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

Laura Lorés-Motta[a], Freekje van Asten[a], Philipp S. Muether[d], Dzenita Smailhodzic[a], Joannes M. Groenewoud[b], Amer Omar[e], John Chen[f], Robert K. Koenekoop[g], Sascha Fauser[d], Carel B. Hoyng[a], Anneke I. den Hollander[a,c] and Eiko K. de Jong[a]

Objective **The aim of the study was to investigate the role of single-nucleotide polymorphisms (SNPs) located in the neuropilin-1 (*NRP1*) gene in treatment response to antivascular 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 with baseline.**

Results **SNP rs2070296 was associated with change in visual acuity after 3 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 ($P = 3 \times 10^{-3}$). The combined effect of these two SNPs on the response was also seen after 6 and 12 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.**

Pharmacogenetics and Genomics 2016, **26**:20–27

Keywords: age-related macular degeneration, antiangiogenic drugs, choroid neovascularization, lucentis, neuropilin-1, pathologic neovascularization, personalized medicine, single-nucleotide polymorphism

Departments of [a]Ophthalmology, [b]Health Sciences, [c]Human Genetics, Radboud university medical center, Nijmegen, The Netherlands, [d]Department of Ophthalmology, University Hospital of Cologne, Cologne, Germany, [e]Montreal Retina Institute, Westmount, Departments of [f]Ophthalmology and [g]Pediatric Surgery, Human Genetics, and Ophthalmology, McGill University Health Centre, Montreal, Quebec, Canada

Correspondence to Anneke I. den Hollander, PhD, Department of Ophthalmology, Radboud university medical center, Philips van Leydenlaan 15, 6525 EX Nijmegen, The Netherlands
Tel: +31 243687075; fax: +31 243540522;
e-mail: anneke.denhollander@radboudumc.nl

Received 13 February 2015 **Accepted** 1 September 2015

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 antivascular 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 10% of the treated patients do not respond to anti-VEGF therapy and still lose more than 15 Early Treatment Diabetic Retinopathy Study (ETDRS) letters 2 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 common variants in VEGF family members, cytokines, and proteins involved in the development and maintenance of the retinal vasculature have also been explored. Not all studies showed

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (*www.pharmacogeneticsandgenomics.com*).

DOI: 10.1097/FPC.0000000000000180

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 coreceptor 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 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 treatment-naive 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 committee 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; 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, Surrey, UK). VA was assessed in all cases before treatment (baseline) and after the three loading monthly injections. After the loading dose, patients were followed up 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 five 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 VA after the three first months of treatment compared with baseline. Long-term treatment response was defined as the change in VA after 6 and 12 months of treatment. Age at first ranibizumab injection, sex, 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 Table, Supplemental digital content 1, *http://links.lww.com/FPC/A912* 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 Table, Supplemental digital content 1, *http://links.lww.com/FPC/A912*, 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 11 patients for which genotyping by KASPar of SNP rs2070296 was not successful. Primers were designed using Primer3 software [27] (see Table, Supplemental digital content 1, *http://links.lww.com/FPC/A912*, which describes the primers used). PCR was performed, and the amplicons were sequenced using an automated sequencer (BigDye Terminator, version 3, 3730 DNA analyzer; Applied Biosystems, Waltham, Massachusetts, USA). 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 (IBM Corp., Armonk, New York, USA). ETDRS and Snellen VA records were converted to the logarithm of minimal angle of resolution (logMAR) for the purpose of statistical analysis. Change in VA after 3, 6, and 12 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 means of a χ^2-test. To determine the influence of the baseline variables on the change in VA after 3 months Spearman's 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 3, 6, and 12 months was assessed using Mann–Whitney *U*-tests. Bonferroni's procedure was applied to correct for four tests ($P \leq 0.01$ were considered statistically significant).

