristics are shown in Table 3. Visual acuity measurements after 12 months were available for 254 patients and showed that non-response at three months (in 30 of 254 patients) was strongly associated with non-response at 12 months (in 43 of 254 patients) ($P < 0.001$). Eight of the non-responders at 3 months, no longer met the criteria after 12 months, but only three of these patients actually gained visual acuity from baseline.
Results of multivariable logistic regression analyses are presented in Table 4. The final reduced regression model included four independent predictive variables, that is age, baseline visual acuity, diabetes mellitus and accumulation of risk alleles in the *CFH*, *ARMS2* and *VEGF-A* genes. Other risk allele combinations of were not of added value (data not shown). Though CNV lesion size was significant and CNV type was marginally significant in univariable analyses, these variables were no longer significantly associated with non-response in the multivariable regression analysis and were not of added value to the model and hence not included in the prediction model.

The Hosmer-Lemeshow goodness-of-fit test indicated an acceptable fit of the final prognostic model (P = 0.69), and the AUC was 0.77 (95% CI: 0.70 - 0.84) (Figure 1). This equals a discriminative ability of 77%, meaning that by using this prediction model 77% of patients are correctly classified as responder or non-responder. Internal validation

Table 2. Univariable analysis of predictor variables for non-response: demographic and disease characteristics

Variables	**Responders** n (%)		**Non-responders** n (%)		**OR** (95%-CI)		**P-value**	
Total	344	(88.0)	47	(12.0)				
Age >80 years	117	(34.0)	25	(53.2)	2.2	(1.2-4.1)	0.012	*
Male gender	151	(43.9)	20	(42.6)	0.9	(0.5-1.8)	0.862	
VA prior to treatment (Snellen)								
> 20/63	142	(41.3)	7	(14.9)	1.0		< 0.001	*
20/63 - 20/200	124	(36.0)	20	(42.6)	3.3	(1.3-8.0)	0.009	*
20/200 - 20/640	74	(21.5)	14	(29.8)	3.8	(1.5-9.9)	0.006	*
≤ 20/640	4	(1.2)	6	(12.8)	30.4	(7.0-133.0)	< 0.001	*
Disease history								
Hypertension (n=273)	143	(58.6)	18	(66.7)	1.4	(0.6-3.3)	0.420	
Diabetes (n=273)	39	(16.0)	10	(37.0)	3.1	(1.3-7.3)	0.009	*
Myocardial infarction (n=122)	10	(9.2)	1	(7.7)	0.8	(0.1-7.0)	0.860	
Angina pectoris (n=122)	9	(8.3)	1	(7.7)	0.9	(0.1-8.0)	0.944	
Stroke or TIA (n=122)	9	(8.3)	0	(0.0)	0.0	(0.0-inf.)	0.999	
Environmental factors								
Smoking ≥ 20 packyears (n=149)	33	(25.2)	7	(43.8)	2.3	(0.8-6.7)	0.123	
BMI > 30 (n=272)	38	(15.6)	5	(20.0)	1.4	(0.5-3.8)	0.566	
Angiographic characteristics								
Occult with no classic	190	(55.2)	18	(38.3)	1.0		0.177	
RAP	20	(5.8)	2	(4.3)	1.1	(0.2-4.9)	0.945	
Minimally classic	34	(9.9)	8	(17.0)	2.5	(1.0-6.2)	0.050	*
Predominantly classic	65	(18.9)	11	(23.4)	1.8	(0.8-4.0)	0.156	
Unknown	35	(10.2)	8	(17.0)	2.4	(1.0-6.0)	0.057	
Lesion size (DA) (n=295)								
< 2	93	(35.9)	6	(16.7)	1.0		0.058	
2 - 4	78	(30.1)	14	(38.9)	2.8	(1.0-7.6)	0.045	*
4 - 6	40	(15.4)	4	(11.1)	1.6	(0.4-5.8)	0.515	
> 6	48	(18.5)	12	(33.3)	3.9	(1.3-11.0)	0.011	*

OR=odds ratio; VA=visual acuity; TIA=transient ischemic attack; BMI=body mass index; RAP=retinal angiomatous proliferation; DA=disk areas.
Values from certain variables were missing for some of the patients; n=total number of patients included in analysis.
*P-value ≤ 0.05, indicating statistical significance. The P-values behind the reference categories, represent the overall P-values for the prognostic factor. All P-values are derived from univariable logistic regression analysis.

Table 3. Univariable analysis of predictor variables for non-response: genotype

Genotype variables	**Responders** n (%)		**Non-responders** n (%)		**OR** (95% CI)		**P-value**	
***CFH* (Y402H)** (n=391)								
TT	67	(19.5)	7	(14.9)	1.0		0.646	
CT	165	(48.0)	22	(46.8)	1.3	(0.5-3.1)	0.594	
CC	112	(32.6)	18	(38.3)	1.5	(0.6-3.9)	0.361	
***ARMS2* (A69S)** (n=391)								
GG	115	(33.4)	12	(25.5)	1.0		0.558	
TG	144	(41.9)	22	(46.8)	1.5	(0.7-3.1)	0.316	
TT	85	(24.7)	13	(27.7)	1.5	(0.6-3.4)	0.368	
***VEGF-A* (rs699947)** (n=375)								
CC	75	(22.7)	8	(18.2)	1.0		0.738	
CA	158	(47.7)	21	(47.7)	1.2	(0.5-2.9)	0.616	
AA	98	(29.6)	15	(34.1)	1.4	(0.6-3.6)	0.436	
***VEGF-A* (rs833069)** (n=368)								
TT	143	(44.0)	15	(34.9)	1.0		0.528	
TC	137	(42.2)	21	(48.8)	1.5	(0.7-3.0)	0.290	
CC	45	(13.8)	7	(16.3)	1.5	(0.6-3.9)	0.420	
***KDR* (rs2071559)** (n=369)								
AA	72	(22.2)	15	(34.1)	1.0		0.133	
AG	166	(51.1)	16	(36.4)	0.5	(0.2-1.0)	0.046	*
GG	87	(26.8)	13	(29.5)	0.7	(0.3-1.6)	0.419	
***KDR* (rs7671745)** (n=364)								
GG	109	(33.7)	16	(39.0)	1.0		0.771	
GA	176	(54.5)	20	(48.8)	0.8	(0.4-1.6)	0.473	
AA	38	(11.8)	5	(12.2)	0.9	(0.3-2.6)	0.841	
***LPR5* (rs3736228)** (n=364)								
TT	191	(59.5)	24	(55.8)	1.0		0.829	
CT	83	(25.9)	13	(30.2)	1.2	(0.6-2.6)	0.550	
CC	47	(14.6)	6	(14.0)	1.0	(0.4-2.6)	0.974	
***FZD4* (rs10898563)** (n=372)								
AA	114	(34.8)	15	(34.1)	1.0		0.826	
AG	159	(48.5)	20	(45.5)	1.0	(0.5-1.9)	0.901	
GG	55	(16.8)	9	(20.5)	1.2	(0.5-3.0)	0.630	
Risk allele accumulation in *CFH*, *ARMS2*, *VEGF-A* (n=375)								
mean number of risk alleles ± SD	3.1	± 1.2	3.4	± 1.0	1.3	(1.0-1.7)	0.105	

Values from certain variables were missing for some of the patients; n=total number of patients included in analysis.
*P-value ≤ 0.05, indicating statistical significance. The P-values behind the reference categories, represent the overall P-values for the prognostic factor. All P-values are derived from univariable logistic regression analysis.

using bootstrapping techniques yielded an over-optimism of 0.03. The shrunk AUC was 0.75 (shrinkage factor of 0.86).

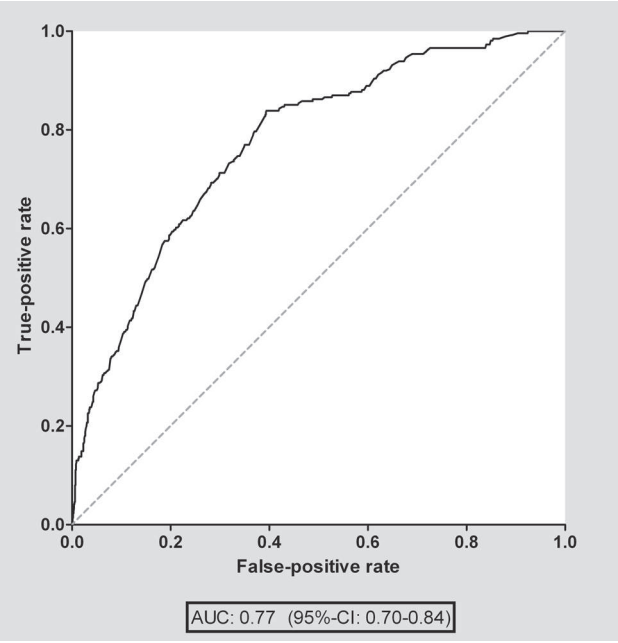

Figure 1. Area under the curve of the receiver operating characteristic curve of the prediction model for non-response.
The ROC curve is a plot of the true-positive rate (sensitivity) versus the false-positive rate (1 – specificity) evaluated at consecutive cut-off points for the predicted probability. The AUC provides a quantitative summary of the discriminative ability of a predictive model. A useless predictive model, such as a coin flip, would yield an AUC of 0.50. When the AUC is 1.00, the model discriminates perfectly between subjects who do and subjects who do not develop a prognostic outcome. The AUC of our prediction model for non-response was 0.77. The prediction model contains the predictors: age, diabetes, accumulation of risk alleles in the *CFH*, *ARMS2* and *VEGF-A* genes and baseline visual acuity.

We developed a clinical prediction rule by assigning points to each predictive factor based on its regression coefficient (Table 4). We assigned 1 point for each risk allele in the *CFH*, *ARMS2* or *VEGF-A* genes, 2 points for diabetes mellitus, 2 points for age of over 80 years, 3 points for a baseline visual acuity between 20/63 and 20/200, 4 points for baseline visual acuity between 20/200 and 20/640 and 9 points for a baseline visual acuity of 20/640 or less. We divided risk scores into 3 groups.
The last group consisted of the 5% highest scoring individuals. The remaining 95% was divided into two groups consisting of the 50% lowest scoring individuals and the individuals in between the lowest and highest scoring groups. This resulted in the

following 3 risk score groups: 0-6, 7-10 and 11-19 points. The absolute risk for being a non-responder increased as the risk score increased (Figure 2).

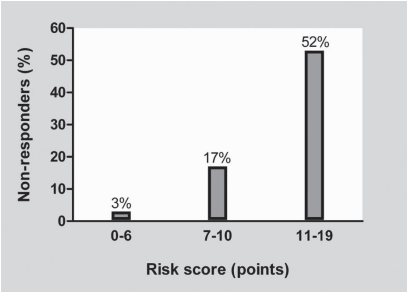

Figure 2. Absolute risk of non-response per risk score group

Discussion

Health care costs are ever rising and to reduce the number of costly and unnecessary treatments we need reliable prediction rules. The current study describes the first step towards a clinical prediction rule that helps identifying non-responders to intravitreal injections with ranibizumab for age-related macular degeneration.

In this study, the independent predictors of non-response were: age, diabetes mellitus, baseline visual acuity and accumulation of risk alleles in the *CFH*, *ARMS2* and *VEGF-A* genes. The association of advanced age and certain risk alleles with a reduced treatment response is in line with earlier reports [17, 18, 20, 21]. New correlations include the association of non-response with a low baseline visual acuity as well as with diabetes mellitus. The relation with a low baseline visual acuity is in contrast with other reports [18, 20, 21] and is most likely a consequence of the novel definition of non-response in this study that corrects for the floor-ceiling effect. The reason for this association is probably a greater degree of photoreceptor damage in the group of patients with low visual acuity. The increased risk for non-response to ranibizumab for diabetes patients has not been shown before although earlier reports suggested an association between diabetes and incident geographic atrophy secondary to AMD as well as incident neovascular AMD [27, 28]

In previous subgroup analyses of the large ranibizumab trials, total CNV lesion size and CNV type were considered important predictors of treatment response [18, 20, 21]. In this study, however, lesion size and CNV type were only associated with non-response in univariable analyses. The adding of baseline visual acuity to the model seemed to take away much of the effect size, indicating that baseline vision already accounts for much of the predictive value of these factors. Considering visual acuity at baseline is a much

more convenient measure in clinical practice and less prone to inter-observer variation, the inclusion of baseline vision into a prediction model instead of the CNV lesion type or the lesion size seems an obvious choice.

This study focussed on a prediction model of functional non-response. Hence, we used visual acuity to define non-response. We defined non-response as a loss of 30% of letters from baseline. When using definitions of non-response employed in other studies, such as for example a loss of 15 letters, the results from the model were very similar, yielding the same predictive factors as in our prediction model. Only baseline visual acuity showed a reversed association, which most probably represents the neglected floor-ceiling effect, underestimating non-response in patients with low visual acuity.

This study is based on clinical practice and therefore included patients with low baseline visual acuity. In most trials these patients are not included because the anticipated treatment effect is small. Our prediction model shows baseline VA as a strong predictor of non-response, which supports the notion that patients with low baseline vision indeed benefit less from treatment. However, the possibility exists that there is an over-representation of individuals with low vision in our non-responders group due to how we defined non-response. To assess the effect of baseline vision on the non-response prediction model, we repeated the analyses with the exclusion of patients with baseline visual acuity <20/400. This did not lead to the selection of different predictors for the final model, nor did it change the parameter estimates of the univariable and multivariable analyses. The accompanying AUC was now 0.73. The absolute risk of non-response in the highest risk group decreased to 26% due to the removal of 10 non-responders on account of the new exclusion criterion. This indicates that the prediction model presented here is applicable to populations with various ranges of vision and that the other predictors presented here are related to non-response independent of baseline vision.

Non-response in this study was determined after 3 months of treatment, because previous studies clearly demonstrated that virtually all visual gain can be expected in this period[5, 6]. Furthermore, response after 3 months appears to be strongly correlated with long-term response[19]. The current study corroborated the correlation between non-response after 3 and 12 months. Also, the factors associated with non-response after 3 months were similarly associated with non-response after 12 months. The associations from this study likely translate to long-term predictions, although 8 out of 47 non-responders at 3 months could be considered responders at 12 months. Therefore, validation of the model with long-term outcomes is warranted.

This study has some potential limitations that should be discussed. The presence of subretinal tissue, cystoid macular edema and reduced central retinal thickness on OCT has been linked with an increased risk for non-response to anti-VEGF [29, 30]. Unfortunately, baseline OCT scans were not available for the majority of patients in

this study. We believe that these predictive factors should also be included in a future improved version of the prediction rule.

A loss of vision of 30% is comparable to the natural course of disease, however, we cannot be sure that the non-responders in this study would not have lost more vision without treatment. To estimate the consequences of such potential misclassification of non-response, we will need efficiency trials that compare conventional treatment strategy to treatment strategy based on a prediction model, once more advanced prediction models become available.

A major strength of the current study is the combination of various new and known predictors of non-response into a prediction model for non-response. There have been many previous studies focusing on single predictive factors. This study incorporates many of these predictor variables into a practical tool. The current model is not yet advanced enough to be the determining factor to discontinue treatment. The chance of non-response for high-risk individuals (namely, older patients with low baseline vision, diabetes and an unfavourable genetic profile), is approximately 50%, compared to the generally accepted 10% in the overall population. This absolute increase is of such significance that it should be taken into account by the clinician and the patient in their shared decision making on the treatment strategy.

We need to continue the search for additional risk factors to refine the prediction model. In that sense, this model might very well serve as a guideline for future prediction models. Every predictor of response adds to a more accurate identification of patients with a heightened risk of non-response and a prediction rule can assist in making the decision to stop treatment. This is important not only to restrict the burden of a prolonged unsuccessful anti-VEGF treatment for patients and their family members, but also to apply a very costly treatment to those patients most likely to benefit. Finally, clinical prediction rules will become even more useful with the availability of new treatment modalities. Patients with a high-risk for non-response to ranibizumab may be offered an alternative therapy at an early stage.

In conclusion, the combination of different genetic and environmental risk factors from this study can predict non-response to intravitreal ranibizumab treatment to certain extent. This study is an important step towards developing a straightforward prediction rule to help identify those AMD patients that are at an increased risk for non-response to ranibizumab treatment.

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Chapter 3b

Polymorphisms in vascular endothelial growth factor receptor 2 are associated with better response rates to ranibizumab treatment in age-related macular degeneration.

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Purpose: Intravitreal anti–vascular endothelial growth factor (VEGF) injections are currently the standard treatment for neovascular age-related macular degeneration (AMD), but a broad range of response rates has been observed. We evaluated the association of single nucleotide polymorphisms (SNPs) in VEGF genes and their receptors (VEGFR) with the response rate to ranibizumab in 366 patients with neovascular AMD.

Methods: Design: Case series study. Participants: A total of 366 eyes of 366 patients with neovascular AMD. Visual acuity (VA) was determined at baseline, after 3 monthly ranibizumab injections, and after 1 year of treatment. Genotyping of 126 SNPs in the genes encoding VEGF family members VEGFA, VEGFB, VEGFC, VEGFD (*FIGF*), and placental growth factor (*PGF*); VEGF receptors VEGFR1 (*FLT1*), VEGFR2 (*KDR*), and VEGFR3 (*FLT4*); and the gene encoding pigment epithelium-derived factor (PEDF) (*SERPINF1*) was performed. Main Outcome Measures: The changes in VA after 3 injections and after 1 year of treatment and their association with VEGF and VEGFR genotypes.

Results: Univariate analyses of variance (ANOVAs) revealed a significant effect of SNP rs4576072 in the *VEGFR2* gene on VA change after 12 months ($F_{[1,235]}$ = 14.05; P = 0.02). A stepwise linear regression analysis returned a model (P = 0.01) with SNPs rs4576072 and rs6828477 in the *VEGFR2* gene as independent predictors for VA change after 12 months, with a mean increase in VA of 0.26 on the logarithm of the minimum angle of resolution (logMAR) scale in patients with 3 contributing minor alleles compared with a loss of 0.03 logMAR in patients with no minor allele.

Conclusion: Polymorphisms in the *VEGFR2/KDR* gene significantly influence visual outcome in patients receiving ranibizumab treatment for neovascular AMD. This study shows that genetic variation partially explains the wide range of response to ranibizumab treatment, which in the future might help clinicians tailoring medical interventions to individual needs.

Introduction

In neovascular age-related macular degeneration (AMD), newly formed choroidal blood vessels (choroidal neovascularization [CNV]) invade the subretinal and intraretinal spaces, causing exudation, hemorrhage, and subsequently visual loss. Drugs such as ranibizumab targeting vascular endothelial growth factor (VEGF) are effective in preserving and improving visual acuity (VA). Although many patients respond favorably to anti-VEGF treatment, some patients lose vision despite optimal therapy. Several studies have suggested that genetic factors influence the response to anti-VEGF treatment.[1-15] For example, poor response rates were demonstrated for patients carrying the complement factor H (*CFH*) Y402H genotype[5-7, 9, 10, 16] or polymorphisms in age-related maculopathy susceptibility 2 (*ARMS2*).[1, 12] Moreover, a cumulative effect of high-risk alleles in the *CFH*, *ARMS2*, and *VEGFA* genes was associated with poor response rates to ranibizumab treatment and a younger age of onset of neovascular disease.[11] However, the effect of genetic variants on treatment response is still not clear.

Ranibizumab is a humanized monoclonal antibody fragment that binds all isoforms of VEGFA, which is an important pro-angiogenic factor that plays a central role in the development of CNV. In addition to VEGFA, the VEGF family comprises VEGFB, VEGFC, VEGFD (FIGF), and placental growth factor (PGF). The VEGF members are ligands of 3 tyrosine kinase receptors: VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4). Vascular endothelial growth factor A binds to VEGFR1 and VEGFR2; VEGFB and PGF bind only to VEGFR1. Vascular endothelial growth factor R2 mediates most cellular responses to VEGF, whereas VEGFR1 might modulate VEGFR2 signaling and act as a decoy receptor competing with VEGFR2 for VEGF. Vascular endothelial growth factor R2 and VEGFR1 are both inhibited by the anti-angiogenic pigment epithelium-derived factor (PEDF). Vascular endothelial growth factor C and VEGFD are ligands for VEGFR3 involved in lymphangiogenesis.[17]

The purpose of this study was to determine whether polymorphisms in genes encoding VEGF family members, VEGF receptors, and PEDF influence visual outcome in patients treated with ranibizumab for neovascular AMD. Although several studies have analyzed a limited number of single nucleotide polymorphisms (SNPs) in the *VEGFA* and *KDR* genes[2, 4, 6, 7, 14] a comprehensive analysis of SNPs across all VEGF and VEGFR genes has not been performed. Therefore, we genotyped 126 tag-SNPs in the *VEGFA, VEGFB, VEGFC, VEGFD (FIGF), PGF, VEGFR1 (FLT1), VEGFR2 (KDR), VEGFR3 (FLT4),* and *PEDF (SERPINF1)* genes in 366 patients with AMD who have been treated with ranibizumab for at least one year.

Methods

Study Population

This multicenter study included 366 eyes of 366 unrelated patients aged 50 years or older with active subfoveal CNV secondary to AMD. All participants were enrolled in the European Genetic Database (EUGENDA), a multicenter database for the clinical and molecular analysis of AMD, between 2008 and 2010. The study was performed in accordance with the tenets of the Declaration of Helsinki. The approval of the local ethics committee was obtained for both centers, and written informed consent was provided by all participants.

Inclusion and Exclusion Criteria

All patients had active subfoveal or juxtafoveal CNV due to AMD confirmed by spectral-domain optical coherence tomography and fluorescein angiography (FA) with indocyanine green. Further criteria in the study eye were a best-corrected VA equivalent to ≥20 Early Treatment of Diabetic Retinopathy Study (ETDRS) letters and no previous treatment for exudative AMD, such as photodynamic therapy or intravitreal injections in the study eye. Exclusion criteria included any previous ophthalmic surgery, except for cataract removal, diabetic retinopathy, and progressive glaucoma.

Diagnostics and Treatment

All patients were treated for at least 12 months with ranibizumab on a pro re nata regimen. Patients initially received 3 consecutive, monthly intravitreal injections of 0.5 mg ranibizumab. After this first series of treatments, patients were monitored in monthly visits. Evaluations included spectral-domain optical coherence tomography, best-corrected VA, and fundus examination. Visual acuity was measured with Snellen charts in 288 patients and with ETDRS charts in 78 patients. A logarithm of the minimum angle of resolution (logMAR) score was recorded alongside each ETDRS measurement. Fluorescein angiography and indocyanine green were used only in unclear cases. Recurrence or persistence of CNV activity was defined as fluid seen by optical coherence tomography or leakage seen on FA, loss of ≥5 letters in ETDRS VA, or new macular intraretinal or subretinal hemorrhage. Recurrences were treated again with a series of 3 consecutive, monthly ranibizumab injections. For lesion type, all FA performed at baseline was graded by 2 independent graders.

Genotyping

The Tagger algorithm[18] was used to select tag SNPs from HapMap to capture all SNPs of minor allele frequency 0.05 with an r^2 of 0.8 in the *VEGFA*, *VEGFB*, *VEGFC*, *VEGFD* (*FIGF*), *PGF*, *VEGFR1* (*FLT1*), *VEGFR2* (*KDR*), *VEGFR3* (*FLT4*),

and *PEDF* (*SERPINF1*) genes. Tag SNPs were genotyped with 4 multiplex iPLEX Gold SNP Genotyping assays (Sequenom Inc., San Diego, CA).

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (version 21; IBM Corp., New York, NY). Visual acuity assessed with Snellen charts was converted to logMAR for statistical analyses. Levene's test for equality of variances was used to test variability of VA changes between Snellen and ETDRS measurements. Improvement in VA was calculated for each patient as the increase of VA between baseline and after 3 months (1 month after the third injection) and after 12 months' follow-up. To identify potential confounders, we computed univariate analyses of variance (ANOVAs) for the dependent variable "change in VA after 12 months" with the factors age ($P > 0.89$), gender ($P > 0.97$), baseline VA ($P > 0.27$), smoking status ($P > 0.86$), lesion type ($P = 0.096$ after Bonferroni correction), and number of injections within 12 months ($P > 0.48$). Only the factor of lesion type showed a trend toward statistical significance and was kept as a potential confounder. For each of the 126 tag SNPs, we computed ANOVAs with the factor "minor allele" (present, absent) and the dependent variable "change in VA after 3 months/12 months." Lesion type (occult, predominantly classic, minimally classic, retinal angiomatous proliferation) was used as a covariate to control for lesion type–related effects on the outcome variable. The threshold for statistical significance was set to $P < 0.05$. The resulting P values were corrected for multiple comparisons using Bonferroni's approach.

As a second step to identify SNPs with an influence on visual outcome, we performed a multivariate stepwise linear regression analysis. Accordingly, we defined change in VA after 12 months as the dependent variable and the 126 analyzed SNPs (coding for the presence [1] or absence [0] of minor alleles of the respective SNP) as the independent predictor variables.

Results

The characteristics of the 366 patients included in the study are summarized in Table 1. All patients were treated for neovascular AMD with ranibizumab on a pro re nata regimen. After 3 initial injections, retreatment followed an optical coherence tomography–guided pro re nata regimen. Visual acuity was measured at baseline, at 3 months (1 month after 3 injections), and at 12 months.

Table 1. Clinical Characteristics of the Study Population (n = 366)

Characteristics	
Sex	164 men (44.8%)
	202 women (55.2%)
Eyes (n = 366)	51% right, 49% left
Age, yrs	
mean ± SD (range)	76.8±7.5 (54–97)
Type of CNV	60% occult
	12% predominantly classic
	21% minimally classic
	7% retinal angiomatous proliferation
No. of intravitreal injections	
Mean ± SD (range)	6.0±3.3 (3–12)
VA (logMAR) at baseline	
Mean ± SD	0.64±0.36
VA after 3 intravitreal injections (logMAR)	
Mean ± SD	0.55±0.41
VA after 1 yr (logMAR)	
Mean ± SD	0.59±0.42

CNV = choroidal neovascularization; logMAR = logarithm of the minimum angle of resolution; SD = standard deviation; VA = visual acuity.

After correcting for multiple testing, univariate ANOVAs revealed a significant effect of SNP rs4576072 in the *VEGFR2* gene on VA after 12 months ($F_{[1,235]} = 14.05$; $P = 0.02$, Bonferroni corrected for multiple comparisons). The analysis of all remaining SNPs did not yield a significant effect on the outcome variable at Bonferroni-corrected thresholds (Table 2, available at www.aaojournal.org).

