**Aims:** To determine whether allelic variants of the cystatin C gene CST3 are genetically associated with exudative age related macular degeneration (ARMD). Cystatin C is a cysteine protease inhibitor that regulates the activity of cathepsin S, a protease with central regulatory functions in retinal pigmented epithelial cells.

**Methods:** CST3 of 167 patients with exudative ARMD was genotyped by using polymerase chain reaction of genomic DNA and restriction enzyme digestion with Kspl and compared with those of 517 control subjects. Patients and controls were white.

**Results:** There was a significant difference in genotype counts between patients and controls ($\chi^2 = 7.158$, df = 2; Fisher’s exact test: $p = 0.037$). There was no significant difference in allele frequencies between patients and controls and between controls from Germany, Switzerland, Italy, and United States. The significant difference in genotype counts between patients and controls could be explained completely by an excess of the homozygous CST3 genotype B/B in patients with exudative ARMD (6.6%) over controls (2.3%), suggesting an odds ratio for ARMD in association with CST3 B/B of 2.97 (95% CI: 1.28–6.86). The results also suggest a stronger association of B/B with ARMD in males than in females. However, in both males and females there was a similar and significant effect of CST3 B/B on disease free survival assessed by Kaplan-Meier analysis. The mean disease free survival time in pooled males and females with genotypes A/A or A/B was 85 years (SE 1; 95% CI: 83–86) and 76 years (SE 2; 95% CI: 72–79) respectively in B/B homozygotes (log rank $p = 0.0006$).

**Conclusion:** Genotyping data, the absence of a significant difference in allele frequencies between patients and controls, and survival analyses suggest an increased susceptibility for ARMD in CST3 B/B homozygotes. Therefore, CST3 B may be a recessive risk allele, significantly contributing to disease risk in up to 6.6% of German ARMD patients. Functional correlates of the allelic CST3 variants A and B remain to be investigated.
with ARMD\(^2\); however, other studies have given negative results.\(^3\)\(^4\) Together, these studies illustrate the challenge to identify susceptibility genes in a most likely complex genetic disorder with the influence of unknown extents of environmental factors.

Familial forms of another macular dystrophy point to the role of the extracellular matrix (ECM) in the pathophysiology of the disease. Mutations in the \(TIMP3\) gene, which encodes a metalloprotease inhibitor that is involved in ECM degradation, are linked to Sorsby's fundus dystrophy, a rare hereditary disease with striking similarities to ARMD in clinical phenotype.\(^19\) However, \(TIMP3\) is not associated with ARMD.\(^20\) Proteases and protease inhibitors are good candidates for pathophysiological factors since extracellular deposits may be related to impaired ECM turnover. One of these protease inhibitors is cystatin C, a ubiquitous secretory cysteine protease inhibitor which is present in various tissues and body fluids.\(^21\) Cystatin C is a strong inhibitor of several cathepsins, among them cathepsin S, a lysosomal enzyme present in retinal pigment epithelial cells where it supposedly functions in the processing of rod outer segments through an incompletely understood mechanism. Inhibition of cathepsin S has been shown to lead to accumulation of debris, when RPE cells are challenged with rod outer segments.\(^22\)

A mutation of the cystatin C gene (\(CST3\)) causes the Icelandic form of hereditary cerebral haemorrhage with amyloidosis (HCHWA-1), a dominant disease characterised by brain haemorrhage and death in young adults. In this disease, a leucine to glutamine mutation at position 68 gives rise to a mutant variant of cystatin C which readily forms amyloid deposits in the walls of cortical arteries and causes these fatal manifestations.\(^23\)

\(CST3\) maps to chromosome 20p11.2. Two \(KspI\) polymorphisms are known in the 5' untranslated sequence, a further \(KspI\) polymorphism results in an amino acid substitution in the penultimate position of the signal peptide.\(^24\) Through a strong linkage disequilibrium between the three polymorphisms only two haplotypes are observed: \(CST3\) A and \(CST3\) B. The \(CST3\) B/B genotype has recently been shown to be associated with late onset Alzheimer's disease.\(^25\) Allelic variation of a gene may be related to the biological function of its encoded protein. We therefore hypothesised a potential association of allelic variants of \(CST3\) in patients with exudative ARMD and determined if any of the \(CST3\) genotypes are associated with exudative ARMD.

**METHODS**

**Patients and controls**

Patients (\(n = 167\), age range 51–94 years), 114 females and 53 males, with mean ages at presentation of 75.3 (SD 7.6) and 73.6 (7.4) years, respectively, were recruited between February and October 1998 at the University Eye Hospital Hamburg-Eppendorf, Germany. Comprehensive ophthalmological examinations in all patients included visual acuity measurements, fundus examination, and fluorescein angiography. All patients were diagnosed with unilateral or bilateral neovascular ARMD based on fluorescein angiography findings and had no other retinal dystrophy or disease that may be associated with the development of CNV. Fluorescein angiographic photographs were evaluated by two independent graders (JZ, GR) according to the guidelines of the international classification and grading system of the International ARM Epidemiological Study Group.\(^26\) Grading results were subsequently reviewed by an additional independent grader. Inclusion criteria for the study were the presence of any form of neovascular maculopathy secondary to ARMD on fluorescein angiography including classic CNV, occult CNV, and disciform scarring, or a combination of any of the above in one or both eyes. If only one eye was affected by neovascular ARMD, the other eye had to present with phenomena of early ARMD including soft drusen and pigmentary abnormalities, or with advanced atrophic ARMD (geographic atrophy) to ensure that ARMD was the cause of neovascular maculopathy. In cases of disagreement between the two initial graders the conclusive grading was done by the reviewing grader. All graders were masked with respect to the genotyping results.