To analyze the combined effect of *NRP1* rs2070296 and *KDR* rs4576072 on the change in VA after 3, 6, and 12 months, patients were combined into three groups of approximately equal size (carriers of less than two risk alleles, of two risk alleles, or of more than two risk alleles), and a Mann–Whitney *U*-test was performed. Only the patients who 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 ($P = 0.01$), having a better baseline VA ($P < 10^{-3}$), and having diabetes mellitus ($P = 0.02$) were associated with worse response after 3 months of treatment (see Table, Supplemental digital content 2, *http://links.lww.com/FPC/A913*, which describes the results of the association tests). The type of choroidal neovascularization showed a trend towards statistical significance ($P = 0.06$). These baseline variables were not associated with the SNPs of interest ($P > 0.05$, lowest $P = 0.22$) (see Table, Supplemental digital content 3, *http://links.lww.com/FPC/A914*, 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 sex [*n* (%)]	215 (57.0)
Disease history [*n* (%)][a]	
Hypertension ($n = 259$)[b]	154 (59.5)
Diabetes mellitus ($n = 259$)[b]	47 (18.1)
Other environmental factors [median (quartiles)]	
BMI (kg/m^2) ($n = 258$)[b]	25.39 (23.52–28.49)
Ophthalmological details [median (quartiles)]	
Baseline VA (logMAR)	0.543 (0.398–1.000)
Equivalent baseline VA (ETDRS letters)[c]	57.9 (35.0–65.1)
Change in VA after 3 months (logMAR)[d]	0.097 (0.000–0.259)
Equivalent change in VA after 3 months (ETDRS letters)[c,d]	4.8 (0.0–12.2)
Change in VA after 6 months (logMAR)[d] ($n = 262$)	0.090 (−0.097 to 0.223)
Equivalent change in VA after 6 months (ETDRS letters)[c,d]	4.5 (−4.9 to 11.2)
Change in VA after 12 months (logMAR)[d] ($n = 240$)	0.040 (−0.192 to 0.204)
Equivalent change in VA after 12 months (ETDRS letters)[c,d]	2 (−9.6 to 10.2)
Type of CNV ($n = 335$)[b] [*n* (%)[a]]	
Occult with no classic	199 (59.4)
RAP	21 (6.3)
Minimally classic	42 (12.5)
Predominantly classic	73 (21.8)
Lesion size (DA) ($n = 285$)[b] [*n* (%)[a]]	
< 2	92 (32.3)
2–4	91 (31.9)
4–6	43 (15.1)
> 6	59 (20.7)

CNV, choroidal neovascularization; DA, disk areas; ETDRS, Early Treatment Diabetic Retinopathy Study; logMAR, logarithm of the minimum angle of resolution; *n*, number of patients; RAP, retinal angiomatous proliferation; VA, visual acuity.
[a]Valid percentage.
[b]For the remaining patients no data were available.
[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 before 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 ($P = 0.81$, 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 3 months ($P = 0.01$) compared with the GG genotype, showing a linear trend for the three genotype groups (Fig. 1a). The SNPs rs2229935, rs2248383, and rs2804495 were not found to be associated with treatment response (Table 2).

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 (Fig. 1b). Patients who carried two risk alleles responded significantly worse to

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 to 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 to 0.273)	Reference
CT	169 (48.4)	0.097 (0.000–0.242)	0.94
TT	57 (16.3)	0.090 (−0.064 to 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 to 0.195)	0.04
AA	9 (2.4)	0.000 (−0.097 to 0.040)	0.04
GA or AA	107 (28.4)	0.040 (−0.097 to 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 to 0.300)	0.84
TG or GG	173 (50.9)	0.097 (0.000–0.279)	0.72

logMAR, logarithm of the minimum angle of resolution; *N*, number; SNP, single-nucleotide polymorphism; VA, visual acuity.
[a]Change in VA after 3 months (logMAR) was calculated in the following manner: VA before treatment − VA after 3 months of treatment.
[b]*P*-values were calculated using the Mann–Whitney *U*-test.

therapy than did carriers of one or zero allele (median of 0.090 logMAR or 4.5 ETDRS letters gained vs. 0.196 logMAR or 10 ETDRS letters gained, $P=0.03$), and

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 to 0.180)	3×10^{-3}

logMAR, logarithm of the minimum angle of resolution; *N*, number of patients; VA, visual acuity.
[a]Change in VA after 3 months (logMAR) was calculated in the following manner: VA before treatment − VA after 3 months of treatment.
[b]*P*-values were calculated using the Mann–Whitney *U*-test.