The stepwise multivariate regression analysis returned a regression model ($R = 0.21$; adjusted $R^2 = 0.04$; $P = 0.010$) with the SNPs rs4576072 (standardized regression coefficient beta = 0.174; $P = 0.012$) and rs6828477 (beta = 0.148; $P = 0.032$) as only contributing predictor variables (Table 2, available at www.aaojournal.org). All remaining SNPs did not significantly improve the model ($P > 0.1$) and were removed. The influence of the contributing rs4576072 and rs6828477 SNP genotypes on VA improvement at 3 and 12 months is shown in Table 3. For each patient, we also calculated the number of minor alleles (0–4) in the 2 SNPs that were identified by multiple regression to influence VA improvement after 12 months. None of the patients had a genotype with 4 minor alleles. The mean increase in VA on the logMAR scale was 0.26 (±0.30) in patients with

3 contributing minor alleles (n = 18), 0.08 (±0.35) in patients with 2 minor alleles (n = 91), and 0.02 (±0.32) in patients with 1 minor allele (n = 178) (Fig 1). Patients with no minor allele contributing had an average decrease of VA of 0.03 (±0.36).

Table 3. Improvement of Visual Acuity on the Logarithm of the Minimum Angle of Resolution Scale Depending on the Genotype of Vascular Endothelial Growth Factor Receptor 2 Single Nucleotide Polymorphisms rs4576072 and rs6828477.

SNP Genotype	No. (%)	Age, yrs (Mean ± SD)	Baseline VA (Mean logMAR ± SD)	VA Improvement after 3m (Mean logMAR ± SD)	VA Improvement after 12m (Mean logMAR ± SD)
rs4576072					
TT	259 (71)	77.1±7.5	0.63±0.37	0.07±0.27	0.00±0.33
CT	96 (26)	76.4±7.9	0.66±0.33	0.12±0.31 (P = 0.019)	0.13±0.35 (P = 0.004)
CC	11 (3)	74.3±5.6	0.65±0.33	0.18±0.27 (P = 0.058)	0.14±0.28 (P = 0.182)
CT or CC	107 (29)	76.2±7.7	0.66±0.33	0.13±0.30 (P = 0.007)	0.13±0.34 (P = 0.002)
rs6828477					
TT	122 (33)	77.3±8.6	0.58±0.33	0.09±0.27	0.00±0.35
CT	193 (53)	76.4±7.5	0.65±0.33	0.07±0.29 (P = 0.779)	0.06±0.33 (P = 0.147)
CC	51 (14)	77.0±8.6	0.75±0.39	0.10±0.30 (P = 0.991)	0.06±0.36 (P = 0.035)
CT or CC	244 (67)	76.5±7.8	0.67±0.36	0.08±0.29 (P = 0.815)	0.06±0.35 (P = 0.137)

logMAR = logarithm of the minimum angle of resolution; SD = standard deviation; SNP = single nucleotide polymorphism.
P values given (Mann–Whitney *U*) when compared with TT genotype.

A more detailed analysis (Table 4) revealed that the presence of at least 1 minor allele of rs4576072 or rs6828477 was associated with an improvement of VA that further increased with the presence of a second minor allele of rs6828477 but not of rs4576072. This was also confirmed when formally testing for differences in VA improvement after 12 months between different combinations of genes. Mann–Whitney *U* tests revealed significant differences (corrected for multiple comparisons). Differences in VA improvements after 3 months did not pass the statistical threshold after correction.

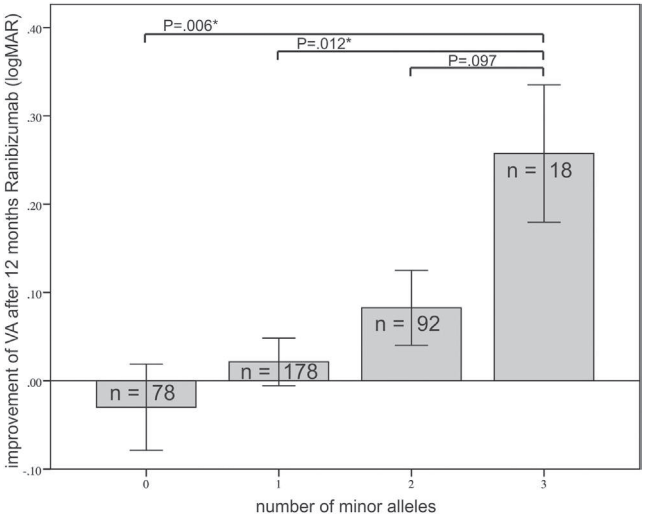

Figure 1. Improvement of visual acuity (VA) on the logarithm of the minimum angle of resolution (logMAR) scale after 12 months in patients receiving ranibizumab for neovascular age-related macular degeneration depending on the number of minor alleles in the single nucleotide polymorphisms rs4576072 and rs6828477 in the *VEGFR2* gene. *$P < 0.05$ obtained by nonparametric testing (Mann–Whitney *U* test).

Table 4. Improvement of Visual Acuity after 3 and 12 Months on the Logarithm of the Minimum Angle of Resolution Scale for All Allele Combinations of the Vascular Endothelial Growth Factor Receptor 2 Single Nucleotide Polymorphisms rs4576072 (Minor Allele T) and rs6828477 (Minor Allele T).

	rs4576072			
rs6828477		TT	CT	CC
TT	0.07±0.26	0.11±0.28	0.26±0.24	
CT	0.06±0.27	0.11±0.33	0.13±0.31	
CC	0.07±0.29	0.22±0.31	./.	
	VA improvement after 3 mos (mean logMAR ± SD)			
	rs4576072			
rs6828477		TT	CT	CC
TT	−0.03±0.36	0.03±0.33	0.13±0.25	
CT	0.02±0.31	0.16±0.35	0.15±0.31	
CC	−0.02±0.34	0.33±0.29	./.	
	VA improvement after 12 mos (mean logMAR ± SD)			

logMAR = logarithm of the minimum angle of resolution scale; SD = standard deviation; VA = visual acuity.

Other individual factors, such as age, sex, smoking, baseline VA, and number of injections, did not show significant effects on VA improvements after 3 or 12 months ($P > 0.1$ for each comparison).

Discussion

In this cohort study, we evaluated the association of SNPs in VEGF and VEGFR genes with the response to ranibizumab treatment in patients with neovascular AMD. We identified 2 SNPs (rs4576072 and rs6828477) in the *VEGFR2* gene that were independently associated with a significantly better visual outcome after 1 year. The difference in VA between patients with minor alleles and those with no minor allele was already apparent after 3 injections but increased to approximately 3 lines after 1 year. These patients gained initially more letters and did not lose vision in the course of treatment within the first year. Visual acuity increased gradually with the number of minor alleles of both SNPs. The differences in VA, which were in the range of 1 to 3 lines, were clinically relevant because a gain of 1 line on the ETDRS chart is perceived by most patients as a subjective improvement.[19]

The 2 SNPs had a significant influence on the variability in treatment response to ranibizumab. Therefore, they represent predictive factors for the therapeutic response to anti-VEGF treatment beyond other individual factors, such as CNV characteristics, diagnostics, adherence to treatment, underdosing, or delay of treatment, which all interfere with the therapeutic outcome.[20, 21] Therefore, this is the first study of AMD to show a correlation between polymorphisms in the *VEGFR2* gene and visual outcome in ranibizumab therapy.

Considered separately, the presence of at least 1 C-allele of SNP rs4576072 led to an improvement of 0.13 logMAR compared with wild-type. This trend toward improved visual outcome was already observed after 3 injections and not influenced by age or VA at baseline. A similar but weaker effect was seen for SNP rs6828477. In line with previous findings, the CNV type showed no association with response to ranibizumab treatment.[7, 11]

Other pharmacogenetic studies of anti-VEGF therapy in AMD have demonstrated that risk alleles in *CFH* and *ARMS2* are associated with vision loss or a higher number of injections,[3, 7, 10, 11, 13, 16] although other studies could not confirm these findings.[9, 12, 15] Although the *VEGFA* gene confers only a minor genetic risk for the development of AMD,[22] genetic variants in *VEGFA* have been demonstrated to significantly influence the outcome of ranibizumab treatment in AMD.[2, 4, 6] The latter association was not confirmed in this study because none of the tag-SNPs across the *VEGFA* gene were significantly associated with change in VA after treatment.

The effect of ranibizumab treatment is mediated primarily through VEGFA and its main receptor VEGFR2. Ranibizumab binds and inactivates VEGFA, which constitutes a key component of neovascularization. In this study, we identified 2 polymorphisms located in different haplotype blocks of the *VEGFR2* gene that are independently associated with the outcome of ranibizumab treatment. Several studies have evaluated SNPs in *VEGFR2* but so far failed to find significant associations with treatment response. This may be due to the limited sample size in those studies or the limited number of SNPs that have been analyzed.[4, 7, 11, 14] In contrast, the present study tested for a broad range of SNPs in a relatively large cohort of subjects (n = 366 eyes), which facilitated the identification of significant associations between genetic factors and treatment response. We did not detect a significant association with genetic variants in other VEGF or VEGFR genes. Although this finding is in agreement with other studies,[7, 14] there is evidence that variants in the *VEGFA* gene affect treatment outcome.[2, 8]

This is the first study to demonstrate a highly significant effect of polymorphisms in VEGFR2 on the therapeutic outcome of ranibizumab treatment by systematically analyzing tag SNPs across the VEGF and VEGFR genes. Further studies are now needed that clarify the pathophysiology underlying these gene-treatment associations with respect to the 2 candidate SNPs and to other genetic variants that are in high linkage disequilibrium with these SNPs. Hints for a putative pathophysiologic mechanism stem from data obtained in patients with metastatic pancreatic adenocarcinoma, in whom response to anti-VEGF therapy was related to VEGFR gene polymorphisms. Here, a synonymous SNP in *VEGFR1* caused increased VEGFR1 expression and increased downstream signaling by a shift in codon use.[23] It has been suggested that increased VEGFR1 concentrations can sequester VEGF, thereby decreasing its proangiogenic effects transduced via VEGFR2 and subsequently limiting the benefits of additional VEGF neutralization by bevacizumab. In line with these findings, we hypothesize that the *VEGFR2* SNPs identified in this study may lead to altered expression or functional activity of VEGFR2, leading to an increased benefit of VEGF neutralization by ranibizumab on visual outcome. Whether such effects are responsible for treatment effects in AMD needs to be elucidated in future studies.

The findings need to be replicated in other cohorts and in a larger sample of subjects to confirm a putative diagnostic predictor for treatment response. In this study, VA at baseline and the initial gain of VA after 3 injections were comparable to other trials.[24] However, we observed some loss of VA after this initial gain after 12 months.

In conclusion, this study is the first to systematically analyze tag SNPs across the VEGF and VEGFR genes. This approach identified 2 SNPs in *VEGFR2* that are independently associated with improved treatment response to ranibizumab in neovascular AMD. In the future, such data may help to identify high-risk patients and to individualize therapy.

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Chapter 3c

A genetic variant in *NRP1* is associated with worse response to ranibizumab treatment in neovascular age-related macular degeneration.

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Purpose: To investigate the role of single nucleotide polymorphisms (SNPs) located in the neuropilin-1 (*NRP1*) gene in treatment response to anti-vascular endothelial growth factor (VEGF) therapy for neovascular age-related macular degeneration (nvAMD).

Methods: Four SNPs in the *NRP1* gene (rs2229935, rs2247383, rs2070296 and rs2804495) were genotyped in a study cohort of 377 nvAMD patients who received the loading dose of three monthly ranibizumab injections. Treatment response was assessed as the change in visual acuity after three monthly loading injections compared to baseline.

Results: SNP rs2070296 was associated with change in visual acuity after three months of treatment. Patients carrying the GA or AA genotypes performed significantly worse than individuals carrying the GG genotype (p=0.01). A cumulative effect of rs2070296 in the *NRP1* gene and rs4576072 located in the VEGF receptor 2 (*VEGFR2* or *KDR*) gene, previously associated with treatment response, was observed. Patients carrying two risk alleles performed significantly worse than patients carrying zero or one risk allele (p=0.03) and patients with more than two risk alleles responded even worse to the therapy ($p=3x10^{-3}$). The combined effect of these two SNPs on the response was also seen after six and twelve months of treatment.

Conclusion: This study suggests that genetic variation in *NRP1*, a key molecule in VEGFA-driven neovascularization, influences treatment response to ranibizumab in nvAMD patients. The results of this study may be used to generate prediction models for treatment response, which in the future may help tailor medical care to individual needs.

Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the Western world.[1] The neovascular, or wet, form of AMD (nvAMD) is the most aggressive, being responsible for around 90% of the vision loss caused by the disease.[2]

The first choice therapy for nvAMD consists of intravitreal injections of anti-vascular endothelial growth factor (VEGF) drugs. Although this treatment has dramatically changed the prognosis of the disease with a significant mean improvement in visual acuity (VA),[3] a high variability in response rates has been described. Approximately ten percent of the treated patients do not respond to anti-VEGF therapy and still lose more than 15 Early Treatment Diabetic Retinopathy Study (ETDRS) letters two years after the start of treatment,[3, 4] which is comparable to the natural course of the disease.[5]

To date, several studies have suggested that genetic variants can influence this variability in treatment response.[6-16] These studies have mainly focused on single nucleotide polymorphisms (SNPs) located in AMD-associated loci, but also common variants in VEGF family members, cytokines and proteins involved in development and maintenance of the retinal vasculature have been explored. Not all studies showed consistent results,[9, 16] however, due to a high variability in study designs it is difficult to reliably compare the outcomes of these studies. Therefore, the relevance and basis of the genetic component of this diverse response to treatment still needs to be elucidated.

Recently, two SNPs in the VEGF receptor 2 (*VEGFR2* or *KDR*) gene, which encodes the main receptor of VEGFA on vascular endothelial cells,[17] have been associated with better anti-VEGF response rates.[12] Consequently, other molecules involved in this pathway are also potential candidates to influence treatment response. Neuropilin-1 (NRP1) is a co-receptor of VEGFA that binds to the predominant isoform, $VEGFA_{165}$,[18] and forms a complex with VEGFR2, which enhances the transduction of downstream signaling.[19-22] Recent studies have implicated NRP1 signaling pathways in pathological neovascularization of the retina[23] and NRP1 has been described to be involved in VEGFA-mediated vascular leakage.[19] Indeed, NRP1 has been shown to affect the evolution of the choroidal neovascularization in AMD[24] and has been proposed as a new target molecule for AMD treatment.[25] Moreover, NRP1 seems to play a role in the cancer prognosis when treated with anti-VEGF compounds,[26] which makes this molecule a compelling candidate for being involved in response variation.

This study aimed to determine whether genetic variants in the *NRP1* gene influence treatment response to anti-VEGF therapy in patients with nvAMD.

Patients and Methods

Study Population

The study cohort comprised 377 eyes of 377 Caucasian treatment-naïve patients aged 50 years or older with active choroidal neovascularization secondary to AMD. A total of 145 patients were treated at the Department of Ophthalmology of the Radboud university medical center, Nijmegen, the Netherlands; 182 at the University of Cologne, Germany and the remaining 50 patients at the McGill University Health Center, Montreal, Canada. The patients from the German and Dutch clinics were enrolled between 2008 and 2010 in the European Genetic Database (EUGENDA), a multicenter database for the clinical and molecular analysis of AMD.

The study was performed in accordance with the tenets of the Declaration of Helsinki (7th revision). Approval of the local ethics committees was obtained for all three centers and written informed consent was acquired from all participants.

The diagnosis of active nvAMD was determined by retinal specialists based on ophthalmic examination, spectral-domain optical coherence tomography (OCT) (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) or fluorescein angiography (FA) (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany; or Imagenet, Topcon Corporation, Tokyo, Japan). Exclusion criteria included any previous ophthalmic surgery, except for cataract removal, and retinal disorders other than AMD. If both eyes received treatment, the first eye to receive treatment was chosen as the study eye. If treatment started simultaneously, the study eye was chosen randomly. All patients were treated between 2007 and 2009 with three consecutive monthly intravitreal injections of 0.5 mg ranibizumab (Lucentis; Novartis Pharmaceuticals UK Limited, Frimley, Surrey, UK). Visual acuity was assessed in all cases before treatment (baseline) and after the three loading monthly injections. After the loading dose, patients were followed on a monthly basis and treated on a pro re nata regimen at the clinics of Nijmegen and Cologne. At the clinic of Montreal, the patients were further managed through a treat-and-extend regimen. OCT, best-corrected VA, fundus examination and FA were used alone or in combination to evaluate the effectiveness of the treatment. Recurrence or persistence of the choroidal neovascularization was defined as fluid seen by OCT, loss of VA of 5 ETDRS letters or more, leakage seen on FA, or new macular hemorrhage or fluid. In case of persistence or recurrence of the choroidal neovascularization, patients received three consecutive monthly ranibizumab injections. If available, VA was collected after 6 and 12 months of treatment. For 304 patients, Snellen VA measurements were collected retrospectively and 73 patients were followed up prospectively using ETDRS VA. Treatment response was defined as the change in visual acuity after the three first months of treatment compared to baseline. Long-term treatment response was defined as the change in visual acuity after six and twelve months

of treatment. Age at first ranibizumab injection, gender and other baseline variables were collected using questionnaires or retrieved from the patient files.

Genotyping

The SNPs rs2229935, rs2247383, rs2070296 and rs2804495 were selected from the major haploblocks of the *NRP1* gene for genotyping (see Supplemental table 1, which details the chromosomal location of the SNPs). Two SNPs, rs2070296 (p.Ala179=) and rs2229935 (p.Tyr422=), were located in the coding region of *NRP1*.

Genotyping of the SNPs was performed using competitive allele-specific KASP genotyping chemistry (LGC, Hoddesdon, UK). Primers and probes were developed by LGC, (see Supplemental table 1, which describes the probes used). Quality control of the genotyping assays was assessed using duplicate DNA samples in each run, achieving a concordance of 100% of the results.

Sanger sequencing of exon 4 of the *NRP1* gene (*NM_003873.5*) was performed in eleven patients for which genotyping by KASPar of SNP rs2070296 was not successful. Primers were designed using Primer3 software (see Supplemental table 1, which describes the primers used).[27] Polymerase chain reaction was performed, and the amplicons were sequenced using an automated sequencer (BigDye Terminator, version 3, 3730 DNA analyzer; Applied Biosystems). Sequences were assembled and analyzed using ContigExpress (Vector NTI Advance, Version 11.0, Life Technologies).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp). ETDRS and Snellen visual acuity records were converted to the logarithm of Minimal Angle of Resolution (logMAR) for the purpose of statistical analysis. Change in VA after three, six and twelve months was calculated as the difference between VA at baseline and VA at the different time points.

Deviation of the genotype frequencies from those expected under Hardy-Weinberg equilibrium was assessed by a χ^2 test. To determine the influence of the baseline variables on the change in VA after three months Spearman correlation was used for the continuous variables, and Kruskal-Wallis or Mann-Whitney U tests were performed for the categorical variables.

The association of the different SNPs with the change in VA after three, six and twelve months was assessed using Mann-Whitney U tests. Bonferroni procedure was applied to correct for four tests (p-values ≤ 0.01 were considered statistically significant).

In order to analyze the combined effect of *NRP1* rs2070296 and *KDR* rs4576072 on the change in VA after three, six and twelve months, patients were combined into three groups of approximately equal size (carriers of less than two, two risk alleles, or more than two risk alleles), and a Mann-Whitney U test was performed. Only the patients that were successfully genotyped for rs4576072 in a previous study[12] were included in

the analysis (n=353). The rs4576072 major allele (T) has been reported to lead to a worse response to therapy,[12] therefore, this allele was considered the risk allele.

Results

Demographics and ophthalmological details of the patients are described in Table 1. Older age at first injection (p-value=0.01), having a better baseline VA (p-value<10^{-3}) and having diabetes mellitus (p-value=0.02) were associated with worse response after three months of treatment (see Supplemental table 2, which describes the results of the association tests). The type of choroidal neovascularization showed a trend towards statistical significance (p-value=0.06). These baseline variables were not associated with the SNPs of interest (p-value>0.05, lowest p-value=0.22) (see Supplemental table 3, which describes the results of the association tests).

Table 1. Characteristics of the study cohort.

Demographics		
Age at first injection (years), mean (SD)		77.11 (7.46)
Female gender, n (%)		215 (57.0)
Disease history		
Hypertension (n=259)[a], n (%)[b]		154 (59.5)
Diabetes mellitus (n=259)[a], n (%)[b]		47 (18.1)
Other environmental factors		
BMI (kg/m^2) (n=258)[a], median (quartiles)		25.39 (23.52 - 28.49)
Ophthalmological details		
Baseline VA (logMAR), median (quartiles)		0.543 (0.398 - 1.000)
Equivalent baseline VA (ETDRS letters)[c], median (quartiles)		57.9 (35.0 - 65.1)
Change in VA after 3 months (logMAR)[d], median (quartiles)		0.097 (0.000 - 0.259)
Equivalent change in VA after 3 months (ETDRS letters)[c,d], median (quartiles)		4.8 (0.0 - 12.2)
Change in VA after 6 months (logMAR)[d], median (quartiles) (n=262)		0.090 (-0.097 - 0.223)
Equivalent change in VA after 6 months (ETDRS letters)[c,d], median (quartiles)		4.5 (-4.9 - 11.2)
Change in VA after 12 months (logMAR)[d], median (quartiles) (n=240)		0.040 (-0.192 - 0.204)
Equivalent change in VA after 12 months (ETDRS letters)[c,d], median (quartiles)		2 (-9.6 - 10.2)
Type of CNV (n=335)[a]		
	Occult with no classic, n (%)[b]	199 (59.4)
	RAP, n (%)[b]	21 (6.3)
	Minimally classic, n (%)[b]	42 (12.5)
	Predominantly classic, n (%)[b]	73 (21.8)
Lesion size (DA) (n=285)[a]		
	<2, n (%)[b]	92 (32.3)
	2-4, n (%)[b]	91 (31.9)
	4-6, n (%)[b]	43 (15.1)
	>6, n (%)[b]	59 (20.7)

SD, standard deviation; n, number of patients; VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution; ETDRS, Early Treatment Diabetic Retinopathy Study; CNV, choroidal neovascularization; RAP, retinal angiomatous proliferation; DA, disk areas.

[a] For the remaining patients no data were available.

[b] Valid percentage.

[c] ETDRS letters equivalents were calculated in the following manner: ETDRS letters = 85 – logMAR/0.02 for logMAR values.

[d] Change in VA after 3, 6 and 12 months was calculated in the following manner: VA prior to treatment - VA after 3, 6 or 12 months of treatment.

Over 90% of patients were successfully genotyped for SNPs rs2229935, rs2247383, rs2070296 and rs2804495 (Table 2). None of the SNPs showed deviations from Hardy-Weinberg equilibrium in the study cohort (p-value=0.81, 0.93, 0.98 and 0.41 respectively). The GA or AA genotypes of SNP rs2070296 were found to be associated with a significantly reduced improvement in VA after three months (p-value=0.01) compared to the GG genotype, showing a linear trend for the three genotype groups (Figure 1A). The SNPs rs2229935, rs2248383 and rs2804495 were not found to be associated with treatment response (Table 2).

Table 2. Association of genotypes in *NRP1* with response to ranibizumab treatment.

SNP	n (%)	ΔVA after 3 months (logMAR), median (quartiles)[a]	*P-value*[b]
rs2229935			
CC	203 (57.2)	0.100 (0.000 - 0.301)	Reference
CT	132 (37.2)	0.079 (0.000 - 0.198)	0.12
TT	20 (5.6)	0.085 (-0.075 - 0.273)	0.50
CT or TT	152 (42.8)	0.079 (0.000 - 0.198)	0.11
rs2247383			
CC	123 (35.2)	0.097 (-0.077 - 0.273)	Reference
CT	169 (48.4)	0.097 (0.000 - 0.242)	0.94
TT	57 (16.3)	0.090 (-0.064 - 0.238)	0.69
CT or TT	226 (64.8)	0.097 (0.000 - 0.242)	0.84
rs2070296			
GG	270 (71.6)	0.100 (0.000 - 0.287)	Reference
GA	98 (26.0)	0.079 (-0.097 - 0.195)	0.04
AA	9 (2.4)	0.000 (-0.097 - 0.040)	0.04
GA or AA	107 (28.4)	0.040 (-0.097 - 0.184)	0.01
rs2804495			
TT	167 (49.1)	0.098 (0.000 - 0.240)	Reference
TG	147 (43.2)	0.097 (0.000 - 0.273)	0.74
GG	26 (7.6)	0.138 (-0.088 - 0.300)	0.84
TG or GG	173 (50.9)	0.097 (0.000 - 0.279)	0.72

SNP, single nucleotide polymorphism; N, number; VA, visual acuity; logMAR = logarithm of the Minimum Angle of Resolution.
[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment. [b] P-values were calculated using Mann-Whitney U tests.

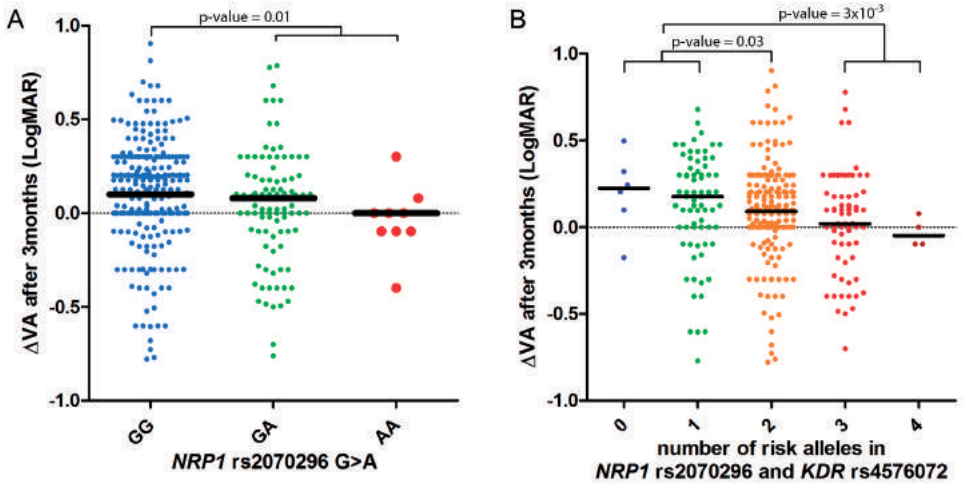

Figure 1. Effect of genetic variants in *NRP1* and *KDR* on response to ranibizumab treatment in nvAMD. (A) Change in visual acuity after three months of ranibizumab treatment stratified by *NRP1* rs2070296 genotype. (B) Change in visual acuity after three months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T). The median change in visual acuity for each group is depicted in both figures.

A combined analysis of *NRP1* rs2070296 and the previously associated SNP rs4576072 in *KDR*[12] revealed a decrease in the change in VA after 3 months depending on the number of risk alleles (Figure 1B). Patients who carried two risk alleles responded significantly worse to therapy than carriers of one or zero alleles (median of 0.090 logMAR or 4.5 ETDRS letters gained versus 0.196 logMAR or 10 ETDRS letters gained, p-value=0.03), and carriers of more than 2 alleles had even worse response rates (median of 0.020 logMAR or 1 ETDRS letter gained, p-value=$3x10^{-3}$) (Figure 1B, Table 3).