The 167 patients with advanced exudative ARMD represented a subset of 200 ARMD patients that included patients with geographic atrophy (\(n = 26\)) or early ARMD (\(n = 7\)) in one or both eyes and did not have CNV. They were therefore excluded from this study. Thus, 88% of all ARMD patients had advanced exudative ARMD in at least one eye, a typical rate for our tertiary care hospital.

We also genotyped 517 unrelated white control subjects (age range 19–99 years), 283 females and 234 males with mean age of 69.5 (12.7) and 66.3 (11.5) years, respectively. In order to allow the assessment of possible regional or ethnic differences in allele frequencies, the controls consisted of an international collection of adult volunteers originating from Hamburg (\(n = 235\)), Basle, Switzerland (\(n = 164\)), Brescia, Italy (\(n = 56\)), and Boston, USA (\(n = 62\)). Control subjects were recruited in several hospitals in these four centres without specific requirements in order to form a large control group that can be used for different association studies. The controls were not examined for ophthalmological disorders and were expected to develop ARMD at the population rate, and there were no exclusion criteria with respect to macular appearance in the control group.

The study was conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

**Genotyping**

Genomic DNA was isolated from peripheral blood leucocytes using a standard salt precipitation technique.\(^27\) Polymerase chain reaction (PCR) products (318 bp) from genomic DNA were generated by using primer 024, TGGAGGGG-CAGGGGTTCC, and 1206R, TCCATGGGCGCTCCACCGA. A 10 µl polymerase chain reaction was performed, containing 0.4 µl of suspended genomic DNA, 500 nM of each forward and reverse primer, 1.5 mM magnesium chloride, 1 µl 10× buffer, 200 µM deoxyribonucleoside triphosphate, 0.4 units Taq DNA polymerase (Gibco, Gaithersburg, MD, USA), 0.5 µl of 5% dimethyl sulphoxide, and 6.7 µl water. The thermostable was 95°C 45 seconds, 13 × (95°C 15 seconds, 68°C 30 seconds –1°C per cycle, 72°C 30 seconds), 23 × (95°C 15 seconds, 55°C 30 seconds, 72°C 30 seconds), and 72°C 5 minutes. Three polymorphic \(KspI\) restriction sites in the 5’ region of \(CST3\) were covered by the 318 bp PCR fragment. Through a strong linkage disequilibrium between the three polymorphisms only two haplotypes were observed. The haplotypes are defined by either concomitant \(KspI\) restriction endonuclease cleavage both 80 bp upstream of the mRNA transcription start site and in the penultimate codon of the signal peptide (haplotype A), or by an exclusive cleavage downstream of the transcription start site (haplotype B). Haplotypes were confirmed by direct sequencing of PCR products from individuals with the genotypes A/A, A/B, and B/B. Restriction digestion of the PCR product with \(KspI\) (MBI Fermentas, Vilnius, Lithuania) at 37°C overnight revealed fragment sizes of 41/226/51 bp (homozygote haplotype A), or 127/191 bp (homozygote haplotype B), or all five fragments in A/B heterozygotes. The digestion products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualised under ultraviolet light.

**Statistical analysis**

All statistical association analyses were done with spss, version 8.0 (SPSS Inc, Chicago, IL, USA). \(p\) Values less than 0.05 were considered significant. Statistical analyses of deviations from Hardy Weinberg equilibrium (HWE) were done by
RESULTS

There was no significant difference in allele frequency of haplotype B (F\(_B\)) in the control groups of the four centres Hamburg, Basle, Brescia, and Boston with F\(_B\) of 0.18, 0.20, 0.21, and 0.12, respectively (p = 0.22, df = 3). The similar F\(_B\) between the German controls (F\(_B\) = 0.181) and those pooled from the other three centres with a mean F\(_B\) = 0.184 (p = 0.88, df = 1) suggested widespread population similarity of F\(_B\) and for age matching, among those expected under HWE. There was a significant difference in genotype counts between patients and controls (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037).

Table 1 shows the genotype counts of all patients and controls. None of the genotype counts significantly deviated from those expected under HWE. There was a significant difference in genotype counts between patients and controls (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037).

For age matching, among those expected under HWE. There was a significant difference in genotype counts between patients and controls (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037). The strongest difference between patients and controls was observed in the B/B homozygotes with 6.6% and 2.3% (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037).