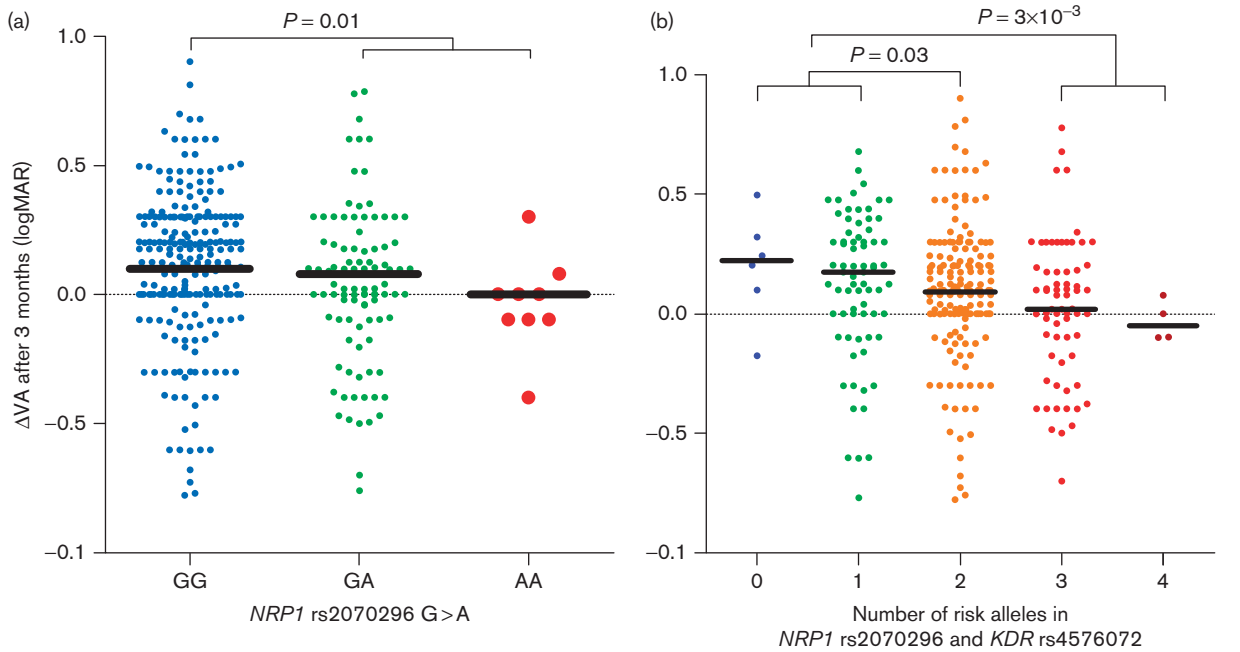
carriers of more than two alleles had even worse response rates (median of 0.020 logMAR or 1 ETDRS letter gained, $P=3\times10^{-3}$) (Fig. 1b and Table 3).

Besides the variability in treatment regimens after the first loading injections, we evaluated whether the effect of rs2072096 in *NRP1* remained significant after 6 and 12 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 (Fig. 2 and Table 5).

Discussion

We evaluated the role of four SNPs located in *NRP1* (rs2229935, rs2247383, rs2070296, and rs2804495) in

Fig. 1



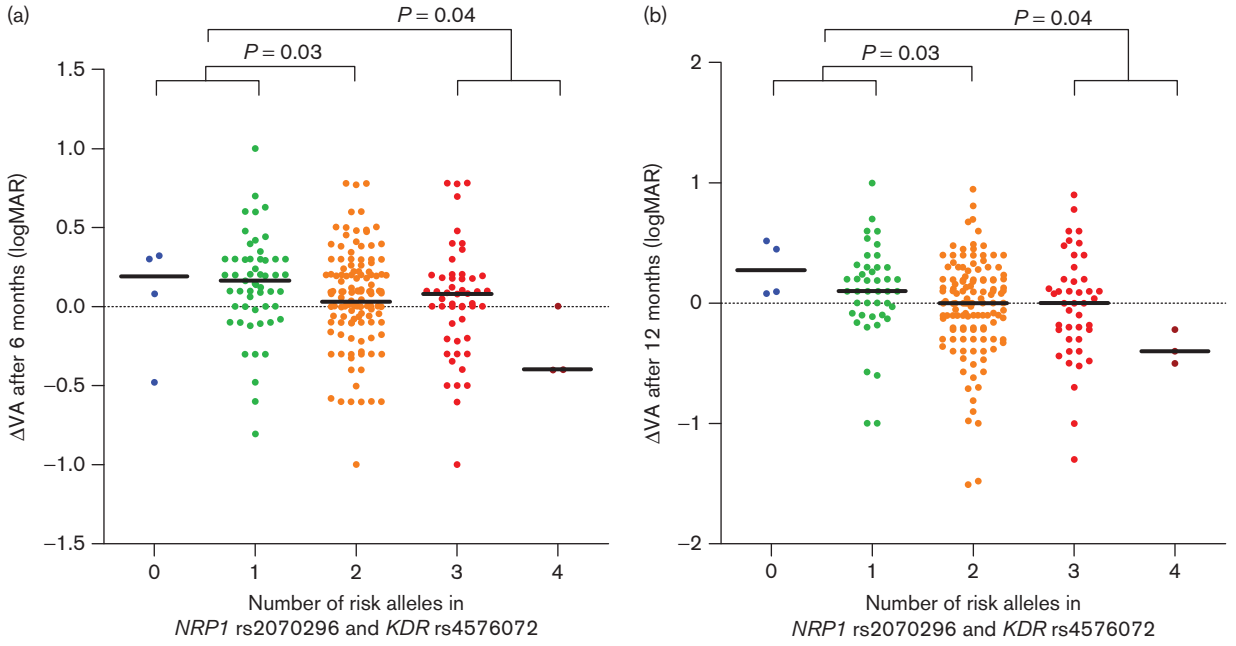
Effect of genetic variants in *NRP1* and *KDR* on response to ranibizumab treatment in nvAMD. (a) Change in visual acuity after 3 months of ranibizumab treatment stratified by *NRP1* rs2070296 genotype. (b) Change in visual acuity after 3 months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T). The median change in visual acuity for each group is depicted in both figures. logMAR, logarithm of minimal angle of resolution; nvAMD, neovascular age-related macular degeneration; VA, visual acuity.