Besides the variability in treatment regimens after the first loading injections, we evaluated if the effect of rs2072096 in *NRP1* remained significant after six and twelve months of treatment. This SNP was not associated with the change in VA after 6 and 12 months (Table 4). However, the combined effect of this SNP in *NRP1* and rs4576072 in the *KDR* gene did influence long term response (Figure 2, Table 5).

Table 3. Combined effect of the risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576072 (T) on response to ranibizumab treatment.

Number of risk alleles	N (%)	ΔVA after 3 months (logMAR), median (quartiles)[a]	*P-value*[b]
<2	79 (22.4)	0.196 (0.000 – 0.321)	Reference
2	201 (56.9)	0.090 (0.000 – 0.204)	0.03
>2	73 (20.7)	0.020 (-0.097 – 0.180)	$3x10^{-3}$

N, number of patients; VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution.
[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment. [b] P-values were calculated using Mann-Whitney U tests.

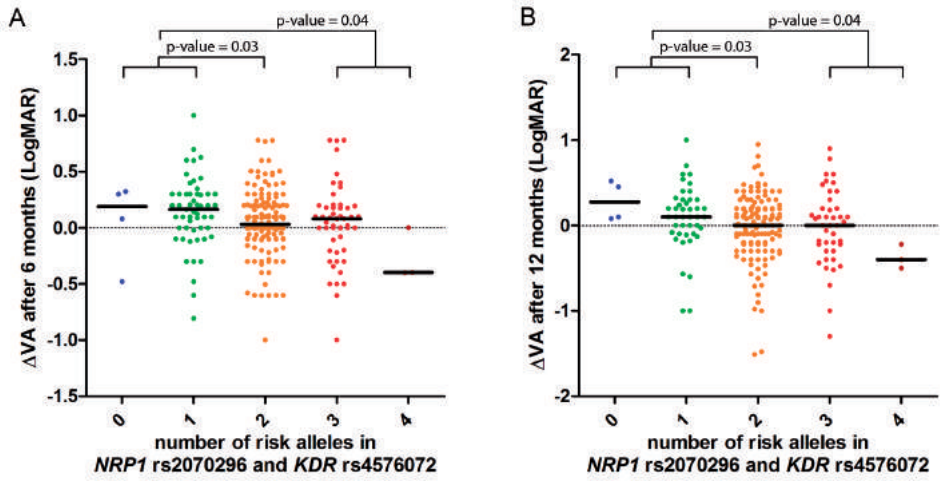

Figure 2. Effect of genetic variants in *NRP1* and *KDR* on long term response to ranibizumab treatment in nvAMD.
(A) Change in visual acuity after six months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T). (B) Change in visual acuity after twelve months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T). The median change in visual acuity for each group is depicted in both figures.

Discussion

We evaluated the role of four SNPs located in *NRP1* (rs2229935, rs2247383, rs2070296 and rs2804495) in response to anti-VEGF treatment. The SNP rs2070296 was found to be significantly associated with a fewer gain in letters. Depending on the genotype, patients showed a different response following an additive model in which the minor allele (A) leads to worse response to treatment. In median, the nine patients that carried the homozygous AA genotype didn't improve their VA and performed five ETDRS letters (one line) worse than the homozygous GG group. Since a recent study showed that most patients perceive one line of the ETDRS chart as an improvement,[28] this difference could be clinically relevant. This effect was not seen after six and twelve months of treatment. Nevertheless, the dilution of the effect seen in the change of VA after the loading dose of three ranibizumab injections, could be due to variability in the treatment regime and progression of the disease, which makes the comparison of the long term response difficult.

We defined treatment response as change in visual acuity after three consecutive loading injections compared to baseline. Visual acuity is an important functional outcome measure, which is most relevant for patients, and therefore, it has been extensively used to evaluate treatment response in nvAMD.[7, 12, 29-36] Most patients achieve the largest change in VA after the three first monthly injections[3] and this time interval can be predictive of long term response.[37] Therefore, this finding not only expands the knowledge of the mechanisms that underlie the variability in the response, but also could be implemented in future prediction models. Despite that, we encourage the evaluation of the effect of this SNP using also anatomic features defined by OCT. The patients from our study cohort were treated between 2007 and 2009, and at that time, OCT scans were not implemented routinely during treatment regimes.

Although our study detected a significant association of rs2070296 with anti-VEGF treatment response, further studies are required to confirm our findings and to determine whether this SNP or other genetic variants in *NRP1* are driving the effect. A more extensive analysis of additional genetic variants in *NRP1* could reveal other SNPs associated with variability in the response. Furthermore, examination of low frequency and rare variants could reveal variants with a higher impact on the trait and major clinical relevance.

The *NRP1* gene has also been implicated in treatment response to anti-VEGF therapy in cancer. A SNP in the 3'UTR of *NRP1* has been associated with better progression-free survival in recurrent ovarian cancer treated with bevacizumab (Avastin; Genentech Inc., San Francisco, CA, USA),[38] an anti-VEGF drug also used off-label for the treatment of nvAMD. NRP1 is expressed in endothelial cells and up-regulated in numerous tumor cell types,[39-48] which has been associated with poorer outcomes in several cancers such as breast cancer,[42] osteosarcoma[46] and nasopharyngeal carcinoma.[48] Therefore, the interest

in developing new therapies targeting NRP1 in cancer is increasing.[49] Moreover, an improved effect of an anti-VEGF drug combined with anti-NRP1 antibodies has been described in tumor treatment.[50] In addition, NRP1 has been proposed as a potential biomarker for treatment response in advanced gastric cancer treated with bevacizumab.[26] In a recent study, Raimondi *et al.* described that NRP1 promotes angiogenesis in a VEGFR2/VEGFA independent manner. In this novel mechanism, NRP1 forms a complex with ABL1 that leads to the activation of paxillin in a fibronectin dependent manner which enhanced motility *in vitro* and angiogenesis *in vivo*. Moreover, in a mouse model of oxygen-induced retinopathy, treatment with Imatinib (an ABL1 inhibitor used for the treatment of leukemia) reduced angiogenesis. Consequently, Imatinab was proposed as a new therapy for nvAMD targeting NRP1.[25]

The wide range of response to anti-VEGF therapy observed in nvAMD patients has drawn much attention in the pharmacogenetic research field. The findings described in this study, together with the findings of Hermann *et al.*[12] and Lotery *et al.*,[9] suggest that variants in components of the neovascularization pathways play an important role in treatment response to anti-VEGF therapy in AMD. The study by Hermann *et al.* showed that rs4576072 in *KDR* is associated with response after twelve months of treatment.[12] In the current study we demonstrated a significant cumulative effect of this SNP and SNP rs2070296 in *NRP1* in the response to ranibizumab treatment after the three loading injections, and also after six and twelve months of treatment. This finding is specifically interesting for the development of prediction models based on relevant clinical parameters, environmental and genetic factors, which would allow patients to be grouped for different regimen doses or therapies.

In summary, our findings suggest that genotyping of SNPs in *NRP1*, in combination with SNPs in other genes as *KDR*, could be used as a rapid preclinical tool for selection of the optimal treatment for individual patients, which besides anti-VEGF treatment could also involve targeting of NRP1. In the future, genetic testing of such variants may help to predict outcome of nvAMD treatment, and to tailor medical care to individual needs.

Supplemental tables

Supplemental table 1. Chromosomal location of the *NRP1* SNPs and primers used for genotyping.

SNP	Chromosomal location (chr, bp)[a]	Primers KASPar sequencing	Primers Sanger sequencing
rs2229935	10, 33510663	Primer Allele FAM (C): AGCTTACCTGTTATCTTGCAACCG Primer Allele HEX (T): CAGCTTACCTGTTATCTTGCAACCA Primer Common: GGGAAACTGGCATATCTATGAGATTTGAA	NA
rs2247383	10, 33489052	Primer Allele FAM (C): CAGAATTGGAGGGAGGCCAGG Primer Allele HEX (T): CAGAATTGGAGGGAGGCCAGA Primer Common: CCACTGGGAACAGAACGCTAATGTA	NA
rs2070296	10, 33552695	Primer Allele FAM (A): CCAGGATAATCTCTGACATCTTTGGT Primer Allele HEX (G): CAGGATAATCTCTGACATCTTTGGC Primer Common: ATCCCAACAGCCTTGAATGCACTTATATT	Fw: CTGAACTTGACTTTCCATACCC Rv: TGCTTTGTTTTCCAGTGTCC
rs2804495	10, 33612500	Primer Allele FAM (G): ACTACTAACTGCCCTAGATACCAG Primer Allele HEX (T): CACTACTAACTGCCCTAGATACCAT Primer Common: CTCTTCTCTGGTTGATTGGCCTGTA	NA

SNP, single nucleotide polymorphism; chr, chromosome; bp, base pair; NA = not applicable; Fw, forward; Rv, reverse. [a] The chromosomal location is based on the assembly of February 2009 (GRCh37/hg19).[51]

Supplemental table 2. Influence of the baseline variables on response to ranibizumab treatment.

	ΔVA after 3 months (logMAR)[a]	
	P-value[b]	***Correlation coefficient/ median (quartiles)***
Demographics		
Age at first injection	0.01	-0.130
Gender	0.38	Female: 0.079 (0.000 - 0.222) Male: 0.100 (-0.020 - 0.300)
Disease history		
Hypertension (n=259)[c]	0.91	Yes: 0.104 (0.000 - 0.250) No: 0.079 (0.000 - 0.301)
Diabetes mellitus (n=259)[c]	0.02	Yes: 0.000 (-0.204 - 0.176) No: 0.107 (0.000 - 0.301)
Other environmental factors		
BMI (kg/m^2) (n=258)[c]	0.22	-0.077
Ophthalmological details		
Baseline VA (logMAR)	$<10^{-3}$	0.195
Change in VA after 3 months (logMAR)	NA	NA
Type of CNV (n=335)[c]	0.06	Occult with no classic: 0.097 (0.000 - 0.204) RAP: 0.107 (-0.048 - 0.301) Minimally classic: 0.000 (-0.099 - 0.176) Predominantly classic: 0.100 (0.045 – 0.311)
Lesion size (DA) (n=285)[c]	0.23	<2: 0.100 (-0.015 – 0.296) 2-4: 0.090 (0.000 – 0.222) 4-6: 0.100 (0.000 – 0.300) >6: 0.000 (-0.097 – 0.176)

VA, visual acuity; logMAR = logarithm of the Minimum Angle of Resolution; NA, not applicable; n, number of patients; CNV, choroidal neovascularization; RAP, retinal angiomatous proliferation; DA, disk areas.

[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment.

[b] P-values were calculated using Spearman correlations for the independent continuous variables and Kruskal-Wallis tests or Mann-Whitney U tests for the categorical variables.

[c] For the remaining patients no data were available.

Supplemental table 3. Influence of *NRP1* SNPs on potential confounding factors.

	Potential confounding factors of ΔVA after 3 months (logMAR)			
	Age at first injection (years)	**Baseline VA (logMAR)**	**Diabetes mellitus**	**Type of CNV**
	***P-value*[a]**	***P-value*[a]**	***P-value*[a]**	***P-value*[a]**
rs2229935	0.23	0.23	0.38	0.22
rs2247383	0.89	0.30	0.37	0.93
rs2070296	0.50	0.34	0.30	0.30
rs2804495	0.85	0.47	0.79	0.39

VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution; CNV, choroidal neovascularization.

[a] P-values were calculated using Kruskal-Wallis tests for continuous outcome variables and χ^2 test for categorical outcome variables.

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Switching treatments after non-response to anti-VEGF therapy

Chapter 4a

Switching to aflibercept in patients with neovascular age-related macular degeneration not responding to bevacizumab: a pilot study.

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Anti-vascular endothelial growth factor (VEGF) therapy has become the mainstay of neovascular age-related macular degeneration (nAMD) treatment and has substantially improved visual prognosis. The first-line anti-VEGF agent used in the Netherlands is bevacizumab because of its superior cost-effectiveness compared to ranibizumab and aflibercept.[1] Even though bevacizumab is generally effective, approximately 10% of patients are non-responders. The effectiveness and working mechanism of bevacizumab is comparable to ranibizumab, both being VEGF-A antibodies.[2] The therapeutic mechanism of aflibercept is slightly different, which functions as a decoy receptor for VEGF-A, VEGF-B and placental growth factor. Therefore, aflibercept seems like a more promising alternative than ranibizumab in case of non-response to bevacizumab. Although aflibercept is an effective treatment for nAMD,[3] its role as a secondary treatment option requires further investigation. Here we report the treatment response to aflibercept in nAMD-patients who did not respond to bevacizumab treatment.

This prospective, single-arm, open-label, clinical trial was approved by the local ethical committee (NL44122.091.13) and was registered at the Dutch trial register (NTR4188). We included 10 eyes of 9 patients (table) that were non-responder to bevacizumab. Inclusion criteria were: inadequate response to bevacizumab treatment defined as a persistent central retinal thickness (CRT) of ≥300 μm on optical coherence tomography (OCT); having received at least 3 bevacizumab injections within 1 year before inclusion in this study; active nAMD as seen on fluorescein angiography and OCT; maximally 2 years since start of bevacizumab treatment; 1- 3 months since the last bevacizumab injection; and best-corrected visual acuity (BCVA) at baseline between 20/25 and 20/320. Patients were excluded if they had: signs of subretinal fibrosis, scarring or geographic atrophy involving the center of the macula; pigment epithelial detachment with a height of ≥150 μm; or any other retinal diseases.

Aflibercept was administered as 3 consecutive monthly injections at a dose of 2 mg (0.05 ml). At every visit we measured CRT on OCT (Spectralis HRA+OCT, Heidelberg Engineering) and ETDRS BCVA. One month after the last injection response was evaluated. All statistical comparisons were made in SPSS using a paired t-test.

CRT decreased significantly after switching to aflibercept with a mean of 102 ± 96 μm after 3 months (p=0.008) (figure). The largest decrease was seen after 1 month (90 ± 83 μm, p=0.007). BCVA increased correspondingly with 6.7 ± 11.4 ETDRS letters, this was however not significant (p=0.096). The change in CRT was substantial in 7 eyes, with a decrease of >50 μm. In 7 out of 10 eyes there was a functionally relevant improvement of >5 ETDRS letters.

Table. Baseline characteristics

n=10 eyes of 9 patients	
Male, n (%)	4 (44%)
Age, median (range)	75 (55-87)
Eye included, n (%)	
Right eye	4 (40%)
Left eye	6 (60%)
Nr of bevacizumab injections, median (range)	6 (3-11)
CRT at baseline in µm, mean (SD)	540 (110)
BCVA at baseline in letters, mean (SD)	57.6 (18.7)

CRT=central retinal thickness; BCVA=best corrected visual acuity

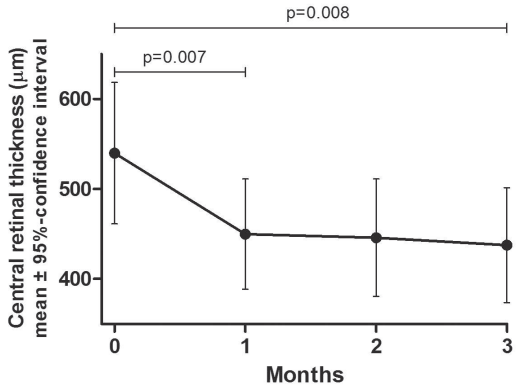

Figure. Course of central retinal thickness after switching to aflibercept

In this study there was a clear anatomical benefit of switching to aflibercept. Most eyes also improved functionally, however not significantly, likely due to a lack of power. The 3 month BCVA changes we found were good compared to other prospective studies on switching to aflibercept after non-response to previous anti-VEGF treatment, where it ranged between 0 and +7 letters after 6 months (3 month data not available for most studies).[4] In these previous studies anatomical response varied between -127 and -15 μm, however, most find CRT changes of <50 μm. An explanation for our good results could be that patients in our study were switched relatively early, sometimes as soon as after 3 months of bevacizumab treatment, leaving more room for improvement. In conclusion, this study provides additional evidence that switching to aflibercept may be beneficial after non-response to bevacizumab, resulting is anatomical as well as functional improvement.

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Chapter 4b

Bevacizumab for diabetic macular edema and management of non-responders in daily clinical practice.

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Manuscript in preparation

Purpose: Treatment of diabetic macular edema (DME) has moved from laser towards anti-VEGF therapy. However, it is unclear how frequent non-response to anti-VEGFs is and what is the best management for these non-responders. We assessed the effectiveness of primary bevacizumab treatment for DME in daily clinical practice and of alternative treatments in non-responders.

Methods: We reviewed the medical charts of DME-patients treated with bevacizumab and recorded whether patients switched treatment during the first year. The course of visual acuity (VA) and central retinal thickness (CRT) was determined. In non-responders, VA and CRT were assessed prior to and following alternative treatment.

Results: We included 94 eyes of 69 patients. Mean VA improved from 20/76 to 20/62 ($p=0.124$) and CRT decreased by 36 μm ($p<0.001$) over 1 year. Nineteen eyes (20%) switched treatment due to non-response. Six eyes switched to ranibizumab. Two responded with ≥50 μm decrease in CRT. Eight eyes switched to intravitreal triamcinolone. Seven responded well, decreasing ≥50 μm in CRT.

Conclusions: The effectiveness of bevacizumab in DME-patients in daily clinical practice is moderate. About 20% of patients require alternative treatment due to non-response. Compared to ranibizumab, switching to triamcinolone was the preferred alternative treatment with clear improvement in most patients.

Introduction

Diabetic retinopathy is a common complication of diabetes mellitus and is the leading cause of visual impairment in the working-age population.[1] Approximately 64% of vision-threatening diabetic retinopathy is due to diabetic macular edema (DME), making it the primary cause of vision loss in diabetics.[2] The global prevalence of DME is ~7% in all diabetic patients and the cumulative incidence can reach up to 29% in long-term type I diabetic patients.[2, 3] The number of people affected with diabetes mellitus is rising each year making DME an increasingly important health concern.[4]

DME results from a disruption of the blood-retina barrier at the level of the retinal capillaries resulting in excessive vascular permeability and leakage of fluid into the retina. Retinal hypoxia leading to increased vascular endothelial growth factor (VEGF) levels and inflammation may both contribute to DME.[5] The role of VEGF in the etiology of DME led to a number of clinical trials evaluating anti-VEGFs in the treatment of DME. From 2007 to 2014 several randomized controlled trials (RCTs) have demonstrated that anti-VEGF therapy is more effective in improving visual acuity (VA) and reducing retinal thickness than laser surgery alone.[6-13] Moreover, there appears to be no added benefit of laser surgery in combination with anti-VEGF therapy [ref BOLT, DRCRN(Protocol I), Pan-American, Solaiman, Restore].[8, 10, 11, 14, 15] Consequently, anti-VEGF therapy has replaced laser surgery as the primary treatment for center-involved DME.

Three anti-VEGF agents are currently available for treatment of DME: ranibizumab, bevacizumab and aflibercept. The protocol T study, a large RCT from the Diabetic Retinopathy Clinical Research Network (DRCRN), compared the effectiveness of these three agents and concluded that for patients with baseline VA of over 20/40, the three agents were equally effective in improving vision. For patients with visual acuity below 20/50 aflibercept resulted in better visual improvement, yielding 5-6 ETDRS letters more than ranibizumab or bevacizumab.[16] Regarding anatomical effects, both aflibercept and ranibizumab were significantly better at reducing central retinal thickness (CRT) compared to bevacizumab and more often resulted in CRT of <250 μm. Before the protocol T study had shown the superiority of aflibercept in DME-patients with visual acuity below 20/50, bevacizumab was the first choice treatment for all DME patients in the Netherlands due to its favorable cost-effectiveness profile.[17]

Although all three anti-VEGF agents have improved visual prognosis of the average DME-patient, there are still patients with suboptimal response or non-response. In the protocol T study 1/3 of aflibercept and ranibizumab treated eyes and almost 2/3 of bevacizumab treated eyes had not achieved a CRT of <250 μm after 1 year, indicating persistent edema.[16] The protocol I study that evaluated the effectiveness of ranibizumab showed that of the patients with persistent edema after 6 months, 40% still have persistent DME after 3 years.[18] So it seems there is a substantial number of patients not responding, or only partially responding, to anti-VEGF therapy.

Non-response is an everyday problem in clinical practice and many ophthalmologists are faced with the dilemma of what to do in case DME persists despite continuous anti-VEGF treatment. At the moment, there are no clear guidelines for defining non-response. This means it is unclear to many ophthalmologists which patients are the non-responders requiring an alternative treatment strategy and it is also unclear how frequent non-response is in clinical practice. Several studies have tried to address the issue of non-response, but even within the same study the frequency of non-response may vary greatly depending on de definition used.

Although there is no consensus as to what should be considered non-response, there are some alternative treatments to consider when non-response is suspected. Mainly, the choice is between switching to a different anti-VEGF agent or switching to steroids. The prospective REEF study showed that switching from bevacizumab to ranibizumab in refractory DME patients resulted in anatomical improvement of >10% CRT reduction in 75% of patients after 3 months.[19] Retrospective studies have shown similar benefit of switching to ranibizumab in refractory DME cases.[20, 21] Intravitreal steroids, such as triamcinolone acetonide (TAC) or long-acting dexamethasone implants may pose another alternative treatment.[22, 23] Steroids are able to improve visual acuity and CRT compared to sham treatment, but the effect is mostly seen in pseudophakic patients.[10] In phakic patients anti-VEGF results in superior visual acuity due to cataract formation in the steroid treated group. Phakic patients treated with TAC require cataract surgery in ~60% of cases. Also, 30-40% of TAC treated patients experience a rise in intraocular pressure of ≥10 mmHg within 2 years.[10, 24] Because of these common side-effects, intraocular steroids are usually not a first choice treatment, but may be considered in non-responders. Switching from bevacizumab to steroids in non-responders has been evaluated in several small sized studies.[25-27] These studies have shown good short-term anatomical response, however, the effect on visual acuity in all studies was less outspoken and changes in vision could not be sustained over the study periods.

So far, no clear guidelines exist for secondary treatment and it is unclear whether non-responders should be switched to a different anti-VEGF or to a steroid. Here we assess the response to primary bevacizumab treatment in DME-patients in daily clinical practice, report the frequency of treatment switching and describe the effect of switching non-responders to the alternative treatments ranibizumab or TAC.

Methods

Population

We reviewed the charts of patients that were treated with bevacizumab for DME at the department of ophthalmology of the Radboud university medical center in Nijmegen between October 2009 and March 2014. Inclusion criteria were: central diabetic macular

edema, no previous intravitreal treatment for DME in the study eye, a completed loading dose of three consecutive bevacizumab injections 4-6 weeks apart and optical coherence tomography (OCT) imaging available at baseline and at least one follow-up moment.

Treatment

All patients were treated with intravitreal injections (IVI) of 1.25 mg in 0.05 ml bevacizumab. Patients received a loading phase of 3 consecutive injections 4-6 weeks apart, followed by a monitoring phase where injections could be administered on a pro re nata (PRN) basis. The decision for re-injection with bevacizumab was based on persistent fluid or retinal thickening on OCT. Patients switched treatments at the treating physicians discretion after non-response to bevacizumab, which entailed an increase or insufficient decrease in CRT despite treatment. Change in VA was not considered in determining non-response. Focal/grid photocoagulation for microaneurysms or panretinal photocoagulation for proliferative diabetic retinopathy could be performed at the treating ophthalmologist's discretion. The studied alternative treatments were ranibizumab and TAC. The choice for either drug was not based on any predefined criteria but according to the preference of the ophthalmologist. Ranibizumab was administered as 3 consecutive monthly IVIs of 0.5 mg in 0.05ml and TAC as a single dose of 2mg in 0.05.

Clinical data collection

We extracted patient characteristics such as type and duration of DM, insulin use and duration of DME from the medical history. CRT was measured on OCT (Spectralis HRA+OCT, Heidelberg Engineering) using automatic volume measurements with manual correction of retinal boundary segmentation errors. CRT and best corrected Snellen VA were assessed at baseline, 3, 6 and 12 months. At every time point we recorded whether a patient had switched treatments and the nature of the alternative therapy. For patients that switched treatments, VA and CRT were also collected prior to and after the alternative treatment.

Statistical analysis

Snellen VA was converted to the logarithm of the maximum angle of resolution (logMAR) to enable statistical analysis. Descriptive statistics were used with eyes as independent units. All other analyses were performed using linear mixed models to account for the dependency between eyes of the same patient.[28, 29] The primary outcome was change in CRT over 1 year of treatment. The difference in course of CRT and VA between non-responders and the remaining patients was assessed by testing for interaction between switching treatment and time. Finally, we evaluated CRT and VA before and after switching to a different treatment modality. All analyses were performed in SPSS version 22 and a p-value of <0.05 was considered statistically significant.

Results

Overall response to bevacizumab

We included 94 eyes of 69 patients. The baseline characteristics are presented in table 1. The complete cohort showed a mean decrease in CRT of 36 ± 127 μm over 1 year (p<0.001). VA improved from 0.58 ± 0.38 logMAR (Snellen 20/76) to 0.49 ± 0.40 logMAR (Snellen 20/62), albeit not significantly over time (p=0.124). Response to treatment of the complete cohort is summarized in table 2. The median number of injections was 6 (interquartile range 3-9).

Table 1. Patient baseline characteristics

Variable	
94 eyes of 69 patients	
Male, n (%)	37 (54%)
Age at first injection in years, mean (SD)	63 (13)
Eye included, n (%)	
OD	28 (41%)
OS	16 (23%)
ODS	25 (36%)
Type of diabetes mellitus, n (%)	
DM I	8 (12%)
DM II	59 (86%)
Unknown	2 (3%)
Duration DM in years, median (IQR)*	12 (5-21)
Duration of DME in months, median (IQR)	8 (2-21)
Insulin user, n (%)	42 (61%)

*duration DM missing for n=7 eyes; SD= standard deviation; OS= oculus sinister; OD= oculus dexter; DM= diabetes mellitus; IQR= inter quartile range; DME= diabetic macular edema

Table 2. Treatment outcomes

	Baseline	3 months	6 months	1 year	*p-value*
	n=94 eyes	*n=83 eyes*	*n=74 eyes*	*n=66 eyes*	
CRT in µm, mean (SD)	474 (124)	426 (139)	437 (136)	441 (154)	<0.001
Change from baseline in µm, mean (SD)		-49 (85)	-42 (89)	-36 (127)	
	n=94 eyes	*n=89 eyes*	*n=81 eyes*	*n=74 eyes*	
logMAR, mean (SD)	0.58 (0.38)	0.51 (0.36)	0.51 (0.36)	0.49 (0.40)	0.124
Snellen equivalent	20/76	20/65	20/65	20/62	
Change from baseline in logMAR, mean (SD)		-0.07 (0.24)	-0.06 (0.27)	-0.07 (0.34)	
Improvement >= 0.3 logMAR, n (%)		14 (16%)	13 (16%)	15 (19%)	
Worsening >= 0.3 logMAR, n (%)		2 (2%)	6 (7%)	6 (9%)	

CRT= central retinal thickness; SD=standard deviation; 0.3 logMAR is equivalent to 15 Early Treatment Diabetic Retinopathy Study (ETDRS) letters.