For age matching, among those expected under HWE. There was a significant difference in genotype counts between patients and controls (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037). The strongest difference between patients and controls was observed in the B/B homozygotes with 6.6% and 2.3% (\(\chi^2 = 7.16, p = 0.007\), df = 2) and more than expected (B/Bobs = 11) B/B homozygotes in the controls (\(\chi^2 = 2.53, p = 0.093\), df = 2; two sided Fisher’s exact test: p = 0.037).
control subjects males less than 64 years and females less than 62 years were excluded. In addition, the association was reanalysed for the age matched controls from Hamburg only as well as for all controls from Hamburg since these control subjects represent the best ethnic match available. Among the age matched controls from Hamburg, males less than 64 years and females less than 58 years were excluded. The results shown in Table 2 suggest a stronger association of B/B with ARMD in males than in females. Little difference was found between the whole control group and the control group from Hamburg, again suggesting population similarity and that ethnic

Figure 2. Composite image shows late phase fluorescein angiography of both eyes of all 11 patients carrying the CST3 genotype B/B: choroidal neovascularisation or a disciform scar secondary to ARMD is present in all patients in at least one eye.
However, to conclude an unambiguous male predominant females. Our male patient sample may have been too small, was significantly reduced in both B/B homozygous males and appeared to be stronger in males, but disease free survival time susceptibility gene for exudative ARMD. The association provide a clinical explanation for a possible sex related differ-

**DISCUSSION**

The homozygous CST3 genotype B/B was associated with exudative ARMD. These results establish CST3 as an interesting susceptibility gene for exudative ARMD. The association appeared to be stronger in males, but disease free survival time was significantly reduced in both B/B homozygous males and females. Our male patient sample may have been too small, however, to conclude an unambiguous male predominant effect of CST3 genotype on susceptibility for ARMD. On the other hand, natural history and clinical appearance are similar between male and female patients and therefore fail to provide a clinical explanation for a possible sex related difference.

For this study, we selected a clinically well defined group of patients with the exudative form of ARMD. We confirmed the diagnosis of neovascular ARMD at the time of presentation. Unfortunately, it is not possible to explore the exact age at onset of the initial signs because of the high variability of subjective histories provided by the patients. Therefore, our objective was to form a homogeneous patient group affected by advanced exudative ARMD proved by fluorescein angiography irrespective of the subjective patient history. If reliable data were available it would be tempting, however, to use them for the differentiation of specific subtypes, courses, or onset of the disease. Nevertheless, the presented theoretical survival analysis based on an assumed 3 year interval between onset and presentation also revealed a similar strong effect of CST3 B/B on disease free survival, suggesting a robust effect of CST3 in a subset of patients.

Genotype distributions and allele frequencies in control groups are critical for candidate gene analyses in case-control association studies. The similar frequency of the allelic variants A and B in control samples from four different countries suggests a homogeneous distribution among white people and allowed us to pool control samples from several centres in order to increase the power to detect an association between ARMD and a relatively rare genotype. In addition, we observed no difference between the whole multicentre control group and the controls from Hamburg which lends support to the assumption of widespread population similarity as described above.

We decided not to chose an ARMD free control population, because the distinction between early ARMD and normal age-

This variation alters the hydrophobicity of the signal sequence near the signal peptidase cleavage site and could be associated with changes in secretory processing of the peptide. These changes are clearly different from the leucine to
glutamine substitution at position 68 seen in the Icelandic form of hereditary cerebral haemorrhage with amyloidosis (HCHWA-1). The resulting aberrant cystatin C in HCHWA-1 has a stronger tendency to dimerise and form aggregates, especially if the temperature is elevated. These aggregates then deposit as amyloid in these patients whereas the spinal fluid level is abnormally low. Deposits in brain vessels cause cerebral haemorrhages, strokes, paralysis, and death in young adults. The point mutation of the cystatin C gene in HCHWA-1 resulting in this aberrant protein is different from the three polymorphisms of CST3 resulting in the haplotypes A and B. Instead, our genetic data provide evidence for a contribution of CST3 B/B to disease risk in the heterogeneous and multifactorial aetiology of exudative ARMD.

The exact impact of cystatin C in the RPE degradation process is not well known. Preliminary data indicate that cellular levels of cystatin C in fibroblasts differ among the genotypes A/A, A/B, and B/B (RM Nitsch, unpublished data), but it is unknown if RPE cells show the same behaviour. Functional correlates of the allelic CST3 variants A and B in the human eye remain to be investigated.

The results of our study imply a role of cystatin C in the pathophysiology of ARMD. They open novel avenues for the study of cathepsins and their inhibitors in ARMD.

ACKNOWLEDGMENTS

The authors are grateful to Dr FG Holz, Department of Ophthalmomology, University of Heidelberg for helping reading and classifying the fluorescein angiograms, and to Drs C Hock, H Staehelin, G Binetti, and JH Growdon for providing control samples.

Acknowledgments

The authors gratefully thank Dr FG Holz, Department of Ophthalmomology, University of Heidelberg for helping reading and classifying the fluorescein angiograms, and to Drs C Hock, H Staehelin, G Binetti, and JH Growdon for providing control samples.

REFERENCES