Table 4 Association of genotypes in rs2070296 *NRP1* with long-term response to ranibizumab treatment

	ΔVA after 6 months (logMAR)			ΔVA after 12 months (logMAR)		
SNP	*N* (%)	Median (quartiles)[a]	*P*-value[b]	*N* (%)	Median (quartiles)[a]	*P*-value[b]
rs2070296						
GG	188 (71.8)	0.097 (−0.092 to 0.257)	Reference	180 (75.0)	0.078 (−0.120 to 0.218)	Reference
GA	67 (25.6)	0.040 (−0.080 to 0.194)	0.38	53 (22.1)	0.020 (−0.222 to 0.252)	0.64
AA	7 (2.7)	0.000 (−0.398 to 0.090)	0.21	7 (2.9)	−0.097 (−0.400 to 0.100)	0.33
GA or AA	74 (28.2)	0.020 (−0.098 to 0.194)	0.25	60 (25.0)	0.010 (−0.222 to 0.203)	0.46

logMAR, logarithm of the minimum angle of resolution; *N*, number; SNP, single-nucleotide polymorphism; VA, visual acuity.
[a]Change in VA after 6 or 12 months (logMAR) was calculated in the following manner: VA before treatment−VA after 6 or 12 months of treatment.
[b]*P*-values were calculated using the Mann–Whitney *U*-test.

Fig. 2



Effect of genetic variants in *NRP1* and *KDR* on long-term response to ranibizumab treatment in nvAMD. (a) Change in visual acuity after 6 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 12 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. logMAR, logarithm of minimal angle of resolution; nvAMD, neovascular age-related macular degeneration; VA, visual acuity.

Table 5 Combined effect of the risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576072 (T) on long-term response to ranibizumab treatment

Number of risk alleles	ΔVA after 6 months (logMAR)			ΔVA after 12 months (logMAR)		
	N (%)	[median (quartiles)][a]	*P*-value[b]	*N* (%)	[median (quartiles)][a]	*P*-value[b]
<2	57 (22.7)	0.164 (−0.010 to 0.301)	Reference	47 (20.4)	0.100 (−0.079 to 0.301)	Reference
2	140 (55.8)	0.031 (−0.097 to 0.203)	0.03	137 (59.7)	0.000 (−0.199 to 0.201)	0.03
>2	54 (21.5)	0.034 (−0.209 to 0.184)	0.04	46 (20.0)	0.000 (−0.325 to 0.200)	0.04

logMAR, logarithm of the minimum angle of resolution; *N*, number of patients; VA, visual acuity.
[a]Change in VA after 6 and 12 months (logMAR) was calculated in the following manner: VA before treatment−VA after 6 or 12 months of treatment.
[b]*P*-values were calculated using the Mann–Whitney *U*-test.

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 did not improve their VA and performed five ETDRS letters (one line) worse than the homozygous GG group. As 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 6 and 12 months of treatment. Nevertheless, the dilution of the effect seen in the change in 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 VA after three consecutive loading injections compared with baseline. VA 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 in 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. An 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, California, USA) [38], an anti-VEGF drug also used off-label for the treatment of nvAMD. NRP1 is expressed in endothelial cells and upregulated 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 and colleagues 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.* [12] showed that rs4576072 in *KDR* is associated with response after 12 months of treatment. 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 6 and 12 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.

Acknowledgements

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 317472 (EyeTN).

R.K. Koenekoop is supported by the Foundation Fighting Blindness Canada and the Canadian Institutes for Health Research.

Conflicts of interest

There are no conflicts of interest.

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