Bevacizumab non-responders

During one year of bevacizumab treatment, the non-response rate was 20% with 19 eyes of 17 patients switching treatment. Three eyes switched after 3 months, another 3 eyes after 6 months and the remaining 13 eyes after 1 year. These 19 eyes experienced a mean increase in CRT of 23 ± 101 µm from baseline during bevacizumab treatment up until the moment they switched (p=0.277) (figure 1). This was significantly worse compared to patients that did not switch, who experienced a CRT reduction of 50 ± 127 µm after 1 year (p=0.001) (figure 1). However, when correcting for baseline CRT this was no longer significant, but a clear trend was still present (p=0.077). The 19 non-responder eyes had higher baseline CRT (534 ± 130 µm compared to 459 ± 119 µm for the remaining eyes; p=0.018). At the time of switching, the mean CRT had increased to 557 ± 116 µm. The non-responders lost 0.03 ± 0.17 logMAR (from Snellen VA 20/74 to 20/80) during bevacizumab treatment, while the remainder that did not switch treatments improved 0.09 ± 0.36 logMAR over 1 year (from Snellen VA 20/78 to 20/59) (figure 2). This change in VA was not significantly different between both groups (p=0.472). There was also no difference in baseline VA between switchers and non-switchers (p=0.918).

In most non-responder eyes that switched treatment (16 of 19) CRT had not decreased more than 50 µm despite bevacizumab treatment. One eye did show a good response during the loading phase of bevacizumab (reduction from 658 to 456 µm in CRT), however, because further decrease in CRT could not be achieved, this patient was switched to ranibizumab after 1 year of treatment. Two eyes decreased 62 µm and 58

μm in CRT after the bevacizumab loading phase respectively, but still had active and extensive macular edema (CRT of 524 μm and 766 μm respectively). One eye was therefore switched to ranibizumab after 1 year and the other to subconjunctival TAC after 6 months.

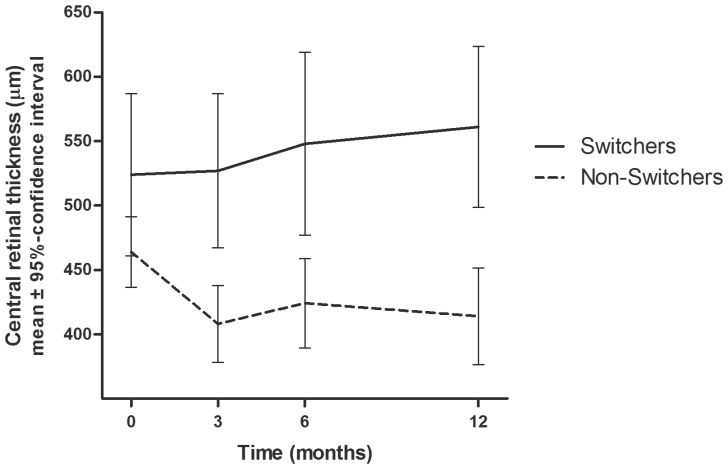

Figure 1. Course of central retinal thickness for non-responders that switched treatment versus those that did not switch treatment

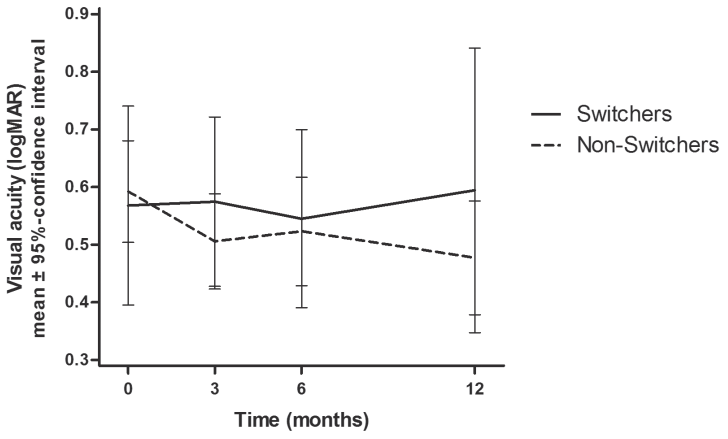

Figure 2. Course of visual acuity for non-responders that switched treatment versus those that did not switch treatment

Switch to ranibizumab

Twelve non-responders were switched to ranibizumab. Six of these eyes were lost to follow-up after referral to an independent outpatient clinic. In general, the response to ranibizumab was poor as shown in figure 3. Two of 6 eyes responded well to ranibizumab with a reduction in CRT of more than 50 μm (eye 2 and 6 in figure 3). The mean decrease in CRT after 3 ranibizumab injections was limited (-10 ± 100 μm (p=0.900)). One eye that initially responded well during the bevacizumab loading phase did not respond to ranibizumab (eye 4 in figure 3) and increased in CRT. The other eye that had responded favorably to bevacizumab also responded to ranibizumab (eye 6 in figure 3). Mean VA change was +0.08 ± 0.51 logMAR (p=0.539). However, only the 2 eyes that showed anatomical response experienced an increase in VA and the increase in mean VA was mostly driven by one patient whose VA increased with 1.30 logMAR (change in Snellen VA from 2/200 to 20/100). With the exclusion of this particular patient, the mean change in VA was -0.10 ± 0.14 logMAR.

Of the 6 eyes that were lost to follow-up, of 3 eyes information could be retrieved. OCT imaging was not performed with Heidelberg OCT, thus they were not included in the analysis. In one eye the fluid had almost completely resolved, with only a few residual cysts left after 3 ranibizumab IVIs. Visual acuity in this eye increased from 20/70 to 20/50. The other 2 eyes showed possible partial resolution of fluid, although different imaging modalities made direct comparison difficult. One of these eyes did not improve in VA, while the other improved from 20/100 to 20/70.

Switch to triamcinolone

Seven non-responders were switched directly to TAC. Of the 6 eyes that switched to ranibizumab, 2 eyes were subsequently switched to TAC because of non-response to ranibizumab (eye 1 and 4 in figure 3 and eye 5 and 6 in figure 4). Thus, a total of 9 eyes were treated with TAC because of non-response to anti-VEGF therapy. One eye received TAC as a subconjunctival injection, so therefore was not included in the analyses. Of the 8 eyes that received intravitreal TAC, 7 responded well with a decrease in CRT of over 50 μm (figure 4). One eye did not respond to TAC (eye 8 in figure 4). The mean change in CRT was a reduction of 295 ± 171 μm (p<0.001). There was a corresponding mean improvement in VA in these patients of 0.11 ± 0.11 logMAR (p=0.045). Six out of 8 eyes experienced an increase in VA, one eye remained stable and one eye lost 0.04 logMAR.

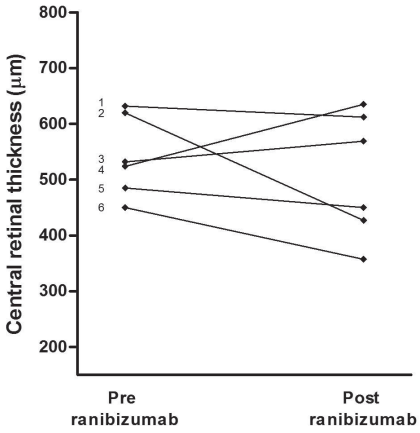

Figure 3. Change in central retinal thickness after switching to ranibizumab

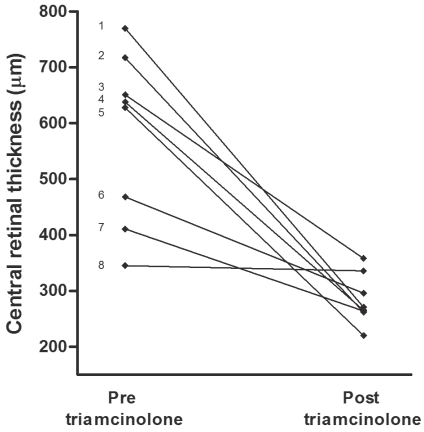

Figure 4. Change in central retinal thickness after switching to triamcinolone

Discussion

Overall, there was a moderate, but significant anatomical as well as a non-significant functional improvement from bevacizumab treatment for DME in this clinical setting. An alternative treatment because of insufficient response to bevacizumab was necessary in 20% of patients. Eyes that switched to TAC responded better with regard to CRT decrease and VA increase compared to eyes that switched to ranibizumab.

In RCTs we usually see a greater effect of bevacizumab on CRT change, as well as VA after 1 year of treatment compared to our retrospective study.[11, 12, 16, 30-33] The RCTs found decreases in CRT ranging between 50 and 180 μm and improved VA by approximately 2 Early Treatment Diabetic Retinopathy Study (ETDRS) lines of vision. In our study the effect of bevacizumab was less successful with a mean CRT decrease of 36 μm and a little under 1 ETDRS line (0.08 logMAR) increase in VA. The limited effect observed in our study compared to RCTs may be caused by the large number of injections given in RCTs, i.e. approximately 9 IVIs on average in RCTs versus 6 in our study. But also the inherent differences between the highly controlled RCT setting and the daily clinical practice population will likely contribute. The improvement in VA we observed was in concordance with retrospective studies evaluating bevacizumab in clinical practice (range of 0-2 ETDRS lines of vision increase).[15, 34-36] However, the effect on the CRT was somewhat lower than these retrospective studies where the reported reduction in CRT varies between 50-150 μm. The fact that our center is a tertiairy referral center with relatively complicated cases may have influenced the outcome.

Some limitations are involved in clinical practice data, as in our study. This study was retrospective and thus uncontrolled. There was a loss to follow up of about 20%, either because the patient refused further treatment or the treatment was continued elsewhere. Also, the non-responder subgroups were small and follow-up was short. In the Protocol I study it was suggested that the effect of TAC may wane over time.[10] Therefore confirmation of results in bigger cohorts with longer follow-up is warranted.

Recent evidence from the protocol T study suggests that aflibercept may be preferable as first-line anti-VEGF for patients with a ETDRS visual acuity below 20/50.[16] In our cohort 59% of eyes presented with a VA of below 20/50 at baseline and therefore it could be expected that they would have fared better on aflibercept. This could potentially influence the percentage of non-responders in the current study and the effect of switching to a different treatment. Based on RCT data, bevacizumab still is the most cost-effective treatment,[17] so studies of aflibercept in daily practice are required before a conscious decision can be made which anti-VEGF agent should be first-line treatment.

In this study patients switched treatments when the ophthalmologist, in consultation with the patient, decided that response to bevacizumab was unsatisfactory. This was not based on predefined response criteria, but did mainly consider the persistence of fluid on OCT. This limitation possibly stems from the inconsistent literature on the definition of

non-response. Several studies have attempted to label lack of response or non-response [ref studies non-response zie samenvatting]. These criteria for non-response in literature may have been created for several reasons: to determine whether retreatment is indicated,[16, 17] as a way to perform statistical analyses comparing responders and non-responders,[26, 37, 38] or as a way to determine treatment effect.[18, 39] Therefore not all published definitions may be suitable for determining non-response in clinical practice. Moreover, response may be based on CRT, VA or a compound measure. Because there is no consensus on which measure should be applied, the percentages of non-response in the same study may vary greatly depending on the definition used. For example in the BOLT study that evaluated bevacizumab, in report number 4[39] they defined non-responders as patients that did not show a reduction of ≥20% CRT at 4 months or 12 months, while in report number 5[40] non-response was defined as never achieving a CRT of <270 μm during 24 months of treatment. The difference in the number of non-responders was 9% in report 4 and 57% in report 5. Therefore the exact definition applied may have quite an impact on clinical practice and non-response management. That this lack of consensus is challenging in clinical practice and could also be noted in this study. Although the group that had switched treatments was different with regard to course of CRT compared to the remaining patients, there was substantial overlap in response. Also, there was no significant difference in change in VA between the two groups, indicating that the non-response criteria based on OCT practiced in this study might not be very relevant to the patient who is mostly concerned with visual gains. OCT features, such as CRT, are often incorporated in non-response definitions. However, the correlation between CRT and VA is only moderate and in some individuals non-response on OCT may even be accompanied by good visual response and vice versa.[37, 41, 42] It is clear there is still room for optimization of secondary DME treatment and a consensus on a relevant definition of non-response would be a good first step.

Regardless of whether the non-response criteria applied here were optimal, it was striking how switching to TAC seemed more effective than switching to ranibizumab. It has been suggested in the FAME study[43] that chronic edema may be especially prone to steroid treatment, however chronic edema was defined as having DME for over 3 years and none of the patients that were switched to TAC had DME for more than 2 years. So it does not seem the effect can be explained by a chronic character of the edema. It could however imply different mechanisms at work as a cause for DME in these bevacizumab non-responders. In DME, fluid leakage originates from the retinal capillaries where change in permeability of retinal endothelial cells results in macular edema.[44] There may be multiple causal pathways for this increased permeability, such as hypoxic or inflammatory pathways, and thus different treatment approaches may be in order. Anti-VEGF therapy targets the hypoxic or VEGF pathway, while steroids target the VEGF and the inflammatory pathway.[45, 46] Depending on which pathway is most active in a patient presenting with DME, one treatment may yield better results

than the other. The study of Jeon et al[26] suggests a beneficial effect of switching to TAC after bevacizumab, and links the response to TAC to the inflammatory marker IL-8 in the aqueous humor. Other inflammatory factors were also tested (VEGF, IL-2, IL-6, TNF-α, TGF-β2), but these were not associated. Possibly, the macular edema in the non-responders in this study was mediated more by inflammation as opposed to VEGF. Future studies are needed to investigate whether systemic inflammatory factors can be measured in advance to determine whether a patient's first line treatment should be anti-VEGF or steroids.

In conclusion we showed a moderate effect on CRT and VA in DME-patients treated with bevacizumab in daily clinical practice. Within 1 year, 20% of patients were considered non-responder and had to be switched to an alternative treatment. In our retrospective study, TAC was a better alternative treatment than ranibizumab.

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Cost-effectiveness of anti-VEGF therapy for AMD

Chapter 5

No excuses. Bevacizumab should be first choice in AMD.

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Purpose: The discussion on the use of bevacizumab is still ongoing and often doctors are deterred from using bevacizumab due to legal or political issues. Bevacizumab is an effective, safe and cheap treatment option for neovascular age-related macular degeneration (AMD). However, bevacizumab is not registered for AMD. Therefore, in some countries ophthalmologists are forced to use the equally effective but expensive drugs ranibizumab and aflibercept. Here, we describe the economic consequences of this dilemma surrounding AMD treatment.

Methods: We modelled cost-effectiveness of treatment with ranibizumab (as-needed), aflibercept (bimonthly) and bevacizumab (as-needed). The drug with the most favourable cost-effectiveness profile compared to bevacizumab was used for threshold analyses. First, we determined how much we overpay per injection. Second, we calculated the required effectiveness to justify the current price and what a reasonable price is for a drug that leads to optimal vision. Finally, we estimated how much Europe overspends if bevacizumab is not first choice.

Results: Bevacizumab treatment costs €27,087 per year, about €4,000 cheaper than aflibercept and €6,000 cheaper than ranibizumab. With similar effectiveness for all drugs as shown by meta-analysis, bevacizumab was clearly the most cost-effective. Aflibercept was chosen for threshold analyses. Aflibercept costs €943 per injection, but we determined that the acceptable price is actually €533. Alternatively, at its current price, aflibercept should yield about twice the visual gain. Even when optimal vision can be achieved, the maximum price for any treatment is €37,453 per year. Most importantly, Europe wastes €335 million on AMD treatment when choosing aflibercept over bevacizumab.

Conclusion: Bevacizumab is undoubtedly the most cost-effective treatment for AMD, yet is not the standard of care across Europe. The registered drugs ranibizumab and aflibercept cause huge overspending with limited health benefits. Health authorities should take steps to implement bevacizumab into clinical practice as first choice.

Introduction

Bevacizumab is a cheap and effective anti-vascular endothelial growth factor (VEGF) drug for neovascular age-related macular degeneration (AMD). Yet, recently it was described how ophthalmologists in the UK are deterred from prescribing this drug.[1] Instead doctors are being forced to prescribe the much more expensive ranibizumab or aflibercept by the General Medical Council, which opposes the use of off-label drugs such as bevacizumab when registered drugs are available. Even the National Institute for Health and Care Excellence (NICE), which holds cost-effectiveness in high regard, recommends the registered drugs over bevacizumab.[2, 3] The article describes the many political and legal issues involved in the development of such guidelines that seemingly ignore cost-effectiveness completely. This, however, does not occur solely in the UK. In fact in many other European countries bevacizumab is not allowed for ophthalmological indications. Europe is wasting money by not addressing this problem adequately as anti-VEGF treatment accounts for a large proportion of total health care expenditures.[4] AMD is a major health concern, affecting almost 200 million people globally within the next five years. Over 11 million will have developed end-stage AMD and about 2.8 million of these people will reside in Europe.[5] Two-thirds will be neovascular AMD cases, requiring multiple anti-VEGF treatments. In the neovascular end-stage of AMD, increased VEGF levels cause newly formed blood vessels to grow into the retina and leak fluid, lipids and proteins, leading to rapid and severe vision loss. Anti-VEGF agents that can be injected in the eye have been developed to inhibit these neovascularisations and with great success. Pivotal randomized controlled trials (RCT) have shown the superiority of anti-VEGF treatment over placebo improving both vision and quality of life in general.[6-8] Now, anti-VEGF therapy has become the mainstay of neovascular AMD treatment. Treatment in clinical practices worldwide is dominated by three anti-VEGF agents: ranibizumab, aflibercept and bevacizumab.

Ranibizumab (Lucentis, Genentech Inc./Novartis) and aflibercept (Eylea, Bayer) are both registered for neovascular AMD. A large non-inferiority RCT comparing aflibercept and ranibizumab showed no discernible difference in effectiveness.[9] Bevacizumab (Avastin, Genentech Inc./Roche) is not registered for use in AMD. The property rights of bevacizumab and ranibizumab are both owned by Roche, the parent company of Genentech, and although bevacizumab was originally developed as a cancer treatment, it has similar properties as ranibizumab, both being VEGF-A antibodies. RCTs have shown that there is no meaningful difference between the two agents in effect on visual acuity.[10-13] However, although the three agents are equally effective in conserving vision, there are large differences in costs. Bevacizumab is a factor 20 cheaper than ranibizumab and aflibercept, i.e. about €50 and €1,000 per injection. As the EU member states are aiming towards containment of expenditures to health care and efficient allocation of resources one would expect that bevacizumab would be first choice in AMD treatment.

However, wide differences exist across Europe in rules and legislations surrounding the use of this off-label drug and so its use in clinical practice is not self-evident.
In this article we describe how much we are overspending on registered anti-VEGF drugs and we provide the economic arguments for AMD treatment decisions, pricing and innovation. We provide insight into the costs involved in ranibizumab, aflibercept and bevacizumab treatment and show the economic consequences of not allowing bevacizumab in ophthalmology. Supported by a cost-effectiveness analysis we will discuss why bevacizumab is the only reasonable first line treatment in AMD from a public health perspective.

Methods

Model development
To estimate the economic consequences for society of prescribing ranibizumab or aflibercept instead of bevacizumab we developed a patient-level decision analytic model evaluating effectiveness, quality of life and costs. Three treatment strategies were compared: ranibizumab administered as-needed, aflibercept administered bimonthly and bevacizumab administered as-needed. The as-needed regimen includes a loading phase of three consecutive monthly injections, followed by injections on an as-needed basis, depending on clinical evaluation. The bimonthly regimen also includes a loading phase, followed by injections every two months. These regimens reflect daily care in clinical practice most closely, but are also supported by evidence from RCTs.
The model estimated the mean costs and benefits for a hypothetical group of patients. AMD patients with specific baseline characteristics entered the model individually and progressed through all three treatment strategies in the model over one year. Each patient was assigned a baseline visual acuity and change in visual acuity for each of the treatment strategies. The outcomes were averaged across a large sample of patients (n=100). We employed a 1-year time horizon since this time span is most extensively studied and allows for the most accurate effect estimates. The model assumed treatment of the best seeing eye, which is known to be most strongly predictive of quality of life.[8, 14]

Effectiveness and quality of life
To determine the effectiveness of each drug a meta-analysis was conducted; seven RCTs were included (table 1).[9-13, 15, 16] The results are presented in table 2. Data were pooled with a random effects meta-analysis. Effectiveness was defined as change in visual acuity from baseline in 'early treatment for diabetic retinopathy study' (ETDRS) letters after 1 year of treatment. ETDRS letters are scored on a letter chart with letters of decreasing size from top to bottom. The number of letters a patient can read on the letter chart determines the visual acuity. Baseline visual acuity was derived from a meta-analysis of the baseline ETDRS letter scores of all included RCTs.

Table 1. Randomized controlled trials used to estimate treatment effect

Study	Drug	Regimen	Number of patients	Mean number of injections	VA at baseline (ETDRS letters) mean (± SD)	Change in VA after 1 year (ETDRS letters) mean (± SD)	Reference
CATT 2012	Ranibizumab 0.5 mg	PRN	285	6.9	61.5 (13.2)	6.8 (13.1)	10
Subramanian 2010	Ranibizumab 0.5 mg	PRN	7	4.0	32.7 (20.9)	6.3 (13.7)	11
Krebs 2013	Ranibizumab 0.5 mg	PRN	263	5.8	56.4 (13.5)	4.1 (7.0)	12
Kodjikian 2013	Ranibizumab 0.5 mg	PRN	183	6.5	55.78 (13.99)	2.9 (15.1)	13
Busbee 2013	Ranibizumab 0.5 mg	PRN	275	7.7	54.5 (11.7)	8.2 (13.3)	15
SAILOR 1 2009	Ranibizumab 0.5 mg	PRN	490	4.9	48.9 (13.8)	2.3 (22.1)	16
CATT 2012	Bevacizumab 1.25 mg	PRN	271	7.7	60.4 (13.4)	5.9 (15.7)	10
Subramanian 2010	Bevacizumab 1.25 mg	PRN	15	8.0	34.93 (14.58)	7.6 (15.3)	11
Krebs 2013	Bevacizumab 1.25 mg	PRN	154	6.1	57.0 (13.0)	4.9 (7.0)	12
Kodjikian 2013	Bevacizumab 1.25 mg	PRN	191	6.8	54.62 (14.07)	4.8 (14.9)	13
VIEW 1 2012	Aflibercept 2 mg	Bimonthly	301	7	55.7 (12.8)	7.9 (15.0)	9
VIEW 2 2012	Aflibercept 2 mg	Bimonthly	306	7	51.6 (13.9)	8.9 (14.4)	9

PRN = pro re nata, injections are administered on an as-needed basis depending on disease activity;
VA = visual acuity; ETDRS = early treatment for diabetic retinopathy study; SD = standard deviation

Table 2. Results of meta-analysis

Variable		
Baseline visual acuity (ETDRS letters) mean (SD)	52.1	(14.1)
Change in visual acuity (ETDRS letters) mean (SD)		
Ranibizumab	4.7	(15.9)
Aflibercept	8.4	(14.7)
Bevacizumab	5.4	(8.8)
Number of injections		
Ranibizumab	6.2	
Aflibercept	7.0	
Bevacizumab	7.0	

ETDRS=early treatment for diabetic retinopathy study;
SD=standard deviation.

Based on their visual acuity after 1 year of treatment, patients were assigned a utility score which was derived from literature (table 3).[17] We assumed that this utility score remained constant throughout the 1-year time horizon of the model. Utility scores represent the quality of life of a patient with a certain health status, or in this case with a certain visual acuity score. Utility scores vary from 1 to 0, with 1 indicating a perfect health state and 0 indicating death. The use of utility scores allows the calculation of quality adjusted life years (QALYs). One QALY equals one year lived in perfect health.

Table 3. Utility value per visual acuity category

Visual acuity category	Visual acuity range	Utility value	Standard error	Distribution
1.	20/20-20/25	0.84	0.027	Beta
2.	20/30-20/40	0.80	0.024	Beta
3.	20/50-20/100	0.71	0.029	Beta
4.	≤ 20/200	0.59	0.027	Beta

Derived from Brown et al. 2002[17]

Costs

Costs were calculated from a societal perspective, including both direct and indirect costs. Direct medical costs included the costs of the drugs, medical visits and ophthalmic examinations. Drug costs were obtained from the Dutch National Health Care Institute[18] and costs for ophthalmic examinations were obtained from the department of Ophthalmology of the Radboudumc. The mean number of injections for ranibizumab and bevacizumab in a year was estimated from published RCTs. The number of injections for aflibercept was set at 7, corresponding with a bimonthly regimen. Indirect costs included the costs of low vision aids and nonmedical costs. The costs of low vision aids were based on a database from the Low Vision Totaal vision clinic containing 550 patients with various forms of maculopathy and their use of vision aids. Per visual acuity category the mean costs for low vision aids per person were calculated. Nonmedical costs, including caregiver costs, adapted housing and transportation, were derived from literature.[19] Table 4 provides an overview of all estimated costs. Costs are estimated in 2014 euro (€), using index prices according to the Dutch pharmaco-economic guidelines[20] and presented in euro or equivalent pound (£) when appropriate (currency rate €/£ January 1st 2015: 0.77661).

Table 4. Direct and indirect costs for neovascular AMD treatment

	Unit	Costs (€)	SE	Distribution	Source
Aflibercept	Per injection Average per year: 7.0	943.48		Fixed	DNHCI
Ranibizumab	Per injection Average per year: 6.2	979.14		Fixed	DNHCI
Bevacizumab	Per injection Average per year: 7.0	44.45		Fixed	DNHCI
Medical visit	Per visit Per year: 12	140.42		Fixed	Radboudumc
Optical coherence tomography	Per measurement Per year: 12	33.39	5.0	Gamma	Radboudumc
Fluorescein angiography	Per measurement Per year: 1	111.33	10.0	Gamma	Radboudumc
Visual acuity measurement	Per measurement Per year: 12	12.19	2.0	Gamma	Radboudumc
Fundus photograph	Per measurement Per year: 1	39.0	10.0	Gamma	Radboudumc
Low vision aids* (One-time)	If 20/20-20/25 If 20/30-20/40 If 20/50-20/100 If ≤20/200	218.24 451.69 1020.00 1065.87	85.95 74.97 53.74 73.76	Gamma	Low Vision Totaal
Nonmedical costs# (One-time)	If 20/20-20/25 If 20/30-20/40 If 20/50-20/100 If ≤20/200	1141.69 6165.32 19477.18 42318.61	327.09 1947.80 2599.00 3822.44	Gamma	Brown et al.[19]

SE=Standard error; DNHCI=Dutch National Health Care Institute, www.medicijnkosten.nl, 2014; Radboudumc = Department of Ophthalmology of the Radboudumc
*Low vision aids includes (electronic) loupes, loupe glasses, filter glasses, loupe lamp, monitor loupes;
#Nonmedical costs include caregivers costs for inside activities of daily living (ADL), outside ADL and transportation costs and residence costs.

Analysis

We developed the decision analytic model using ARENA software (version 14.00.00 Rockwell Automation, Inc). The three treatments were compared in terms of mean costs, mean effects (in QALYs) and incremental cost-effectiveness ratios (ICERs). The ICER represents the extra costs that are incurred in order to gain one additional QALY, comparing one treatment over the other. The ICER is calculated by dividing the estimated difference in costs between two treatments by the difference in QALYs.[21]

Whether a treatment may be considered cost-effective depends on how much a society would be willing to pay for a QALY. We used a willingness-to-pay (WTP) threshold of €80,000 per QALY, the highest cost-effectiveness threshold recommended by The National Health Care Institute.[22] This means that a strategy is deemed cost-effective compared to another strategy when it costs €80,000 or less to gain an extra QALY, i.e. the ICER is lower than €80,000 per QALY. Bevacizumab was used as the comparator in all cases. Probabilistic sensitivity analyses were performed to assess the impact of uncertainty on the outcomes.[23] This means that we assigned distributions to model parameters, to reflect the uncertainty in the estimation of that parameter when possible. All distributions are listed in tables 3 and 4. Parameter values were sampled at random from the assigned distributions, using Monte Carlo simulation. After each sample, 100 patients were simulated using these parameter values, which was repeated 1000 times. Cost-effectiveness acceptability curves were plotted, which show the probability that an anti-VEGF drug is the most cost-effective over a range of WTP thresholds.

To answer our question of what an acceptable price is for innovative treatments in AMD, we performed threshold analyses for the drug with the most favourable cost-effectiveness profile compared to bevacizumab. Threshold analyses determine under what conditions the drug will become the most cost-effective treatment compared to bevacizumab, i.e. under which circumstances the ICER will drop below the WTP threshold of €80,000 per QALY. First, the acceptable price for one injection was calculated by determining the total acceptable costs for a treatment at an ICER of €80,000 and subtracting all remaining costs except for drug costs and dividing by the total number of injections. Second, we determined how effective the drug should be to justify the current difference in costs compared to bevacizumab. Third, we calculated the acceptable costs for an innovative treatment that leads to optimal visual acuity. When varying quality of life gains, indirect costs were reduced as quality of life gains increased. The reduction in indirect costs per 0.01 QALY was estimated through linear regression in the modelled patient population. Similarly, we used linear regression to determine the number of QALYs gained per ETDRS letter to translate quality of life changes to visual acuity changes.

Overspending was defined as the amount of extra money paid for health care that cannot be justified by the WTP threshold of €80,000, i.e additional treatment costs without an equivalent health benefit. Overspending in Europe was calculated based

on the overpricing of the registered drug compared to bevacizumab and the number of expected injections. The number of injections in the UK was derived from incidence data of new patients requiring treatment for neovascular AMD (102 per 100,000)[24] and the assumption that most patients in real-world clinical practice will receive on average 5 injections in the first year.[25-28] From the department of ophthalmology of the Radboudumc, where bevacizumab is first choice, we could estimate that 80% of injections for AMD are with bevacizumab, while the other 20% are mostly with aflibercept reserved for non-responders. All estimates were then extrapolated to the entire European Union, which has a population of approximately 200 million aged over 50 years.[29]
Reporting in this economic evaluation was based on the Consolidated Health Economic Evaluation Reporting Standards (CHEERS) statement.[30]

Results

Costs-effectiveness
Ranibizumab was dominated by bevacizumab in the cost-effectiveness model, meaning it was more expensive without yielding additional QALYs. Aflibercept yielded 0.015 extra QALYs compared to bevacizumab. Assuming a threshold of €80,000 for one QALY gained, bevacizumab was the most cost-effective treatment in 100% of simulations

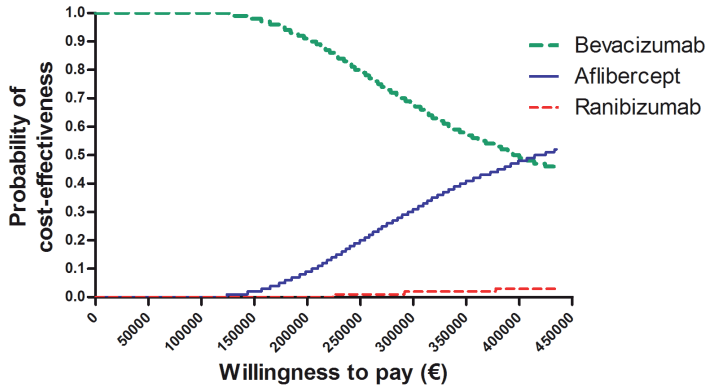

Figure 1. Acceptability curve of the three anti-VEGF treatments
The acceptability curve shows the probability of a treatment being cost-effective over a range of willingness-to-pay thresholds. The curve shows that bevacizumab is the most likely to be cost-effective until a willingness-to-pay threshold of €407,250 is reached, after which aflibercept is most likely to be cost-effective.

(figure 1). Using aflibercept instead of bevacizumab means paying €278,099 per QALY gained (table 5). Using ranibizumab would mean paying money without any health benefits.

Table 5. Differences in effectiveness and costs between treatments
The ICERs show that we pay €278,099 per QALY if we use aflibercept instead of bevacizumab and that ranibizumab is dominated by bevacizumab, meaning it is more costly, but does not yield any health benefit.

	Mean costs € (95-%CI)	Difference in costs Δ€	Mean effectiveness QALY (95-%CI)	Difference in effectiveness ΔQALY	ICER Δ€/ΔQALY
Ranibizumab	€ 33,137 (28,883-37,926)	€ 6,050	0.69 (0.66-0.73)	0.000	Dominated
Aflibercept	€ 31,119 (26,979-35,766)	€ 4,032	0.71 (0.67-0.74)	0.015	€ 278,099
Bevacizumab*	€ 27,087 (22,818-31,789)	-	0.69 (0.66-0.73)	-	-

QALY=quality-adjusted life year; ICER=incremental costs-effectiveness ratio; 95%-CI=95%-confidence interval; *Bevacizumab is comparator

Threshold analyses

Because aflibercept was more promising in terms of cost-effectiveness, we chose this drug to show what would be necessary in order for it to be a justifiable alternative to bevacizumab. The current acquisition costs of aflibercept are €943. For aflibercept to meet the standard criteria of cost-effectiveness, this should be reduced to €533 per injection. This means aflibercept is currently €410 overpriced. Because WTP thresholds may vary across countries, the relation between the price for one aflibercept injection and WTP is shown in figure 2. At a willingness to pay of £30,000 (€38,629), the threshold practised by NICE, aflibercept should cost no more than £347 (€447) per injection.

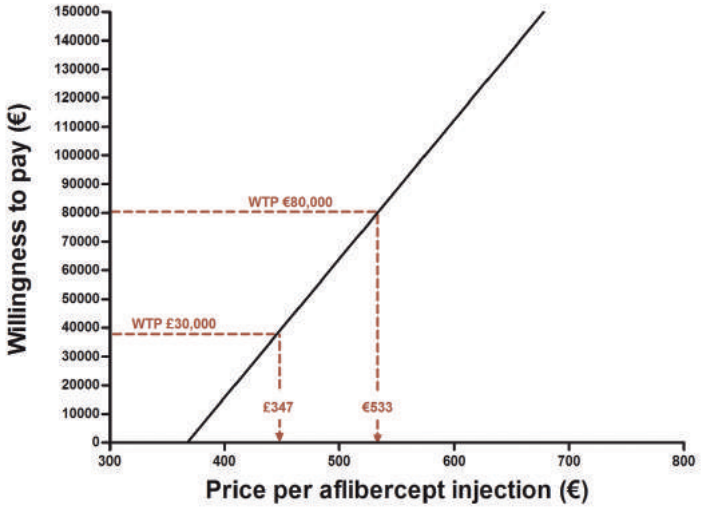

Figure 2. Acceptable costs per aflibercept injection
Which price is acceptable for one aflibercept injection depends on what society is willing to pay. The graph shows that at a willingness-to-pay of €80,000 per QALY aflibercept should cost no more than €533. To reach the NICE threshold for cost-effectiveness, the costs should be reduced to £347.

We also reversed the question by estimating how well aflibercept should work to justify the current pricing. The difference in total costs between aflibercept and bevacizumab was €4,032. Taking into account that indirect costs will decrease as effectiveness increases, we calculated that aflibercept should at least render an additional 0.041 QALYs compared to bevacizumab. Linear regression showed that 0.004 QALYs corresponded to approximately 1 letter on ETDRS chart. This implies that aflibercept should at least render an additional visual gain of 10.3 letters compared to bevacizumab, which is a change from baseline of 15.7 letters instead of the current 8.4 letters.
New drugs are constantly being developed and one day these may be highly effective in restoring vision. The maximum utility to be reached in neovascular AMD was established to be 0.84 in the highest visual acuity group.[17] Bevacizumab treatment leads to a mean utility score of 0.69, implying that at maximum 0.15 QALYs can be gained each year. To remain within the €80,000 WTP threshold, total costs for the new treatment should not exceed €38,813 during the first year. Since indirect costs for this new treatment will be lower when effectiveness increases, we re-calculated the indirect costs to be €1,360 (table 4) when vision is optimally restored. After subtracting indirect costs, the direct medical costs of a treatment that accomplishes perfect vision in AMD should not exceed €37,453 per year.

Financial consequences

In the UK alone the number of eyes with neovascular AMD requiring treatment will rise to 32,000 within the next 5 years.[24] These patients will require on average 5 injections in their first year, summing up to 160,000 injections in total. The UK does not have bevacizumab as their first choice treatment since the NICE guidelines recommend the registered drugs instead. Assuming that 80% of patients could be treated with bevacizumab instead, we have calculated that the UK is overspending over €52.5 million (£38.7 million) each year on new neovascular AMD patients. For Europe we estimate that approximately 204,000 new AMD patients will require roughly 1 million injections. Treating 80% of these people with bevacizumab instead of aflibercept, would save Europe approximately €335 million yearly.

Discussion

Bevacizumab is undoubtedly the most cost-effective treatment for neovascular AMD. That aflibercept and ranibizumab are not cost-effective compared to bevacizumab does not come as a surprise and has been investigated before.[31-33] We estimated that each injection of aflibercept is €410 overpriced and that Europe overspends €335 million on health care if aflibercept is first choice treatment instead of bevacizumab. The waste of implementing ranibizumab as first choice will be even bigger. An important remark here is that these costs are not accompanied by a substantial health benefit. Visual acuity gains of aflibercept were estimated to be approximately 3 letters compared to bevacizumab, corresponding to 0.01 QALYs. A difference this small will not be perceived by the individual patient.[34]

The major strength of our model is that it gives a clear view of the total costs involved in choosing one drug over the other. This article presents the absolute overspending associated with aflibercept treatment and can thus contribute to budgetary discussions. Some possible limitations should also be discussed. First, we assumed treatment of the best-seeing eye in our model since we know that the vision related quality of life in an individual is mostly determined by the vision in the eye that sees best. Treating the worst-seeing eye will have little impact on quality of life.[8] Modelling the best-seeing eye will therefore lead to conservative estimates, and cost-effectiveness is probably even poorer in real-life. Second, we did not consider adverse events of any kind. A recent Cochrane review evaluated the evidence for systemic safety of ranibizumab and bevacizumab (n=3,665) and they found no relevant differences except for gastrointestinal events.[35] Interestingly, the authors recommend that health care authorities refrain from prohibiting bevacizumab on account of theoretical safety issues. Evidence for safety of aflibercept is still lagging behind, but in the large RCT of aflibercept versus ranibizumab no differences in safety were noted.[9] Third, nonmedical costs were derived

from an American source. It is unsure whether these are fully representative of the European setting. Also, the larger the differences of nonmedical costs between vision categories, the more favourable the cost-effectiveness of aflibercept becomes. This is because when the cost differences between the vision categories become bigger, small differences in effectiveness become more valuable. So there will be some variation in results, depending on how you estimate these nonmedical costs. In our model, the cost differences were substantial. Nonetheless, aflibercept did not come close to being cost-effective. Finally, we only estimated costs and effects for the first year of treatment. After this year, patients are still treated, but the number of injections and their effectiveness are unknown. Arguably, in the following years costs are still made, but vision is expected to deteriorate. Hence, the actual overspending is probably underestimated. So regardless of these limitations, it is clear that ranibizumab and aflibercept are grossly overpriced.

Europe is divided when it comes to its health care policies regarding the use of off-label drugs. As we know now, the General Medical Council and the NICE guidelines in the UK dissuade ophthalmologists from using bevacizumab, as it lacks proper appraisal. Unfortunately, the UK is not the only country where the use of this cheap alternative is held back by outmoded legislation. In countries such as Germany, Switzerland, Belgium, and until recently France, the use of bevacizumab in ophthalmology is not allowed because the health authorities declare that off-label use of drugs should be avoided when a registered alternative is available. But in the case of bevacizumab, should there not be an exception to the rules seeing the immense benefits?

The discussion here is not only about overpaying for a drug when a cheaper alternative is available. Some may argue that even a little gain in quality of life is reason enough to use one drug over the other. This seems ethical, but is actually the opposite. Health care funds are not an infinite resource. Investing in one part of health care, means having less resources left for other types of health care. For the National Health Services (NHS) in the UK this has been meticulously researched by Claxton et al.[36, 37] They published new methods for estimating NICE cost-effectiveness thresholds which was accompanied by a calculator to estimate the overall loss of QALY based on the additional costs of approving a new technology. Knowing that a year's worth of aflibercept treatment costs €4,032 more than bevacizumab, this means an additional investment of €129 million to treat the AMD-population. This money has to come from somewhere. The calculator shows us that this additional investment requires redistribution of health care, resulting in a total of 7,346 QALYs lost because of reduction of resources in other types of health such as respiratory diseases and mental health. So not only does the use of aflibercept over bevacizumab lack a substantial health benefit, it in fact results in loss of overall health within the society.

A simple solution would seem to register bevacizumab for use in AMD. However, only Roche, who has the ownership rights to bevacizumab, can request such a registration and Roche also owns its more costly alternative ranibizumab. Roche states that bevacizumab

is not manufactured or approved for intraocular use and they wish to focus on developing bevacizumab further for oncological indications and not for ophthalmology. They do not comment on the fact that registration of the much cheaper bevacizumab would surely cost them money, since this would of course lead to a reduction of their ranibizumab sales. However, we cannot rely on commercial companies to actively contribute to financial sustainability in health care and this should be the task of the health care authorities. The registration and patent policies will probably not change anywhere in the near future, but fortunately ranibizumab's patent will be ending in 2018[38] and if not extended, this will hopefully lead to an influx of cheaper generic alternatives. And who knows, maybe this will impel Roche to register bevacizumab after all.

In conclusion, since AMD is such a prevalent and debilitating disease, there is a large demand for treatments. Value based pricing in health care means that drugs should be priced the same as equivalent drugs that are available, or should at least be priced in accordance with their health benefits.[39] It is clear that aflibercept and ranibizumab do not adhere to those rules. New drugs are continuously being designed and as long as policy makers are negligent of cost-effectiveness and sustainability of health care, it is a lucrative market for pharmaceutical companies. The question remains whether the development of these new agents is truly justified, considering the availability of the cheap and effective bevacizumab. Developing a new drug at the cost of below €40,000 per year will be a challenging task. The awareness of the importance of cost-effectiveness to keep health care sustainable is growing and new drugs should be critically appraised on their added value. In the case of neovascular AMD treatment there are no excuses. Bevacizumab should be first choice.

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Improving the injection procedure

Chapter 6

Are intravitreal injections with ultrathin 33G needles less painful than the commonly used 30G needles?

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Purpose: This study investigated whether pain from intravitreal injections (IVIs) can be reduced by injecting with a 33G needle instead of the commonly used 30G needle. Additionally, several pain-related psychological factors were explored as predictors of outcome.

Methods: This randomized crossover trial included 36 patients who received injections with both needles in randomized order. After the injection, patients rated IVI-pain on a 0-10 scale. Prior to injection, distress and pain expectations were assessed. Afterwards, patients rated the IVI-procedure and anticipated consequences. In addition, we assessed the force necessary to penetrate the sclera for both needles in porcine eyes.

Results: The 33G needle did not result in lower IVI-pain (2.8 vs. 3.1, p=0.758), but tended to cause less vitreal reflux (0 vs. 5 times, p=0.054). Factors related to more pain were: distress, expecting IVI-pain and discomfort, dissatisfaction with the preparation procedure, anticipating negative consequences, and female gender. Patients regarded povidone-iodine disinfection as particularly unpleasant. Exploration of the needles' mechanical properties showed that 33G needles penetrate the sclera more easily.

Conclusion: The thinner 33G needle does not reduce IVI-pain, but may limit scleral damage. Future efforts could be aimed at optimizing patient information, reducing distress, and the use of better tolerable disinfectants.

Introduction

Intravitreal injections (IVIs) are used with increasing frequency in ophthalmic practice and they have become a significant part of standard eye care. It was estimated that over 1 million injections were performed in 2009.[1] At present that number has multiplied and will only increase as the indications for IVIs expand. Currently, the large majority of IVIs relate to anti-VEGF agents that are mainly used in neovascular age-related macular degeneration (AMD), diabetic macular oedema (DMO) and macular oedema secondary to retinal vein occlusion (RVO).

From the patient's perspective, an eye injection can be a frightening prospect. To improve IVI experience, it is important that the injection procedure is as comfortable and pain free as possible. Factors that have been associated with a higher pain experience in IVIs are female gender, older age, and negative treatment results.[2] However, these are non-modifiable factors and efforts to increase patient comfort should focus elsewhere. The needle, and more specifically the size of the needle, seems to be a logical candidate. Currently, a 30G needle, with an outer diameter of 0.31 mm, is most commonly used for intraocular injections with non-viscous, soluble agents such as VEGF-inhibitors. There is evidence suggesting that the size of the needle is associated with pain experience during IVI, but results have been inconsistent.[2-4] One study found no difference in pain of a 33G guarded-needle in comparison to a regular 30G needle[5], however, due to the difference in technique, it is hard to draw conclusions from this study regarding needle gauge. No study up to date has assessed the potential benefit of a 33G needle in pain reduction in a suitable study design. This needle has an outer diameter of only 0.21 mm, which is a reduction of 32% compared to its 30G counterpart. Additionally, it is well recognized that psychological factors such as distress prior to surgical procedures can be a major influence on pain experience.[6-8] Likely, this also plays a role in IVIs and patients regularly mention negative expectations, such as a fear of injection and/or fear of negative results.[9] Controlled efforts to diminish distress in IVIs, however, have been limited,[10] so its role in pain management in IVIs remains unclear.

The goal of this study was to investigate whether IVI pain scores can be reduced by replacing the 30G needle with an ultrathin 33G needle. We employed a crossover design allowing all participants to be their own control to account for interpersonal differences in pain perception. We measured the difference in force necessary to penetrate the sclera between 30G and 33G needles. Finally, we explored effects on secondary outcomes of complications during the IVI and the influence of patient's distress, anticipated pain, anticipated consequences of the IVI, and overall visit experience on the level of pain from an IVI.

Methods

Design and Population

We included 36 patients for this randomized, crossover trial. Patients who were scheduled for at least two consecutive IVIs with VEGF-inhibitors within 6 weeks were eligible. Patients and researchers were both masked for needle size. Because of the nature of the intervention and the difference in appearance of the needles, the surgeon performing the IVI could not be masked. We performed this study in accordance with the tenets of the Declaration of Helsinki and the Dutch Medical Research Involving Human Subjects Act (WMO) and acquired an informed consent from all participants. The local ethics committee declared to have no objection to this trial. This clinical trial was registered at the Netherlands Trial Register (NTR3770).

Injection procedure

All patients received a total of two injections up to 6 weeks apart, one with the 30G needle [0.31x13 mm, BD Medical] and another with the 33G needle [0.21x13 mm, TSK laboratory]. Both needles were of the same tri-beveled design, developed for optimal penetration. We randomized the order in which these needles were used. All IVIs were administered according to clinic protocol and by the same surgeon. Twenty to 30 minutes prior to injection the preparations started with an eye drop of oxybuprocain, povidone-iodine 5% and tetracain 1%. After another 10 minutes patients received an additional povidone-iodine 5% drop and after 20 minutes a tetracain 1% eye drop. Immediately prior to injection patients received a last drop of tetracain 1% and the eyelids were disinfected with povidone-iodine 10%. An eyelid speculum was inserted to keep the eye open during injection. The conjunctiva was gently displaced using a pair of surgical forceps and the injection was placed in a straight angle in the upper temporal quadrant, 3.5 mm from the limbus in pseudophakic eyes and 4 mm from the limbus in phakic eyes. At the end of the procedure the povidone-iodine was rinsed out with sterile saline solution. The surgeon recorded the occurrence of reflux and any irregularities during the procedure.

Data collection

The primary outcome was pain score on a 0-10 numeric rating scale (NRS).[11] Immediately after each injection, patients were asked to score the amount of pain they felt during the intraocular injection with anchors of 0 meaning 'no pain' to 10 'worst pain ever experienced'.

As secondary outcome variables, distress, anticipations regarding pain and consequences of the IVI, and visit experience were assessed. Thirty to 60 minutes prior to the injection procedure, patients were asked to fill out the Hospital Anxiety and Depression Scale (HADS)[12-15] to determine distress over the last week (anxiety and depression subscale

possible scores ranging from 0-21). Internal consistency for composite scores was assessed using Cronbach's α to indicate whether the individual questions measure a similar concept and a composite score is indeed acceptable. Internal consistency of the HADS depression and anxiety subscales was considered good with Cronbach's α of .61 and .71 respectively during the first visit, and 0.75 and 0.81 during the second visit. Additionally, patients were asked to rate several items on 0-10 NRS regarding distress at that moment and expectations of the injection regarding painfulness and discomfort (Table 1a). An overall composite score for distress was calculated by averaging the scores from the tension, anxiety, and nervousness questionnaire items. The items showed good internal consistency, with a Cronbach's α of 0.94 during the first visit and 0.89 during the second. The number of preceding injections and indication for injections were extracted from the patient file.

Following the injection procedure, participants scored on 0-10 NRS how they perceived several aspects of the clinic visit and what they believed the consequences of the IVI on ocular function would be (Table 1b). Two specific aspects of the clinic visit were scored separately. The preparations surrounding the injection included the time spent in the preparation room and administration of the eye drops starting 20-30 minutes prior to injection. The procedure surrounding the injection consisted of the time spent in the procedure room, disinfection with povidone-iodine, insertion of the eyelid speculum, and the injection itself. Regarding the consequences of the injection we distinguished between expectations of negative consequences, such as worsening of disease or risk of complications, and expecting the disease to contribute to the improvement of the disease, either in terms of improved eyesight or slowing down the disease process. Lastly, patients were asked in an open question what aspect of the overall clinic visit they perceived as least pleasant.

Penetration force measurements

We measured the force required for scleral penetration for both needles, to gain insight in mechanically relevant differences between the 33G and the 30G needle. We used a material testing machine Z2.5 (Zwick/Roell, Ulm, Germany) with a load cell of 20 Newton. Recently enucleated porcine eyes were mounted on a custom-built container to prevent excessive movement during needle penetration. We measured penetration force requirements of 33G, 30G and 27G needles. The 27G needle was included as a control since it has previously been shown to require a higher maximum force to penetrate the sclera as compared to the 30G needle.[16] The needle was attached to a syringe with luer lock, which in turn was secured to the load cell. The needle was then lowered unto the porcine eye with a constant speed of 1 cm/second until the sclera was penetrated. Measurements were repeated 10 times for each gauge, using another location on the sclera with a fresh needle. Maximum force to penetration was recorded for each single test.

Statistical analysis

The required sample size was calculated based on a paired-samples t-test on the pain scores of the 33G versus the 30G needle. The standard deviation of the difference was estimated to be 2.0 and the correlation between consecutive pain scores 0.5.[2, 17] In order to demonstrate a difference in pain score of at least 1.0 on a 0-10 NRS, a sample size of 34 was required (α=0.05, β=80%). All variables were checked for normal distribution. The primary outcome measure showed a skewed distribution. The difference in pain score between needles was calculated using the Wilcoxon ranks test. Explorative associations between pain scores and psychological variables were assessed using linear mixed models to account for the dependency between the measurements from the first and second visit. Differences in adverse events were analysed with the Fisher's exact test. Maximum force between the 30G and the 33G needle were compared using an independent t-test. All statistical analyses were performed in SPSS 20.0 (SPSS Inc., Chicago, IL).

Table 1a. Psychological questionnaire prior to injection

Question	Scoring scale
1. Please indicate on the scale **how much tension** you are feeling **right now**?	0 “no tension” - 10 “worst tension ever experienced”
2. Please indicate on the scale **how anxious** you are feeling **right now**?	0 “not anxious” - 10 “most anxious ever experienced”
3. Please indicate on the scale **how nervou**s you are feeling **right now**?	0 “not nervous” - 10 “most nervous ever experienced”
4. Please indicate on the scale **how much pain** you are feeling **right now**?	0 “no pain” - 10 “worst pain ever experienced”
5. Please indicate on the scale **how painful you expect** the **injection** is going to be?	0 “no pain” - 10 “worst pain ever experienced”
6. Please indicate on the scale **how uncomfortable you expect** the **injection** is going to be?	0 “very comfortable” - 10 “very uncomfortable”

Table 1b. Psychological questionnaire after the injection procedure

Question	Scoring scale
1. Please indicate on the scale **how painful** you thought the **injection** was at the moment you received it?	0 “no pain” - 10 “worst pain ever experienced”
2. Please indicate on the scale **how much pain** you are still feeling **right now**?	0 “no pain” - 10 “worst pain ever experienced”
3. Please indicate on the scale how you experienced **your current visit** to the clinic and the approach of the clinic personnel and treating surgeon?	0 “very unpleasant” - 10 “very pleasant”
4. Please indicate on the scale how you experienced the **preparation before the injection**?	0 “very unpleasant” - 10 “very pleasant”
5. Please indicate on the scale how comfortable or uncomfortable your thought the **procedure surrounding the injection** was?	0 “very comfortable” - 10 “very uncomfortable”
6. Please indicate on the scale to what extent you expect the injection is going to have **negative consequences for your health**?	0 “not at all” - 10 “to large extent”
7. Please indicate on the scale to what extent you expect the injection is going to **contribute to curing/improving your eye condition**?	0 “not at all” - 10 “to large extent”
8. Please indicate briefly what you thought was least pleasant regarding the entire procedure surrounding today’s injection, from the moment you entered the clinic.	

All questions were scored on 0-10 NRS. Patients were asked to mark the place on the NRS scale that according to them most represented how they felt.

Results

Patient characteristics

After randomization, 13 (36%) patients received the first injection with the regular 30G needle and the second injection with the ultrathin 33G needle; in the remaining 23 patients, the 33G needle was the first needle used. The baseline characteristics for the two groups are described in Table 2. All patients received treatment with bevacizumab. Common reasons for IVI were neovascular AMD (39%), DMO (22%), RVO (17%), and macular oedema from various causes (11%).

Table 2. Baseline characteristics of the two randomization groups

	Start with 30G needle	**Start with 33G needle**
	(n=13)	**(n=23)**
Gender, n (%):		
Male	9 (69)	14 (61)
Female	4 (31)	9 (39)
Mean age (SD)	71.3 (14.5)	66.3 (11.3)
Eye, n (%):		
OD	5 (38)	7 (30)
OS	7 (54)	15 (65)
ODS	1 (8)	1 (4)
Median nr injections (range)	4 (1-56)	8 (1-29)
Indication for injection, n (%):		
AMD	8 (62)	6 (26)
DME	2 (15)	6 (26)
RVO	3 (23)	3 (13)
Other	0 (0)	8 (35)

SD= standard deviation; OD= oculus dexter; OS= oculus sinister; ODS= oculus dexter and sinister; AMD= age-related macular degeneration; DME= diabetic macular edema; RVO= retinal vein occlusion.

Pain

The mean pain score did not differ significantly between IVIs with the ultrathin 33G needle (2.8 ± 2.3) and the 30G needle (3.1 ± 2.6) (p=0.758). There was no significant order effect, as there was no difference between the first and second injection mean pain scores (3.0 ± 2.4 and 2.9 ± 2.5 respectively, p=0.786), nor was the outcome different between randomization groups. Also, the expectation of pain and distress preceding the second injection was not influenced by the type of needle used in the previous visit (Distress composite score: p=0.739; NRS pain expectation: p=0.361).

Evaluation of IVI procedure

Patients were generally satisfied with the procedure and gave high ratings to the overall experience of the current visit (8.7 ± 1.1). In 37 (51%) of in total 72 visits, patients did not report any negative aspects. The most unpleasant element was the application of povidone-iodine disinfectant in 19 (26%) visits, the IVI itself in 7 (10%) visits, the insertion of the eyelid speculum in 6 (8%) visits and the waiting period was considered most unpleasant in 3 (4%) visits.

Complications

No serious complications were reported during the study. The surgeon did report some minor events: there was a trend towards significant difference in vitreal reflux, which was reported 5 times during injections with the 30G needle compared to 0 times during the 33G needle injections (p=0.054). Secondly, subconjunctival bleeding occurred twice during the 30G needle injections versus 3 times for the 33G needle (p=1.000). The surgeon did not report an influence of needle size on ease of injection. Specifically, the surgeon indicated there was no discernible difference in force required to enter the vitreous cavity, nor did he notice difference in resistance during injection of the drug.

Predictors of pain

Overall, patients reported low levels of distress directly prior to the injection with 2.4 ± 1.9 on the 0-10 distress composite score. Also distress scores in the preceding week were low with 4.5 ± 3.3 on the HADS anxiety subscale. An overview of the scores from the psychological questionnaires per visit are reported in Table 3. The associations between demographic and psychological factors and IVI pain levels are presented in Table 4. Higher pain scores were associated with more distress prior to injection, expecting more pain, female gender (even though distress scores between men and women were similar), a less positive rating of the preparations before the injection procedure, a negative experience from the procedure preparations, expecting more negative consequences from the treatment, and low scores on believing the injections would contribute to health.

Penetration force measurements

The mean maximum force required for penetration was 0.55±0.12 and 0.45±0.06 Newton for the 30G and the 33G needle respectively. This difference was statistically significant with p=0.025. The 27G needle required approximately twice the maximum force to penetration (1.08±0.37 Newton) compared to the 30G needle, this finding is in line with the report by Pulido and co-workers.[16]

Table 3. Psychological factor scores per visit.

Variable	Visit 1 Mean (SD)		Visit 2 Mean (SD)	
Female gender pain score	4.0	(3.0)	4.3	(2.9)
Male gender pain score	2.4	(1.9)	2.2	(2.0)
HADS scores				
HADS depression score	4.2	(2.8)	4.6	(3.3)
HADS anxiety score	4.4	(3.1)	4.5	(3.5)
HADS total score	8.6	(5.0)	9.1	(5.9)
Questions prior to IVI				
Distress composite score	2.8	(2.0)	2.2	(1.8)
1. *Tension prior to injection*	3.2	(2.1)	2.7	(2.2)
2. *Anxiety prior to injection*	2.5	(2.6)	1.7	(1.7)
3. *Nervousness prior to injection*	2.8	(2.3)	2.2	(1.9)
4. Pain at this moment	0.9	(1.3)	0.8	(1.3)
5. Pain expected from injection	3.3	(2.2)	3.2	(2.7)
6. Discomfort expected from injection	4.9	(2.6)	4.7	(2.7)
Questions after IVI				
2. Pain directly after injection	2.7	(2.2)	3.2	(2.6)
3. Experience of current visit	8.8	(1.0)	8.7	(1.2)
4. Experience of preparations before the injection	8.1	(1.9)	8.2	(1.8)
5. Discomfort of procedure surrounding injection	4.3	(2.8)	4.3	(2.1)
6. Expected negative consequences on health	2.3	(2.2)	1.7	(1.8)
7. Contribution to improvement of disease	5.7	(2.2)	5.9	(2.4)

Table 4. Psychological and demographic predictors of pain from intravitreal injections.

Variable	Parameter estimate (SE)	p-value
Age in years	-0.01 (0.03)	0.603
Number of injections	0.01 (0.03)	0.664
Male gender	Ref.	
Female gender	1.84 (0.62)	0.005*
HADS scores		
HADS depression score	0.13 (0.10)	0.197
HADS anxiety score	0.32 (0.09)	0.001*
HADS total score	0.16 (0.05)	0.005*
Questions prior to IVI		
Distress composite score	0.33 (0.16)	0.039*
1. *Tension prior to injection*	0.28 (0.14)	0.046*
2. *Anxiety prior to injection*	0.28 (0.15)	0.081
3. *Nervousness prior to injection*	0.27 (0.14)	0.065
4. Pain at this moment	0.36 (0.24)	0.142
5. Pain expected from injection	0.49 (0.10)	0.000*
6. Discomfort expected from injection	0.28 (0.11)	0.013*
Questions after IVI		
2. Pain directly after injection	0.63 (0.10)	0.000*
3. Experience of current visit	-0.34 (0.27)	0.214
4. Experience of preparations before the injection	-0.65 (0.14)	0.000*
5. Discomfort of procedure surrounding injection	0.20 (0.11)	0.091
6. Expected negative consequences on health	0.38 (0.14)	0.007*
7. Contribution to improvement of disease	-0.27 (0.13)	0.041*

Numbers in front of the variables refer to the question number.
SE= standard error; HADS= Hospital Anxiety and Depression Scale;
IVI= intravitreal injection
All p-values were derived from mixed models with "pain from IVI" as the dependent variable. * p-value < 0.05

Discussion

Previous studies on needle size in diabetic insulin therapy showed that larger needles are associated with more pain.[18-20] Studies comparing needle sizes in IVI have been inconsistent with respect to pain scores. One study found that patients experienced less pain when injected with 29G or 30G needles as compared to 26G or 27G needles[3] and another showed patients rated a 31G needle as less painful than a 30G needle.[4] By contrast, a third study reported no difference in pain levels between 27G, 30G and 32G needles.[2] None of these studies used a crossover design to account for the large variability in pain perception between patients. One study by Eaton et al.[5] did compare a guarded 33G needle with the regular 30G needle in patients who were receiving bilateral injections. This study did not find a significant difference in pain, but used a 0-4 Likert scale to measure pain, which could explain why they noted only small differences in pain score. Moreover, a different technique was employed for each of the needles (use of a guarded 33G needle without speculum versus regular injection technique with a 30G needle and eyelid speculum), which makes it difficult to assess the exact role of needle size in pain perception. The current crossover study comparing a 30G and an ultrathin 33G needle used the same injection technique for both needles. The IVI pain scores were low to moderate and could not be reduced any further with the ultrathin 33G needle.

We subsequently explored which factors were predictive of IVI related pain. Psychological factors such as distress and expectations are known to influence pain experience.[6-8] In this study, we could corroborate the relevance of these factors in IVIs. In general, patients reported low distress scores and patients were overall satisfied with the procedure as a whole. Psychological factors that were associated with higher pain experience were: distress prior to injection, expecting pain and discomfort from the injection, dissatisfaction with the preparations before IVI, expecting negative consequences from the injection, and not expecting the IVI to contribute to improvement of disease. Possibly, in a subset of patients, the subjective experience of the injection procedure could be improved by focussing more on patient communication to reduce distress and manage patient expectations.

A noticeable item that came up in the questionnaires was the povidone-iodine disinfectant. Many patients specifically mentioned this as very unpleasant, which was also reflected in the lower scores given to the procedure surrounding the injection. Despite rinsing the eye after each injection with sterile saline solution, patients still complained about irritation from the disinfectant. Reducing the povidone-iodine concentration from 10% to 5% or possibly even lower may be a straightforward way to reduce patient discomfort without increasing the risk of infection.[21, 22]

Even though the 33G needles did not lower pain scores, these ultrathin needles may offer other advantages. We found no significant difference in complication rates, but we

did find a trend towards more vitreal reflux with the 30G needle. This was also seen in previous studies which noted less reflux with thinner needles.[3, 23] Whether this is clinically relevant remains to be seen, since reflux from IVIs usually consist of liquefied vitreous and hardly affects the quantity of anti-VEGF delivered in the vitreous cavity.[24-26] Reflux could, however, prevent a peak in intraocular pressure (IOP) directly after the IVI.[23, 27] Manual palpation after the procedure did not identify any patients with clear IOP increase in this study and no IOP related adverse events were reported. What the exact consequences are of a transient rise in IOP due to decreased reflux remains unknown. Increased reflux probably does indicate more scleral damage. The substantially smaller diameter of the 33G needle (0.21mm compared to 0.31mm) is surely less damaging to the eye wall, as already demonstrated in porcine eyes.[28, 29] We investigated this assumption further by measuring the difference in maximum penetration force required to puncture the sclera in porcine eyes between both needles. We observed that the 33G needle required less force to penetrate the sclera as compared to the 30G needle. However, this difference was small and could not be felt by the surgeon performing the injection. We should keep in mind that long term studies on anti-VEGF treatment show that 32-65% of patients still require IVIs after 4 years and the number of injections received in that period can accumulate to over 40 injections.[30-33] It would be interesting to explore whether 33G ultrathin needles are better tolerated when such high numbers of IVIs are administered.

An obvious strength in this study is the use of a crossover design where every case served as its own control. This prevents large interpersonal differences in pain perception as a cause of differences in pain scores. Moreover, in crossover studies, differences in pain scores are not dependent on baseline differences that may coincidentally arise from randomization. This crossover trial also includes some potential limitations that should be discussed. An apparent limitation would be that this trial was conducted with a relatively small sample size of 36 patients. This number was big enough to show a difference in pain score of at least 1.0 on a 0-10 NRS, which is the minimum to be considered a clinically relevant reduction in pain score.[34, 35] However, this number is quite low with regard to the explorative analyses for pain correlations with psychological factors and results should be interpreted with caution.

In conclusion, ultrathin 33G needles did not significantly reduce pain scores in this study. For now, there seems to be no clear benefit of IVIs with 33G needles. Also taking into account that 33G needles are more expensive (a price difference varying from €5.00 to €30.00 per 100 needles), there is no immediate reason to start using ultrathin 33G needle for IVIs. The pain scores during IVI were low, and opportunities to improve patient experience appear to lie more in management of patient expectations, stress reduction and the use of better tolerated disinfectants.

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Mechanisms of preventive treatments for AMD

Chapter 7

Zinc supplementation inhibits complement activation in age-related macular degeneration.

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Purpose: Age-related macular degeneration (AMD) is the leading cause of blindness in the Western world. AMD is a multifactorial disorder but complement-mediated inflammation at the level of the retina plays a pivotal role. Oral zinc supplementation can reduce the progression of AMD but the precise mechanism of this protective effect is as yet unclear.

Methods: We investigated whether zinc supplementation directly affects the degree of complement activation in AMD and whether there is a relation between serum complement catabolism during zinc administration and the *complement factor H* (*CFH)* gene or the *Age-Related Maculopathy susceptibility 2 (ARMS2)* genotype. In this open-label clinical study, 72 randomly selected AMD patients in various stages of AMD received a daily supplement of 50 mg zinc sulphate and 1 mg cupric sulphate for three months. Serum complement catabolism— defined as the C3d/C3 ratio—was measured at baseline, throughout the three months of supplementation and after discontinuation of zinc administration. Additionally, downstream inhibition of complement catabolism was evaluated by measurement of anaphylatoxin C5a. Furthermore, we investigated the effect of zinc on complement activation *in vitro.*

Results: AMD patients with high levels of complement catabolism at baseline exhibited a steeper decline in serum complement activation ($p<0.001$) during the three month zinc supplementation period compared to patients with low complement levels. There was no significant association of change in complement catabolism and *CFH* and *ARMS2* genotype. *In vitro* zinc sulphate directly inhibits complement catabolism in hemolytic assays and membrane attack complex (MAC) deposition on RPE cells.

Conclusion: This study provides evidence that daily administration of 50 mg zinc sulphate can inhibit complement catabolism in AMD patients with increased complement activation. This could explain part of the mechanism by which zinc slows AMD progression.
This trial was registered with The Netherlands National Trial Register; NTR2605; http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2605

Introduction

Worldwide, age-related macular degeneration (AMD) affects 30-50 million people and is the leading cause of blindness in the Western world.[1-4] AMD is a complex, multifactorial disease that manifests clinically as a loss of central vision resulting in an inability to read, recognize faces or discriminate colors. The hallmark lesions of early stage AMD are drusen, which are pathological deposits of extracellular material that form between the retinal pigment epithelium and Bruch membrane.[5] The late stages of AMD can be separated into geographic atrophy and neovascular AMD.[5] In patients with neovascular AMD, choroidal blood vessels invade the central retina and subretinal space causing a rapidly progressive loss of vision.[5] Although the neovascular AMD accounts for 10% of all AMD patients, it is responsible for the majority of AMD-related severe visual impairment.[6-9] Despite the beneficial effects of intraocular injections of vascular endothelial growth factor A (VEGF-A) inhibitors,[10, 11] a large percentage of neovascular AMD patients continue to lose vision.[12, 13] In patients with geographic atrophy, loss of the RPE and photoreceptor cells in the central retina result in a progressive decline of vision at a much slower rate than neovascular AMD.[5] Unfortunately, an effective therapy for treating geographic atrophy has yet to be developed.

Pivotal studies performed during the past decade have changed our understanding of the molecular mechanisms underlying AMD. These findings have led to the exploration of a new therapeutic paradigm for managing AMD, namely the targeting of specific molecular components in the complement pathway.[14, 15] The complement system is a major component of innate immunity with crucial roles in the first line defense against invading microorganisms, clearance of the apoptotic cells and modulation of the adaptive immune response.[16] There are three pathways of complement activation: the classical, the lectin and the alternative pathway.[16] The most important step of the alternative complement pathway activation is the formation of unstable C3 convertase C3bBb, which cleaves C3 to generate the active fragment C3b. Deposition of C3b on the target surface triggers the effector molecules C3a, C5a and the membrane attack complex (MAC), resulting in inflammation and cell lysis. The discovery that drusen contain proteins of the alternative complement pathway led to the hypothesis that drusen could be involved in local complement-mediated inflammation.[17, 18] Moreover, the discovery of a strong association between AMD and genetic variants in *CFH* gene, a major inhibitor of the alternative pathway, provided a second line of evidence in support of the inflammation model.[19-22] In addition to *CFH*, several other AMD risk variants have been found in genes underlying the alternative pathway.[23-26] A third line of evidence supporting complement involvement in AMD came from studies that showed that AMD patients have higher levels of complement activation products in their blood.[27-30] However, it is likely to be several years before any of the complement inhibiting drugs

will be approved for routine use in clinical practice, assuming they are eventually found to be safe and effective.

In 2001, the data collected from the Age-Related Eye Disease Study (AREDS) revealed that patients who were treated with zinc—either alone or in combination with vitamins—had reduced progression to advanced AMD.[31] Based on these results, AREDS recommends that persons who are older than 55 years of age and who are at risk for developing advanced AMD should consider taking vitamin supplements plus zinc.[31] A report published by the Blue Mountains Eye Study, a population-based study, confirmed the beneficial effect of zinc in AMD patients.[32] The large population-based Rotterdam Study supported the hypothesis of biological interactions between the *CFH* gene Y402H variant and zinc, β-carotene, lutein/zeaxanthin and omega-3 fatty acids and between the *ARMS2* gene A69S variant and zinc and omega-3 fatty acids.[33] As a result of these findings, the Rotterdam Study recommended that clinicians give dietary advice to young individuals who are at risk for AMD.[33] More recently, AREDS2 demonstrated that addition of lutein, zeaxanthin and omega-3 long-chain polyunsaturated fatty acids to the AREDS formulation, did not further reduce risk of progression to advanced AMD.[34] However, exploratory subgroup analyses demonstrated that addition of lutein and zeaxanthin to the AREDS formulation, resulted in a significant reduction of progression to advanced AMD for persons in the lowest quintile of dietary intake, suggesting different treatment effects within subgroups of AMD patients.[34] Despite the widespread use of zinc and antioxidants among AMD patients, the mechanism by which zinc exerts its beneficial effects in AMD patients has not yet been identified. The design of optimal and appropriate therapies require a comprehensive understanding of the factors that drive and delay pathogenesis of AMD. To add to current knowledge we designed the present study to investigate whether zinc affects the activity of the alternative complement pathway in patients with AMD, which might explain how zinc slows AMD progression in subgroups of patients with AMD. Secondly, we correlate the response to zinc supplements to the *CFH* and *ARMS2* genotype status. Lastly, we conducted an in vitro experiment to evaluate whether there is a direct effect of zinc on complement activation.

Methods

Study population of the clinical study

This study was performed in accordance with the Declaration of Helsinki and the Dutch Medical Research Involving Human Subjects Act. Prior to the study, we obtained approval from the local ethics committee (Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen, April 20th 2010) as well as written informed consent from all participants. This clinical study was registered with The Netherlands National Trial

Register (number NTR2605) shortly after recruitment began due to an administrative error. The authors confirm that all ongoing and related trials for this drug/intervention are registered. The protocol for this trial and supporting TREND checklist are available as supporting infromation (Protocol S1 and Checklist S1). The study participants were enrolled in EUGENDA (www.eugenda.org), a multicenter database for the clinical and molecular analysis of AMD, between March 2006 and August 2009. Patients with various stages of AMD were selected at random from the EUGENDA database and were included between June 2010 and February 2011. Follow-up ranged between 14 and 22 months. All data were collected at the department of Ophthalmology of the Radboud university medical center. We excluded individuals who had a core body temperature above 38°C and/or received antibiotics at baseline. In addition, we excluded patients who were receiving intraocular anti-angiogenic treatment, individuals with atypical hemolytic uremic syndrome or membranoproliferative glomerulonephritis type 2 and patients who received local or systemic steroid therapy within the three months prior to the trial. A total of 72 AMD patients were included in this study (Figure 1).

Study design

To study the effect of zinc on complement activation in patients with AMD, 72 AMD patients received a daily oral supplement containing 50 mg zinc sulphate and 1 mg cupric sulphate in capsule form. The capsules were to be taken at home for a period of three months. These components were donated by Sanmed, Almere, the Netherlands. The 50 mg dose of zinc was lower than in the original AREDS formulation and was chosen to minimize the chance of side-effects. Also, we chose zinc sulphate instead of zinc oxide (as used in the AREDS study), because most over-the-counter supplements contain zinc sulphate and in addition there is evidence that the bioavailability may be higher.[35, 36] The primary endpoint of the study was a change in serum complement catabolism during the three months of zinc supplementation. AMD patients have increased serum levels of C3 and the metabolic byproduct C3d, the most prominent marker of chronic activation of the alternative complement pathway.[30] To correct for individual variations in the level of C3, complement activation was defined as the C3d/C3 ratio as described previously.[30] Anaphylatoxin C5a levels are also elevated in AMD patients and promote choroidal neovascularization.[28, 29, 37] In order to explore downstream inhibitory effects of zinc on complement catabolism, we additionally measured serum C5a levels during the study period. The second objective was to study the association of serum complement catabolism during zinc administration and genotypes of AMD risk variants in *CFH* or *ARMS2.*

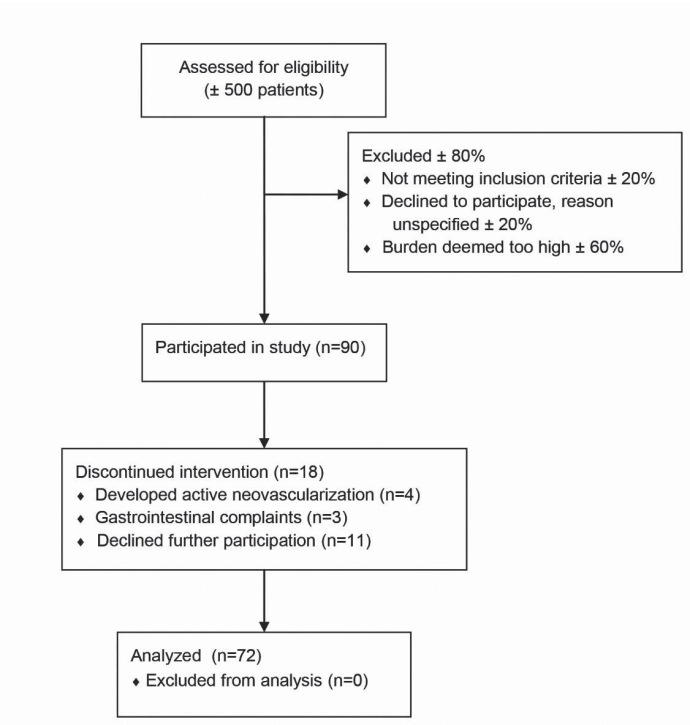

Figure 1. Flow diagram of patient inclusion

During the course of the study, six venous blood samples were collected. One sample was collected prior to zinc supplementation and served as the baseline sample. Three samples were collected at the end of months 1, 2 and 3 of the three-month period of zinc supplementation. We collected a fifth blood sample two months after ending the zinc administration (i.e., at the end of month 5) to check for any reversible effects on complement activation. A final blood sample was collected in months 14-22. From one month prior to zinc supplementation through the end of month 5, the patients were prohibited to take any type of nutritional supplement; from month 5 onwards, the patients were free to take supplements at their own discretion.

To identify clinical manifestations associated with intermittent infections, at every visit, we performed a general physical examination, measured the serum C-reactive protein (CRP) levels and administered a questionnaire that was aimed at identifying clinical manifestations associated with intermittent infections. At every visit, patients were asked whether they had been taking the zinc supplements daily to promote compliance. We also assessed the best-corrected visual acuity using Early-Treatment Diabetic Retinopathy Study (ETDRS) charts at every visit. In addition, we imaged the retinas using high-resolution spectral-domain optical coherence tomography (SD-

OCT) to detect active neovascular manifestation of AMD. We performed color fundus photography at baseline to assist in AMD grading based on the 5-grade Clinical Age-Related Maculopathy Staging (CARMS) classification scale.[38]

Complement measurements and genotyping in AMD patients

Serum was prepared by coagulation at room temperature and after centrifugation the samples were stored at -80 °C within one hour after collection. C3 and C3d were measured in serum samples as described.[39, 40] All C3 and C3d measurements in this study were performed in a single experiment, except for the final sample in months 14-22. C5a was measured by ELISA at a 1/10 dilution using a commercially available development kit (DuoSet) for human complement component C5a (R&D Systems, Minneapolis, USA). All the samples collected at baseline to month 5 were measured in a single run.

The *CFH (*Y402H; rs1061170) and *ARMS2 (*A69S; rs10490924) SNPs were genotyped as described.[41]

Serum zinc concentration was measured by atomic absorption spectroscopy with the spectrophotometer 1100 B from Perkin Elmer. CRP levels were measured by Abbott Architect C16000 system. The immunoturbidimetric test for CRP was provided by Abbott Diagnostics (Abbott Diagnostics).

In vitro hemolytic assays and membrane attack complex (MAC) deposition on RPE cells

We designed in vitro experiments to provide evidence of a direct effect of zinc on the complement pathway. Human serum was prepared from blood of several healthy volunteers after written informed consent had been obtained with the specific permit (418/2008) from the ethics committee of Lund University. Commercially available rabbit erythrocytes (Håtunalab, Bro, Sweden) were washed in 2.5 mM veronal buffer pH 7.3, supplemented with 70 mM NaCl, 140 mM glucose, 0.1% porcine gelatin and 7 mM $MgCl_2$. Different concentrations (0-64 μM) of zinc sulphate (Merck) were pre-incubated with 2% serum in the same buffer for 1.5 h at 37°C, followed by 1 h incubation at 37°C together with the erythrocytes. The amount of lysed erythrocytes was determined from the amount of released hemoglobin at 405 nm using Cary 50 MPR microplate reader (Varian).

To study the effect of zinc on membrane MAC deposition on human RPE cells subjected to oxidative stress, RPE cells (ARPE-19, ATCC) were cultured in DMEM/F12 media (HyClone), supplemented with 10% FCS (Gibco) and antibiotics (HyClone). After detachment using trypsin, the cells were incubated in medium containing 10 mM H_2O_2 for 2 h at 37°C, to mimic oxidative damage and make them amenable to attack from complement.[42, 43] After washing with PBS, the cells were incubated with 5% human serum, together with 0-250 μM zinc sulphate, in the veronal buffer defined above , for 1 h at 37°C. The amount of MAC deposited on the RPE cells was detected using

a monoclonal C9 neoepitope antibody (aE11, Hycult), which only recognizes C9 in the C5b-9 complex, followed by a FITC-conjugated secondary antibody and flow cytometric analysis (Partec).

Statistical analysis

A sample size of 70 was calculated to detect a decrease in serum C3d/C3 of 10% after 3 months, using the complement levels from a previous study to estimate variation,[30] with α=0.05 and a power of 80%.

Change in serum zinc, change in C3d/C3 ratio and change in C5a over the entire study period (0 to 14-22 months) were all modeled separately. Changes in serum zinc concentration were analyzed using a linear mixed-effects model with zinc concentration as the dependent variable. To make optimal use of repeated measures and to allow for correction of baseline differences, changes in C3d/C3 and C5a levels level were analyzed using linear mixed-effects models with C3d/C3 ratio or C5a as the dependent variable. The interaction between time and baseline complement levels was included in a linear mixed-effects model to study any baseline effects. To illustrate the effect of the baseline C3d/C3 ratio we plotted the course of the C3d/C3 ratio for 3 groups with different baseline ratios using the raw data. In a recent study we measured C3d/C3 levels in 150 unaffected control subjects of 65 years and older,[30] and we used the mean value (1.5) and standard deviation (0.6) to determine the cut-off points. The cut-off values for the different groups were selected by taking the mean C3d/C3 ratio and one standard deviation above and below the mean of the healthy control group. Our population was not large enough to create groups of individuals with two standard deviations above and below the mean. This resulted in the following three groups: 1. patients with baseline ratio ≥2.1 (n=16); 2. patients with ratios between 1.5-2.1 (n=29); and 3. patients with ratio <1.5 (n=31). Only very few subjects (n=3) had a baseline ratio more than one standard deviation below the mean, so these individuals were included in group 3. The associations between the complement levels throughout the study and *CFH* and A*RMS2* genotype, age, gender, CRP level and zinc level were also studied using a linear mixed-effects model. In the final models for the change in zinc, C3d/C3 and C5a, only significant predictors were used ($p < 0.05$). For the final serum zinc and C5a models this meant the inclusion of time and baseline values as the independent variables. In the final C3d/C3 model the independent variables were time, baseline C3d/C3 and the interaction between time and baseline C3d/C3.

To further explore the relationship between baseline complement catabolism and other patient characteristics at baseline, we assessed the associations of age and baseline visual acuity with baseline C3d/C3 ratio and C5a using the Pearson correlation and the Spearman's rank correlation coefficient. The difference in baseline complement catabolism between different genotypes was assessed using one-way ANOVA.

Because patients often display different stages of AMD in each eye, we created five groups for both eyes. These groups were based on the CARMS classification as follows: (CARMS grade 2:2), small drusen and/or RPE changes in both eyes; (CARMS grade 3:3), large drusen and/or drusenoid RPE detachment in both eyes; (CARMS grade 2: 4-5), small drusen and/or RPE changes in one eye and geographic atrophy or choroidal neovascularisation in the other eye; (CARMS grade 3:4-5), large drusen and/or drusenoid RPE detachment in one eye and geographic atrophy or choroidal neovascularisation in the other eye; and (CARMS grade 4-5:4-5), geographic atrophy or choroidal neovascularisation in both eyes. The association between baseline systemic complement catabolism and CARMS classification was tested using one-way ANOVA with a post-hoc Bonferroni correction. The correlation between baseline visual acuity and CARMS was tested using the Spearman's rank correlation coefficient.

Visual acuity changes during the course of the study were assessed by generalized estimated equations (GEE). The GEE model estimated the probability of low vision (LogMAR < 0.5) versus high vision (LogMAR > 0.5), with time and baseline C3d/C3 ratio as predictors. Data for the hemolytic assay and the RPE cell assay were analyzed using one-way ANOVA with Dunnett's multiple comparison test.

Reported p-values are two-sided, and differences were considered to be statistically significant if lower than 0.05. All statistical analyses were performed using SPSS, version 18.0.

Results

To evaluate the effect of receiving zinc supplements on systemic complement catabolism, AMD patients received oral zinc sulphate. The baseline characteristics of the study population are presented in Table 1. Serum zinc concentration increased significantly during the supplementation period ($p<0.001$) and returned to baseline levels two months after the zinc supplements were discontinued (Figure 2). The mean complement activation level, defined as the C3d/C3 ratio, in the 72 patients showed tendency to decline (albeit not significantly; $p=0.149$) during the three months of zinc supplementation (Figure 2). From month five onwards, 36 patients indicated they had been using over-the-counter zinc supplements, but generally in lower dosages than used in this study.

Table 1. The baseline characteristics of the study population.

Baseline characteristics	**AMD, n=72**
Mean age — years ± SD	73.9 ± 8.3
Sex, male — No. (%)	29 (40.3)
Visual acuity OD — median (1^{st}-3^{rd} quartile)	20/83 (20/400-20/25)
Visual acuity OS — median (1^{st}-3^{rd} quartile)	20/55 (20/333-20/26)
Mean C3d/C3 ratio ± SD	1.65 ± 0.69
Mean zinc level — µmol/l ± SD	13.33 ± 2.83
CFH (Y402H; rs1061170) genotypes, No. (%)	
CFH TT genotype (wildtype)	1 (1.4)
CFH CT genotype	36 (50.0)
CFH CC genotype	34 (47.2)
ARMS2 (A69S; rs10490924) genotypes, No. (%)	
ARMS2 GG genotype (wildtype)	19 (26.4)
ARMS2 TG genotype	30 (41.7)
ARMS2 TT genotype	22 (30.6)
Serum C-reactive protein (CRP), No. (%)	
< 5 — mg/l	52 (72.2)
5 - 15 — mg/l	18 (25.0)
16 - 45 — mg/l	2 (2.8)

SD=Standard deviation, visual acuity in Snellen.

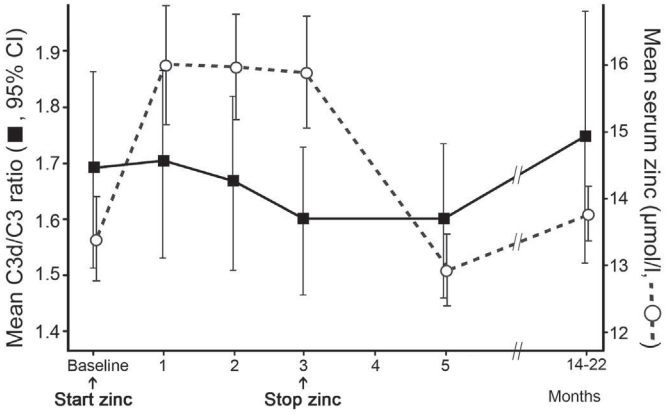

Figure 2. Serum zinc concentration and C3d/C3 ratio throughout the study period.
During the three months daily zinc supplementation, serum zinc concentration increased significantly ($p<0.001$). After zinc supplementation was discontinued, the serum zinc levels returned to baseline levels within 2 months. The C3d/C3 ratio showed non-significant decline during zinc supplementation ($p=0.149$).

Exploration of effect of zinc supplementation on complement catabolism

We conducted further exploratory analyses whether zinc supplementation may have different effects within patients with different levels of baseline complement catabolism defined as C3d/C3 ratio. In this analysis, we observed a strong interaction between baseline C3d/C3 ratio and change in C3d/C3 ratio during zinc supplementation ($p<0.001$). The AMD patients with relatively high baseline levels of serum complement catabolism exhibited a more pronounced decline in their C3d/C3 ratio during the administration of zinc sulphate, compared to those AMD patients with lower baseline levels. After the zinc supplementation period, the decline in C3d/C3 ratio remained at this lower level for the following two months. Measurements performed at least nine months later (in months 14-22) showed that complement activation had returned to baseline levels. The AMD patients who already had a relatively low C3d/C3 ratio at baseline showed no decline in C3d/C3 ratio throughout the treatment period. Figure 3 illustrates the course of serum C3d/C3 ratio over time in three groups with different baseline C3d/C3 ratios. The statistical model was not based on these cut-off points. There was no significant association between C3d/C3 ratio and age or gender throughout the course of the study. C5a levels decreased significantly over the three-month supplementation period ($p=0.019$) (Figure 4). We observed a similar baseline effect for the course of C5a levels, however, the interaction between baseline and time was not significant and therefore not included in the final model ($p=0.065$).

Association between the stage of AMD and serum complement catabolism

We further analyzed the clinical characteristics of AMD patients with a relatively high baseline complement catabolism. Higher baseline C3d/C3 ratio was significantly associated with younger age (r=-0.33, p=0.005) and better visual acuity (OD: r=0.25, p=0.031 and OS: r=0.36, p=0.002). Also, baseline C3d/C3 ratio was associated with the CARMS classification based on both eyes (p=0.010). Post hoc analysis revealed that patients with large drusen and/or drusenoid RPE detachment in one eye and geographic atrophy or choroidal neovascularization in the other eye (3:4-5) had higher baseline complement catabolism compared to geographic atrophy or choroidal neovascularization in both eyes (4-5:4-5) (Table 2). There was no association with baseline C5a and age (r=0.068, p=0.583), visual acuity (OD: r=-0.110, p=0.381 and OS: r=-0.023, p=0.855) or the CARMS classification based on both eyes (p=0.947). C3d/C3 ratio and C5a levels were measured in separate experiments and were not correlated (r=0.086, p=0.490). As expected, baseline visual acuity for each eye was strongly associated with the CARMS classification per eye (OD: r=-0.69, p<0.001 and OS: r=-0.65, p<0.001) (Table 3).

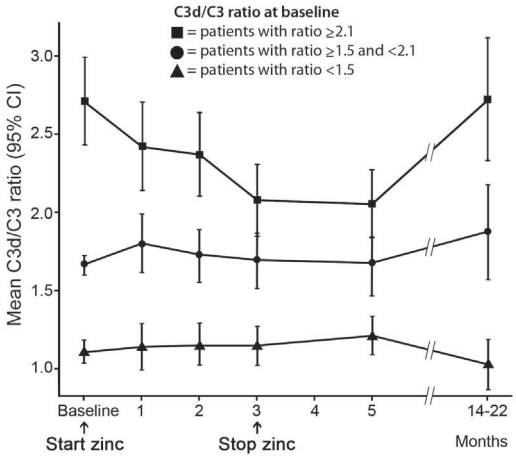

Figure 3. The effect of zinc supplementation on patients with different level of complement catabolism at baseline.

The patients with high serum complement catabolism had a steeper decline in C3d/C3 ratio during the administration of zinc sulphate (p<0.001).

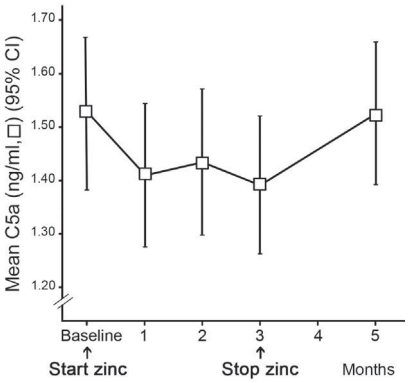

Figure 4. C5a concentration throughout the study period.
The C5a levels decreased significantly during the three months of zinc supplementation and returned to baseline level within 2 months after the cessation of zinc supplementation.

Table 2. Association between the stage of AMD and serum complement catabolism.
Compared to the patients with intermediate AMD in one eye and late AMD in the other eye (CARMS stage 3:4-5), the patients who had late AMD in both eyes (CARMS 4-5:4-5) had significantly lower C3d/C3 levels (p=0.006).

Clinical Age-Related Maculopathy Staging (CARMS) for both eyes	**Mean C3d/C3 ratio (SE)**	**No. (%)**	**p***
Grade 2 in both eyes (2 : 2)	1.64 (0.30)	4 (5.6)	1.000
Grade 2 in the first eye and grade 4 or 5 in the second eye (2 : 4-5)	1.69 (0.31)	9 (12.7)	1.000
Grade 3 in both eyes (3 : 3)	1.86 (0.17)	10 (14.1)	0.263
Grade 3 in the first and stages 4 or 5 in the second eye (3 : 4-5)	2.01 (0.20)	19 (26.8)	0.006
Grades 4 or 5 in both eyes (4-5 : 4-5)	1.32 (0.06)	29 (40.8)	Ref.

* p-value from one-way ANOVA with post hoc Bonferroni correction

Table 3. Visual acuity per CARMS classification grade.
Baseline median visual acuity in Snellen per Clinical Age-Related Maculopathy Staging (CARMS) classification grade for the separate eyes. Visual acuity decreases as CARMS classification increases.

Clinical Age-Related Maculopathy Staging (CARMS)	**Visual acuity, median (1st-3rd quartile)**	
	OD	**OS**
Grade 2	20/20 (20/25-20/20)	20/22 (20/40-20/20)
Grade 3	20/25 (20/40-20/21)	20/25 (20/50-20/20)
Grade 4	20/143 (20/200-20/40)	20/63 (20/200-20/40)
Grade 5	20/400 (20/1000-20/133)	20/267 (20/1000-20/80)

Visual acuity in Snellen

Correlation between the serum complement catabolism and the genotype

The baseline C3d/C3 ratios did not differ significantly between wildtype/heterozygous Y402H *CFH* genotype and the homozygous Y402H genotype (p=0.934) nor between *ARMS2* genotypes (p=0.729). There was also no difference in baseline C5a between *CFH* (p=0.597) and *ARMS2* genotypes (p=0.412). Change in C3d/C3 ratio or C5a levels were not related to *CFH* or *ARMS2* genotypes.

Intermittent infections and the C3d/C3 ratio

Serum CRP levels were measured at every visit and were not significantly associated with the C3d/C3 ratio (p=0.168) nor the C5a levels (p=0.942). Questionnaires demonstrated that antibiotics were prescribed in 10 patients during the study period. Use of antibiotics was not related to increased CRP levels, increased body temperature or elevated C3d/C3 ratio (data not shown) in these individuals.

Effect of zinc on complement catabolism in vitro

To demonstrate the *in vitro* effect of zinc on the complement activity of human serum and to better understand the effect observed in vivo in the patients, we performed an alternative pathway hemolytic assay. Results showed that zinc sulphate inhibits the lysis of rabbit erythrocytes in a dose-dependent manner (Figure 5A). Retina is exposed to high levels of oxidative stress from light exposure and metabolic processes.[44] We tested *in vitro* whether zinc could also protect the RPE from a oxidative stress related damage from the complement system. The test results show that the amount of MAC deposited on RPE cells exposed to oxidative stress can be reduced in a dose dependent manner by zinc sulphate (Figure 5B-C). In the negative controls, zinc and serum were omitted.

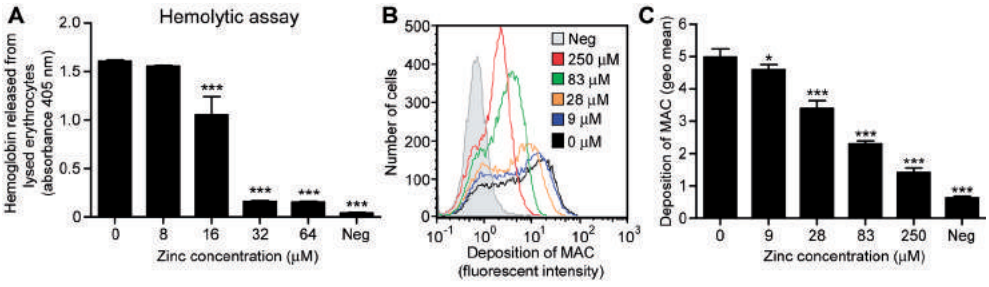

Figure 5. The effect of zinc on the hemolytic activity of human serum and on the membrane attack complex (MAC) deposition on retinal pigment epithelial (RPE) cells. **(A)** Zinc sulphate inhibits the lysis of rabbit erythrocytes in a dose-dependent manner. **(B-C)** the amount of MAC deposited on RPE cells exposed to oxidative stress can be reduced in a dose dependent manner by zinc. *p<0.05 and ***p<0.001.

Discussion

In the past decade, it has become increasingly clear that complement-mediated inflammation plays a fundamental role in the etiology of AMD.[18, 45] All current therapies for treating neovascular AMD are designed to reduce the ongoing VEGF stimulus—and hence inhibit the growth of new vessels—but do not address the underlying pathology. Moreover, no effective therapy has been developed for treating early AMD or geographic atrophy. The discovery of complement as a major contributing factor to AMD pathogenesis has sparked considerable interest in this system as a potential therapeutic target, and various complement inhibitors are currently being tested in clinical trials.[14, 15]

Our findings suggest that increased complement catabolism, defined as the C3d/C3 ratio, in AMD patients can be reduced by the daily oral administration of 50 mg zinc sulphate. However, the effect of complement inhibition seemed to be limited to patients with a high baseline level of complement catabolism. The C3d/C3 ratio returned to its baseline value after the supplementation period, indicating a reversible effect of zinc supplementation on complement activation. Continuous zinc supplementation may therefore be necessary to inhibit complement activity over longer periods of time. Approximately 50% of patients indicated they had been using zinc supplements during the period before the last measurement. However, the zinc dosage and possibly compliance in these patients was apparently too low to exert a clear effect on complement levels.

We then linked the degree of serum complement activation to the clinical stages of AMD and found that the level of serum complement activation is correlated with patients having large drusen and/or drusenoid RPE detachment. It has been demonstrated that 42% of patients with drusenoid RPE detachment progress to end-stage AMD and develop profound and irreversible visual loss within five years.[46] The AREDS1 study showed that this group in particular benefits from zinc plus antioxidant supplementation. Our results suggest that this may be related to increased activation of the alternative complement pathway in this group, which would support the notion that patients with large drusen and/or drusenoid RPE detachment should receive supplements. Although correlation coefficients were modest, higher complement levels at baseline were mostly observed in younger patients with better visual acuity, corresponding with less advanced disease. This could indicate that the use of supplements should not be postponed until more advanced stages of the disease.

C5a showed a significant decrease during zinc supplementation, indicating that zinc inhibition of the complement pathway can also be detected further downstream. We observed a similar pattern of increased inhibitory effect in patients with higher baseline C5a levels, but this was not as profound as for the C3d/C3 ratio. This could be explained by the more unstable nature of C5a as compared to the C3d/C3 ratio which corrects for

intrapersonal fluctuations. C5a returned to its baseline value within 2 months after the supplementation period also suggesting a reversible effect of zinc on C5a.
The exact role of genotype in the response to zinc and antioxidant supplements remains unclear. The Rotterdam study showed that high dietary zinc intake reduces the risk of AMD associated with the *CFH* Y402H variant, suggesting a relationship between zinc and this genotype.[33] A recent subgroup analysis, utilizing data from the AREDS study, showed that the response to zinc and antioxidants may be influenced by *CFH* and *ARMS2* genotype. Their results suggested that patients carrying the *CFH* Y402H risk allele have no benefit from zinc supplementation on the 10-year disease progression.[47] In previous studies by AREDS study researchers on the interaction between genotype and treatment response, they found that the AREDS supplements may be less effective in reducing progression in carriers of the *CFH* risk allele.[48] But in a later publication they could not corroborate the interaction and could not find any relation with response to zinc or antioxidants and genotype.[49] Notably, a biochemical study of zinc and factor H showed that the interaction between zinc and the factor H protein was not influenced by the *CFH* Y402H variant.[50] In our study, genotype did not have an effect on baseline or change of complement activation levels. Given the small number of study participants our study probably lacks the power to detect a possible interaction between *CFH* or *ARMS2* genotype and zinc supplementation.
Changes in C3 activation over time can also be caused by various factors related to immune defense in case of infection.[51] Since serum CRP levels were not significantly associated with C3d/C3 ratio, it is unlikely that the observed change in complement catabolism can be ascribed to an intermittent infection. Data obtained from a general physical examination and a questionnaire aimed at identifying clinical manifestations of intermittent infections also did not point to an infectious cause for the change in complement levels in these AMD patients. Finally, it is unlikely that the study results were influenced by the statistical phenomenon of 'regression to the mean' because the C3d/C3 ratio returned to baseline levels for after discontinuation of zinc administration.
In further support of our hypothesis that zinc administration affects complement catabolism, we demonstrated *in vitro* that zinc sulphate directly inhibits complement activation in human serum in a dose-dependent manner. In addition, we demonstrated that during oxidative challenge the presence of zinc sulphate diminishes MAC deposition on RPE cells, thereby preventing complement-mediated cytolysis and apoptosis. This implies that zinc not only has the ability to reduce systemic activation of the alternative complement pathway, but may also diminish complement activation locally on RPE cells. Important to note is that zinc concentrations were in physiological levels,[44, 52] and therefore have biomedical significance. A previous biochemical study showed that oligomerization of the CFH protein occurs in the presence of zinc, theoretically leading to increased complement activation.[53] A more recent biochemical study from researchers of the same study group demonstrated that factor H-C3b complexes are precipitated by

zinc which would inhibit complement activation.[54] Thus we cannot pinpoint the exact molecular mechanism behind our observations, however, we can conclude that zinc inhibits systemic complement activation and local MAC deposition preventing RPE cell damage.

This study has some limitations that should be addressed. A relatively small number of subjects were included, and zinc was administered for a relatively brief period of time. Patient compliance was monitored through questionnaires, which could potentially underestimate zinc intake. However, a steep increase in serum zinc following the initiation of treatment indicated that supplementation had been successful. Because of the slow natural progression of AMD, this study was never designed to measure a direct protective effect of zinc on visual acuity. Larger patient cohorts and a longer period of zinc supplementation should also be studied to corroborate and extend our findings.

In summary, in our study increased levels of serum complement catabolism correlates with the stage of AMD. Our study demonstrate that increased levels of complement catabolism can be normalized by the daily oral administration of 50 mg zinc sulphate. Findings from the present study might explain how zinc slows AMD progression in subgroups of patients with AMD.

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Chapter 8

Discussion

The general aim of this thesis was twofold: [1] to identify areas in the treatment of neovascular age-related macular degeneration (nAMD) that are arguably in need of improvement, and [2] to assess different strategies to achieve such improvements. To achieve this overall aim, this thesis is structured around three more specific objectives: 1) to predict non-response to anti-VEGF treatment in patients with nAMD, 2) to evaluate and identify new areas for improvement of both the injection procedure and anti-VEGF treatment in clinical practice, and 3) to evaluate the cost- effectiveness of anti-VEGF treatment for nAMD. In this discussion, I will outline the main findings and put the results in a broader perspective. I will discuss what I believe is needed to improve non-response prediction, how differences between clinical practice and randomized clinical trials (RCTs) can affect treatment protocols and policies, and how costs in nAMD treatment may be controlled. Finally, specific recommendations will be given for future research.

The main findings of this thesis were the following:

- To predict non-response to anti-VEGF treatment in patients with nAMD (*objective 1*), we developed a prediction model in chapter 3a which showed that non-response to anti-VEGF drug ranibizumab can be predicted with moderate accuracy on the basis of a specific set of clinical and genetic factors.

- To identify new areas for improvement of both the injection procedure and anti-VEGF treatment in clinical practice (*objective 2*), we showed in chapter 2 and 4b that there is still a gap between efficacy as reported in clinical trials and that achieved in clinical practice, and in chapter 6 we showed that there are specific, practical ways to reduce the burden of the injection procedure.

- As for the cost- effectiveness of anti-VEGF treatment for nAMD (*objective 3*), we showed in chapter 5 that bevacizumab is the most cost-effective first choice treatment for AMD, but that it is not yet considered the standard of care across the globe.

Predicting non-response

In order to achieve objective 1 we developed a prediction model for early identification of non-responders. The main reasons for developing predict models for non-response were to reduce the treatment burden for patients and clinics and to control costs. Individualizing treatment decisions on the predicted level of response could reduce unnecessary, costly injections and help to keep the large patient flows manageable. Patients predicted to be non-responders can be diverted to other treatment strategies,

which can be either a second-line therapy, or withholding intraocular treatment combined with appropriate aftercare such as low vision measures.
We were able to develop a model that predicted non-response with 77% accuracy, meaning that 77% of nAMD patients were correctly classified as either a responder or a non-responder based on several clinical and genetic predictors. Patients in the highest risk score group had a probability of being non-responder of ~50%. Whether this accuracy of prediction is sufficient to change treatment guidelines is not so straightforward. The prediction model from chapter 3a and the identified genetic response predictors in chapter 3b-c make an important contribution to clinical practice by creating the awareness amongst ophthalmologists that non-response is a frequent problem. In addition, they show that non-responders may carry specific genetic and clinical traits. Especially in patients over 80 years old, with diabetes and poor starting vision it may be wise to switch to an alternative treatment more promptly. However, the performance of our prediction model is not 100%, and, therefore, some patients will be falsely classified as non-responder. Withholding treatment from those predicted to be non-responders is not ethical as 50% of those patients will actually benefit from treatment. Hence, although our prediction model comprises an important step towards personalized medicine, further improvements are needed before any type of prediction tool can be implemented into clinical practice, which I will discuss below.

1) Improving management of non-responders
The first improvement relates to what the second-line treatment strategy for non-responders should be. Without knowing what the effect of a second-line treatment is, it is difficult to estimate the value of a prediction model in clinical practice. To decide to withhold treatment when the model predicts non-response, it has to be very accurate as the consequence of not treating a nAMD patient can mean severe vision loss. However, when a good second-line treatment would be available, a non-responder could be offered that option, avoiding treatment delay. We evaluated the second-line treatment aflibercept in chapter 4a and observed adequate response from switching after non-response. However, it is unclear whether the prediction model we generated is specific for ranibizumab or whether it can be applied to all anti-VEGF agents. Therefore, non-responders may not necessarily show a good response to second line anti-VEGFs and all drugs should undergo similar modelling for comparison.

2) Improving accuracy of prediction
The second improvement refers to the accuracy of the prediction model. This means finding new predictors for non-response, but possibly also better understanding the role of known predictors. Understanding how predictors relate to non-response, may help to more accurately measure these predictors. The only truly established factors that predict visual acuity are baseline vision and age.[1-4] Low baseline visual acuity is

predictive of a greater gain in Early Treatment for Diabetic Retinopathy Study (ETDRS) letters, but of less gain in percentage of letters as shown in chapter 3a. Older age has also been consistently associated with poorer response, but the cause for this is unclear. Possibly the consistency of the vitreous plays a role, which is more liquefied in older age, leading to changes in pharmacokinetics. Indeed, vitreomacular adhesion caused by vitreous liquefaction has been associated with poor response in some studies,[5, 6] although others could not corroborate this finding.[7] Possibly, older age simply represents a more advanced disease or a longer disease duration, negatively influencing treatment effect.-
Genetics is an interesting area for finding new non-response predictors. AMD risk is largely genetically determined and many research groups have turned towards pharmacogenetics to explain the high variability in treatment response to anti-VEGF. The vast majority of pharmacogenetic studies have assessed single nucleotide polymorphisms (SNPs) known to be associated with the development of AMD. Of all assessed SNPs, very few have shown consistent associations with treatment response. The *CFH Y402H* SNP is the only SNP that has been evaluated in three separate meta-analyses. Each of these studies concluded that the C risk allele shows a poorer response with an odds ratio of ~1.6 comparing wildtype to homozygous risk allele carriers.[8-10] Many other SNPs have been evaluated,[11] among others in the genes *ARMS2*,[12-14] *HTRA1*,[12, 13, 15] *C3*,[12, 13, 16] and *VEGF*[16, 17] but none of these have been convincingly shown to be independently associated with treatment response. Possibly this is because there truly is no association with response, but it is also likely that any role that these individual SNPs play is small. Still what is lacking in all these pharmacogenetics studies is the mechanism behind their effect on non-response. How would these AMD-susceptibility loci be associated with response to a treatment that is basically symptomatic? Possibly the SNPs are indicative of more severe disease, but some have hypothesized that the aberrant inhibition of the complement system induces VEGF, explaining a resistance to anti-VEGF.[9, 18] Still, the biological relevance is unclear for most SNPs. In chapters 3b and 3c we therefore focused on SNPs with a biological relation to anti-VEGF treatment, namely SNPs in the VEGF pathway. We identified SNPs associated with visual acuity in the *VEGFR2* and *NRP1* genes, coding for the VEGF receptor and the VEGF co-receptor respectively. Interestingly, although these SNPs in itself had small effects, jointly they could make up a difference of 10 letters on average. This may not be enough to turn a responder into a non-responder, but is does indicate how increased burden of the VEGF pathway is involved in treatment response. In combination with other predictors it could improve the accuracy of an updated prediction model. After chapter 3b was published, investigators from the CATT and IVAN study have tried to replicate the results of the identified SNPs in *VEGFR2*,[19] but to no avail. Interestingly, neither the CATT nor the IVAN study has ever identified pharmacogenetic associations. Possibly, the strict treatment regimen leaves very little room for natural variability of response. This could indicate that genetic variants predispose to an increased vulnerability to undertreatment,

which is presumably more common in clinical practice settings as compared to trial settings. However, the possibility of false-negative findings should not be excluded, and all pharmacogenetic associations should be replicated in independent cohorts before including them in prediction models.[20]

3) Improving the definition of non-response

The third and possibly most important issue standing in the way of accurate and meaningful non-response prediction is that non-response itself is poorly defined. There is currently no generally accepted definition of non-response, but two major subtypes may be considered: functional and anatomical. The functional type refers to response in terms of change in function of the retina, usually expressed as visual acuity. Anatomical response is determined by change of neovascular activity on imaging modalities, commonly defined by central retinal thickness (CRT) on optical coherence tomography (OCT).

Functional response determined by visual acuity is the main outcome of RCTs as this outcome is necessary for the registration of a drug.[21-23] Visual acuity is what determines the quality of life of a patient and therefore is the more relevant outcome to the patient.[24] Nevertheless, there are some important limitations to using visual acuity to define response. We discuss in chapter 3a of this thesis how visual acuity is subject to the floor and ceiling effect when using an absolute value. We explain how patients with a low baseline vision are less likely to deteriorate in terms of visual acuity, simply because they have less vision to lose, and are therefore less likely to be classified as non-responder. The opposite holds for patients with high baseline VA. Eventual visual acuity, however, is usually higher in patients with good baseline vision than in patients with low baseline vision,[2, 3] so an absolute cut-off value for non-response will not necessarily select those patients that are worst off.

Another disadvantage of visual acuity as measure of response is that many other factors besides the efficacy of the drug may influence its change over time. Visual acuity can be influenced by scarring,[25] development of geographic atrophy[26] and photoreceptor damage.[27] These different causes of functional non-response likely represent different mechanisms, making accurate response prediction particularly difficult. In addition, contextual factors may play a role, such as treatment delay.[28, 29]

To avoid the difficulties of defining response as a loss of vision, many researchers and ophthalmologists opt for the use of OCT to identify non-responders. OCT features, such as CRT, are considered more objective measures compared to visual acuity. CRT, the mean thickness of the central 1 mm around the fovea, reflects the amount of intraretinal and subretinal fluid and is used to monitor anatomical response. A major drawback of using OCT to predict treatment response is that changes in retinal thickness are poorly correlated with changes in visual acuity.[30-32] As described before, visual acuity is

influenced by other factors than purely presence of fluid on OCT, so even though the treatment is effective on OCT, the patient may not experience any gain in vision.
When choosing critera for response it is also important to consider how the results should be interpreted. OCT measurements can be useful in etiological studies trying to explain the large variability in pharmacological response between patients. Visual acuity is a more appropriate measure to determine the value of a treatment to a patient. Therefore, we chose to use visual acuity as the outcome in chapter 3a for predicting non-response. However, it should be noted that both visual acuity and CRT may influence treatment strategy and thus both should be considered when trying to optimize treatment.
Ultimately, neither functional nor anatomical response is sufficiently accurate to define all non-response. Probably, response is much more complicated than we think. Visual acuity is likely a composite measure, an accumulation of damage to the retina by multiple causes.[33] Anatomical response could be a part of this. Fluid that does not resolve due to drug resistance could impair the ability to regain vision, but fluid itself also damages the retina and the photoreceptors, more so in some than in others. In addition, formation of fibrosis or geographic atrophy during treatment may be the cause of sustained vision loss.[33] Visual acuity will capture all of these entities, but we are not yet sure how all of these fit together. Possibly, all are part of a different mechanism of disease and each leads to non-response, although through a different pathway. When predicting response in a patient cohort, different reasons for non-response will play a role. Likely, one simple prediction model will not suffice to capture the complexity of treatment response. Anatomical response through CRT will not be exhaustive, because it does not reflect all different types of non-response; on the other hand, functional response through visual acuity might be too exhaustive. Maybe we should step away from trying to define non-response as if it were a single entity and start by accurately classifying non-responders in their different subgroups.

Response and non-response in clinical practice

In chapter 2 and 4b we showed that nAMD patients in Dutch clinical practice gain less vision than reported for patients in RCTs. The difference of visual gains is 5 ETDRS letters after 1 year and 10 letters after 2 years.[21, 22, 34] Similarly, for patients with diabetic macular edema (DME), we showed that the difference between RCTs and clinical practice is apparent in terms of change in visual acuity as well as improvement on optical coherence tomography (OCT). Patients in our clinics gained 3.5 letters on average over 1 year of treatment, while patients in RCTs can gain as much as 10 letters.[35, 36] The decrease in central retinal thickness (CRT) was also less prominent.
The observed effectiveness gap between trial data and clinical practice is a relevant difference. A change of 5 letters is the approximate minimum change in vision that can

be perceived by an individual and will likely affect quality of life.[37] Therefore, these large differences between RCTs and clinical practice cannot be ignored. First, establishing that a treatment is not as effective as it could potentially be, means we should be evaluating our treatment procedures carefully to find out why we are not reaching greater effectiveness. Second, management of nAMD patients in clinical practice may be more complicated than anticipated from RCTs. Non-response may be more frequent than anticipated and the search for alternative treatments becomes a more pressing matter. Third, when developing (inter)national treatment guidelines that rely on cost-effectiveness analyses, proper effectiveness estimates may make an important difference in which treatment should be preferred over the other.

We can deduce from chapter 2 and 4b that reasons for underachievement in clinical practice could be that patients tend to receive fewer injection and do not always complete the loading phase. For current clinical practice this means that ophthalmologists should be conscious of how they establish retreatment in a patient. Lowering the threshold for determining active disease would help to avoid undertreatment and should improve vision outcomes for patients. Also, to reduce the chances for a patient of not receiving a completed loading phase, ophthalmologists should schedule three loading phase injections as soon as the diagnosis is made..

Although these are valuable observations for clinical practice, it does not yet fully explain the difference with RCT data. There may be many other reasons why effectiveness is reduced in the real-world. In clinical practice patients may present themselves with AMD or DME who would not fit the criteria for inclusion in RCTs. For example, many RCTs on AMD will not include patients with visual acuity below 20/320, but these patients are treated in clinical practice, albeit with more modest results.[38] Additionally, in clinical practice follow-up may be more inconsistent and there may be more divergence from protocol. These can be related to practical issues leading to treatment delay, which are unfortunately often due to logistical issues such as arranging proper transport and waiting for approval by a third party payer.[28, 29, 39] We were unable to identify such causes from our own study, so this could be a relevant subject for future research.

Cost of anti-VEGF treatment

In chapter 5 we showed that bevacizumab is just as effective for AMD as the other anti-VEGF agents, that there is no proof that bevacizumab is less safe, and that it is substantially more cost-effective than either ranibizumab or aflibercept. Yet, many countries and ophthalmological societies have not adopted bevacizumab as first choice nAMD therapy. This is for an important part because of legislations that state that physicians are not allowed to prescribe off-label medication when equivalent registered alternatives are available.[40] Therefore, our findings should be directed at the policy

makers in health care and not just ophthalmologists who need to operate within legal constraints.

Registration of bevacizumab for AMD would solve much of the problem, but this registration can only be requested by the owner of the drug. Roche indicated that they will not further develop bevacizumab for intraocular use. Large investigator-driven, government-issued trials proving the effectiveness of bevacizumab have already been conducted[41, 42] so maybe a next step could be the formation of an independent government-issued committee to conduct the final assessment of the drug for registration.

Off-label prescriptions are common, not only in ophthalmology, and account for about 20% of prescriptions.[43] In our aging population with increasing need of care, regulating registration independent from pharmaceutical companies could become an important method to limit health care expenses.

Changing drug-registration policy will take time. An alternative solution could be to significantly reduce the price of ranibizumab and aflibercept. Drug manufacturers are free to ask any price for their drug and they are not obligated to be transparent regarding how this price was determined. Actual production costs are usually only a small part of the final price.[44, 45] On the other hand, governments are not obligated to accept any price the manufacturer offers. Lower prices can be negotiated.[46] As a last resort a government can issue a manufacturing licence to a different company, allowing them to produce the same drug at a much lower cost.[46] Cost-effectiveness analyses that include a societal perspective like in chapter 5 can support governments to negotiate fair prices for all parties involved.

Recommendations for future research to optimize AMD treatment

Prediction of non-response

A next step in finding new predictors and elucidating the role of genetics in treatment response could be to perform genome-wide association studies (GWAS). The GWAS assesses SNPs spanning the entire genome and provides a hypothesis free approach to identify genetic associations.[47] It provides the unique opportunity to identify genetic associations in new regions, pointing to previously unknown mechanisms of treatment response. Therefore, the GWAS could be an important tool to help us better understand the underpinnings of treatment response. There are, however, some hurdles to overcome. A GWAS tests a large amount of SNPs at once leading to a high chance of finding false positive associations.[48] To limit this phenomenon and to prevent the literature from overflooding with false associations, the GWAS requires a correction for multiple testing. Usually the significance threshold lies at $p=5x10^{-8}$, assuming there are approximately 1 million independent regions in the human genome. To reach this threshold, at least one of two things is needed: very large effect size, or big sample size. Both will be

challenging. The effect size, or odds ratio, of an average SNP identified in a GWAS is only 1.33.[49] Treatment response in a disease as multifactorial as AMD is unlikely to be associated with SNPs of large effect size. The largest pharmacogenetics study up to date was a collaboration between the CATT and the IVAN study, comprising 1347 patients.[19] Using a cut-off to define non-response will unlikely yield any information as that will leave the study with only ~130-260 (10-20%) of cases. Adapting the classic GWAS that compares cases to controls and utilizing response as a continuous variable may help increase the chance of finding associations.

Defining and better understanding non-response

Besides predictors, it is also important to re-evaluate the outcome of non-response. To develop individualized treatment strategies, we first need a better understanding of why an individual responds poorly in the first place. In DME we are becoming more aware of different pathways influencing response. In chapter 4b we showed that some patients do not respond to anti-VEGF, but do respond to intravitreal steroids. This could point towards different disease mechanisms at play. Although steroids have no particular role in AMD,[50] similarly different biological pathways could be involved in non-response in AMD-patients. We should start by classifying non-responders according to these pathways, such as scar formation or pharmacological resistance, and predictions could in turn become more focused on these specific biological pathways instead of clusters of pathways all leading to vision loss. This could make response prediction more complicated, especially in an ophthalmology clinic where physicians have only 10 minutes per patient, but it will probably better represent the actual biology of non-response, leading to more accurate and more relevant predictions.

Currently, we do not have clear definitions of non-response. Recently, guidelines were published by Amoaku et al. based on literature and expert opinion attempting to label non-response for use in clinical practice.[51] They have chosen a combination of visual acuity and CRT as definition. Moreover, they divide response into good, poor, and non-response. Good response was defined by resolution of fluid, and/or improved visual acuity of more than 5 letters. Poor response was less than 25% reduction from the baseline in CRT, persistent or new fluid, or change in visual acuity between 0 to 4 letters. Non-response was defined as an increase in retinal fluid or hemorrhage and/or loss of over 5 letters. These guidelines were developed in order to facilitate decision making for when to discontinue or switch treatments in clinical practice. It is important to adhere to these guidelines so as to make treatment across clinics more comparable. This definition is not necessarily the best or closest to the biology of non-response, but practically, we need to start somewhere. It would be interesting to see how these specific cut-offs are represented in clinical practice, how many patients would be considered non-responder and what the effect would be of a secondary treatment in this specific

group on visual prognosis. Also, we should evaluate whether quality of life is different between these subgroups to determine whether these are truly relevant distinctions.

Discrepancy between clinical practice and RCTs

We still poorly understand why there are such a large differences between the RCT and clinical practice populations. We saw in chapter 2 that patients presented themselves with relatively low baseline vision. This could be translated into either more severe disease in general or vision loss due to treatment delay. Treatment delay can be dealt with, so it would be important to find out what causes this, especially considering baseline vision is such an important predictor for further response. Although we learn about treatment delay from other research, a lot of these issues may be country- or even clinic-specific. For example, obtaining health insurance approval is currently not an issue in the Netherlands. Possibly, there is a lack of awareness among patients, or a delay in referral from other professionals such as the general physician or the optometrists. A structured mapping of Dutch patients' referral history could help determine what causes this delay, and interventions can be directed accordingly.

Costs assessment

Costs management of anti-VEGF treatment can be improved by including observations from clinical practice. Although we are confident that bevacizumab is rightfully the first choice in nAMD treatment, our cost estimates could still be improved. We know the clinical benefit and differences in efficacy of anti-VEGF drugs through RCTs comparing them to placebo treatment,[21, 22] followed by comparisons amongst the different anti-VEGF agents.[23, 41, 42] RCTs are the best available evidence for drug effectiveness and the randomized controlled design is crucial to avoid any bias that may occur through selection bias or confounding bias. Thus RCTs provide the most reliable information in terms of potential effectiveness and cost-effectiveness. There is however a downside to the use of RCTs in cost-effectiveness analyses. The effectiveness observed in RCTs is often not reached in clinical practice, and thus quality of life of AMD-patients is reduced. Furthermore, in chapter 2 we see that not only the mean effectiveness appears to be lower, but the variability of treatment effect also appears to be larger in the real world, and the amount of people ending up in the expensive non-responder category is much higher. Because we do not know how this affects cost-effectiveness of bevacizumab and aflibercept, it is important to expand cost-effectiveness analyses of anti-VEGF in AMD with observational data. Although observational studies have their own limitations and biases,[52] we should take them into account when estimating a treatment's overall value. For a treatment as vastly used and expensive as anti-VEGF, this could have important implications for health care policy.

Recommendation beyond treatment optimization

This thesis has mainly covered anti-VEGF treatment of nAMD and we have seen how this treatment has realized enormous improvements for millions of nAMD patients worldwide. Anti-VEGF treatment, however, is not a cure. Despite treatment, patients will usually not achieve perfect vision and recurrences of CNV are common.[53] While we are trying to optimize anti-VEGF treatment, the damage is already partly done in the eyes of these patients.

At the moment, there are no cures for AMD, nor can we prevent it. We can slow progression somewhat by addressing modifiable risk factors such as smoking and diet.[54-56] The Age-Related Eye Disease Study (AREDS) study is the only study up to date that was able to prove a benefit from any intervention on AMD progression.[57] It showed that we could lower the risk of progression to advanced AMD by 25% by taking supplements containing 500 mg vitamin C, 400 IU vitamin E, 15 mg beta-carotene, 80 mg zinc oxide and 2 mg copper as cupric oxide. Investigating this treatment effect required over 4000 patients and 10 years of follow-up. Approximately 50% of patients with intermediate AMD advance to CNV or GA within 10 years.[58] This means that a substantial group of AMD patients actually do not progress to the advanced stages. In chapter 7, we delve deeper into the mechanism of progression by assessing the effect of zinc on complement levels in AMD patients. Although we did not have end-points like development of advanced AMD, it gave us an initial understanding of a potential mechanistic approach to reduce progression. Understanding these mechanisms of progression to advanced AMD is important in this multifactorial disease and likely multiple pathways are involved in disease progression.[59] Insight into which pathways lead to progression can help us target and personalize preventive measures. Ultimately, the most optimal way to prevent vision loss in AMD patients and to simultaneously reduce AMD-related costs, is to prevent advanced AMD from developing at all.

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Summary

Wet age-related macular degeneration (AMD) is the most important cause of severe vision loss in the elderly. Due to aging of the population, AMD is becoming an increasing health concern. Within 5 years almost 12 million people worldwide will require treatment for wet AMD. Therefore, we need to work towards optimization of this treatment in the areas of effectiveness, costs and procedure.

Chapter 1 is the introduction of this thesis and serves to familiarize the reader with the treatment of wet AMD with anti-vascular endothelial growth factor (anti-VEGF) injections in the eye. Currently, the three available anti-VEGF drugs are bevacizumab, ranibizumab and aflibercept. Large randomized clinical studies have shown good results of anti-VEGF therapy, however often these levels of effectiveness are not reached in clinical practice. Non-response remains a widespread problem, which raises the question whether we can predict non-response in advance and what is the best treatment strategy.

In **Chapter 2** we evaluate the effectiveness of the anti-VEGF drug ranibizumab as primary treatment for wet AMD in Dutch clinical practice. Although the effectiveness we observed was not of the level as that in randomized controlled trials, ranibizumab was able to prevent a large part of AMD related vision loss.

In **Chapter 3** we looked for different predictors of non-response. Genetic factors that are involved in the mechanisms behind wet AMD seem to be related to treatment response. Especially accumulation of genetic risk factors may have a relevant effect on the eventual vision change after treatment. Different genetic and clinical factors together in a model, may predict non-response in part. A prediction model that we developed based on several of these factors, was able to predict non-response with an accuracy of 77%.

After non-response to anti-VEGF treatment has been established for a patient, it is key to start the appropriate second line treatment. In the first part of **Chapter 4** we evaluate second line treatment with the anti-VEGF drug aflibercept in patients with AMD who did not respond to bevacizumab. Treatment with aflibercept significantly reduced fluid in

the retina and therefore seems an effective alternative for non-responders to bevacizumab. In the second part of this chapter we compared two second line treatments for diabetic macular edema after primary treatment with bevacizumab. We compared second line treatment with ranibizumab to the steroidal anti-inflammatory drug triamcinolone. We concluded that triamcinolone seemed to yield better short-term results and could therefore be preferred as second line treatment.

There are large differences in costs between the anti-VEGF drugs bevacizumab, ranibizumab and aflibercept. In **Chapter 5** we evaluate the cost-effectiveness of these three AMD treatments. Bevaicuzmab is a factor 20 cheaper than the other two drugs, however all of them are equally effective. Therefore, we believe bevacizumab the most appropriate primary treatment of wet AMD. Unfortunately, not everyone in the ophthalmological community agrees and a large subset of the world does not use bevacizumab as primary treatment. In this chapter we explain why bevacizumab should be first choice in AMD and discuss the enormous waste in health care spending that accompanies the use of the other drugs.

In **Chapter 6** we review the treatment procedure and conducted a randomized trial to determine whether an ultrathin needle could reduce the pain associated with the anti-VEGF injection procedure. We saw that pain from the injection was limited and that injecting with an ultrathin needle could not reduce this. Moreover, we studied the influence of psychosocial factors and found that anxiety, negative expectations, and dissatisfaction with the treatment negatively influenced the pain experience.

In **Chapter 7** we try to get a better understanding of the preventive measures in AMD. From previous studies we know that vitamin and zinc supplements slow the progression of AMD. Since the complement system plays such a prominent role in the development of AMD, we hypothesized that supplements with zinc exert their protective action by inhibiting complement activation. In a clinical study we instructed patients to take a daily zinc supplement for four months. We saw that zinc could indeed lower complement activation in patients with high intrinsic complement levels.

Finally, in **Chapter 8** we discuss the findings from this thesis. We discuss the challenges related to predicting non-response to anti-VEGF drugs, the large gap in effectiveness of anti-VEGF treatment between clinical practice and clinical trials, and how to reduce costs of AMD treatment. This section concludes with practical advice for further research.

Samenvatting

Natte leeftijdsgebonden maculadegeneratie (LMD) is de belangrijkste oorzaak van ernstig visusverlies op latere leeftijd. Door vergrijzing is LMD een toenemende zorg voor ons gezondheidsstelsel. Over 5 jaar zullen bijna 12 miljoen mensen wereldwijd behandeld moeten worden voor natte LMD. Daarom is het van belang dat deze behandeling geoptimaliseerd wordt qua effectiviteit, kosten en procedure.

Hoofdstuk 1 vormt de inleiding op dit proefschrift en dient om de lezer bekend te maken met de behandeling van natte LMD met anti-vasculaire endotheliale groeifactor (anti-VEGF) injecties in het oog. Grote medicijnstudies laten goede resultaten van deze behandeling zien, maar in de klinische praktijk worden deze resultaten vaak niet bereikt.

In **Hoofdstuk 2** wordt de effectiviteit van het anti-VEGF medicijn ranibizumab geëvalueerd als primaire behandeling voor natte LMD in de Nederlandse klinische praktijk. Hoewel de resultaten zijn niet zo goed als in de klinische medicijnstudies, is ranibizumab in staat een groot gedeelte van het visusverlies door LMD te voorkomen.

In **Hoofdstuk 3** hebben we gezocht naar verschillende voorspellers voor behandelrespons. Genetische factoren die betrokken zijn bij het mechanisme achter natte LMD blijken geassocieerd met de respons op behandeling. Met name een opeenstapeling van genetische factoren kan een belangrijk verschil maken in de uiteindelijke verbetering van het zicht na behandeling. Verschillende klinische en genetische factoren samen in een voorspellingsmodel kunnen tot op zekere hoogte non-respons voorspellen. Wij zagen dat dit model met een accuraatheid van 77% non-respons van respons kon onderscheiden.

Nadat non-respons op anti-VEGF behandeling is vastgesteld bij een patiënt, moet een juiste tweedelijns behandeling worden ingezet. In het eerste deel van **Hoofdstuk 4** evalueren we tweedelijnsbehandeling met het anti-VEGF middel aflibercept bij personen met LMD die niet goed op bevacizumab hebben gereageerd. Behandeling met aflibercept verminderde significant het vocht in het netvlies en lijkt dus een effectieve tweede keus. In het tweede deel van dit hoofdstuk bekijken we welke tweedelijnsbehandelstrategie het beste is voor diabetisch macula oedeem na primaire behandeling met bevacizumab.

We vergelijken tweedelijnsbehandeling met ranibizumab of de ontstekingsremmer triamcinolon en concluderen dat triamcinolon betere korte termijn resultaten geeft.
Vanwege grote kostenverschillen tussen de anti-VEGF medicijnen bevacizumab, ranibizumab en aflibercept evalueren we in **Hoofdstuk 5** de kosten-effectiviteit van deze drie LMD-behandelingen. Bevacizumab is een factor 20 goedkoper dan de andere twee medicijnen, maar alle drie zijn ze gelijkwaardig in effectiviteit. Daarom beschouwen wij bevacizumab als meest geschikte eerstelijnsbehandeling voor natte LMD. Helaas blijkt dat deze mening niet gedeeld wordt door een groot gedeelte van de wereld en is dit medicijn nog niet overal de eerste keus behandeling. In dit hoofdstuk betogen wij waarom dit wel zou moeten zijn en bespreken hierbij de gigantische kostenverspilling die gepaard gaat bij het gebruik van de andere twee middelen.
In **Hoofdstuk 6** nemen we de behandelprocedure onder de loep en hebben we onderzocht of het gebruik van een ultradunne naald voor anti-VEGF injecties de pijnbeleving bij de injectie doet verminderen. Wij zagen dat de pijn van de injectie beperkt was en dat deze niet verminderd kon worden door een ultradunne naald. Daarnaast bekeken we of psychosociale factoren invloed hadden op de pijn van de injectie en het bleek dat angstgevoelens, negatieve verwachtingen en ontevredenheid met de procedure de pijn negatief beïnvloedde.
In **Hoofdstuk 7** proberen we een beter begrip te krijgen van preventieve behandeling van LMD. Uit voorgaande studies weten we dat voedingssupplementen met vitaminen en zink de progressie van LMD kan verminderen. Omdat het complementsysteem een grote rol speelt bij het ontstaan van LMD, bestudeerden wij of zink supplementen de activatie van het complementsysteem kan verminderen. Zinksuppletie kan inderdaad de complementactivatie verlagen bij LMD patiënten met een intrinsiek verhoogde complementactivatie.
Ten slotte worden in **Hoofdstuk 8** de vondsten van dit proefschrift bediscussieerd. We bespreken de uitdagingen die we tegenkomen bij het voorspellen van non-response bij anti-VEGF middelen, het grote verschil in effect van anti-VEGF tussen de klinische praktijk en klinische medicijnstudies, en hoe we de kosten van de behandeling kunnen verminderen.

Curriculum vitae

Freekje van Asten was born on the 11th of January 1987 in Helmond, the Netherlands. She received her secondary school education at the Dr. Knippenbergcollege, in Helmond, graduating with honors. In 2005 she went on to study Medicine at the Radboud University in Nijmegen, the Netherlands. Here she developed a special interest in ophthalmology, which lead to her doing a senior internship at the department of Ophthalmology of the Radboud university medical center, Nijmegen, under supervision of prof. dr. Carel B. Hoyng. This is where she first came into contact with ophthalmological research, specifically in the field of age-related macular degeneration. After completing her medical studies in 2011 she started with her PhD project "Toward personalized medicine in patients with age-related macular degeneration", which was supported by a grant awarded by the Nijmegen Center for Evidence Based Practice (currently Radboud Institute for Health Sciences). This research project arose from a collaboration between the department of Ophthalmology and the department Health Evidence of from the Radboud university medical center and was supervised by prof. dr. Carel B. Hoyng, prof. dr. Gert Jan van der Wilt, prof. dr. B. Jeroen Klevering and prof. dr. Maroeska M. Rovers. During her PhD research, she studied Epidemiology at the Radboud University for which she received a Master of Science degree in 2015. After finalizing her PhD project in 2016, she decided to continue to research age-related macular degeneration as a postdoctoral fellow at the National Eye Institute/National Institutes of Health, in Bethesda, the United States of America.

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Publication list

In chronological order

Identification of a novel variant associated with anti-VEGF response in age related macular degeneration using exome chip analysis.
Lorés-Motta L, Grunin M, Riaz M, Corominas J, van Asten F, Richardson AJ, International AMD Genomics Consortium, Fauser S, Guymer R, de Jong EK, Heid IM, Hoyng CB, Lotery AJ, Mitchell P, Muether P, Baird PN, den Hollander AI, Chowers I. *Manuscript in preparation.*

Hyperreflective foci on optical coherece tomography as a predictor for treatment response to bevacizumab in patients with diabetic macular edema.
Schreur V, Altay L, van Asten F, Groenewoud JMM, Fauser S, Klevering BJ, Hoyng CB, de Jong EK. *Manuscript in preparation*

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Elshout JA, Bergsma DP, Sibbel J, Baars-Elsinga A, Lubbers P, van Asten F, Visser-Meily JMA, van den Berg AV. *Submitted.*

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van Asten F, Michels CTJ, Hoyng CB, et al. *Submitted.*

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Venhuizen FG, Ginneken B, van Asten F, van Grinsven MJJP, Fauser S, Hoyng CB, Theelen T, Sánchez CI. *Accepted for publication in IOVS.*

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