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 pigment 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 KspI 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 (χ² = 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.

Age related macular degeneration (ARMD) is the leading cause of legal blindness in Western Europe and in the United States. Among the elderly, the overall prevalence is estimated between 5.7% and 30% depending on the definition of early ARMD, and its differentiation from features of normal ageing, a distinction that remains poorly understood. The hallmark of early ARMD is the presence of drusen, yellow deposits beneath the retinal pigment epithelium (RPE), and, on histology, diffuse thickening of Bruch’s membrane because of deposition of debris in the inner collagenous layer. Advanced stages of ARMD can be subdivided into geographic atrophy and exudative ARMD. Geographic atrophy is characterised by progressive atrophy of the RPE. In exudative ARMD the key phenomenon is the occurrence of choroidal neovascularisation (CNV). Eyes with CNV have varying degrees of reduced visual acuity, depending on location, size, type, and age of the neovascular lesion. The development of choroidal neovascular membranes can be considered a late complication in the natural course of the disease possibly due to tissue disruption (Bruch’s membrane) and decompensation of the underlying longstanding processes of ARMD.

Many pathophysiological aspects, as well as vascular and environmental risk factors, are known to be associated with a progression of the disease, but little is known about the aetiology of ARMD itself as well as about the underlying processes of complications like the occurrence of CNV.

Family, twin, segregation, and case-control studies suggest an involvement of genetic factors in the aetiology of ARMD. However, the extent of heritability, number of genes involved, and mechanisms underlying phenotypic heterogeneity are unknown. The search for genes related to ARMD faces challenges: the onset is late in life, and there is usually only one generation available for studies. The parents of patients are often dead, and the children are too young to manifest the disease. Generally, the heredity of late onset diseases has been difficult to estimate because of the uncertainties of the diagnosis in previous generations and the inability to diagnose ARMD among the children of an affected individual. Even in the absence of the ambiguities in the diagnosis of ARMD in previous generations, the late onset of the condition itself, natural death rates, and small family sizes result in underestimation of genetic forms of ARMD, and in overestimation of rates of sporadic disease. Moreover, the phenotypic variability is considerable, and it is conceivable that the currently used diagnostic entity of ARMD in fact represents a spectrum of underlying conditions with various genetic and environmental factors involved. The search for genetic factors related to ARMD has to address these challenges.

Several results on genetic studies with ARMD have been published. In a recent study, a genetic association of the manganese superoxide dismutase with exudative ARMD was reported. Contradictory results exist concerning a possible role of ApoE polymorphisms in ARMD. Some authors have reported a lower frequency of the ε4 allele in subgroups of ARMD, while other reports could not confirm this association.

The ε2 allele was reportedly more frequent in ARMD patients. Further, allelic variations in the ABCR gene were proposed to be associated with advanced atrophic ARMD, but again, other authors found no evidence to support this hypothesis. Recently, Allikmets et al have reported additional data in support of an association of ABCR...
with ARMD; however, other studies have given negative results. 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. However, TIMP3 is not associated with ARMD. 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. 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 retinal pigment cell metabolism and pigment epithelial cell function of its encoded protein. We therefore hypothesised a potential association of allelic variants of CST3 in patients with 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 leukocytes using a standard salt precipitation technique. Polymerase chain reaction (PCR) products (318 bp) from genomic DNA were generated by using primer 024, TGGGAGGGA-CAGGGGTTC, and 1206R, TCCATGGGGCTCCACCAG. 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 10X buffer, 200 µM deoxynucleoside triphosphate, 0.4 units Taq DNA polymerase (Gibco, Gaithersburg, MD, USA), 0.5 µl of 5% dimethyl sulphoxide, and 6.7 µl water. The thermoprofile 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 (β = 0.06, p < 0.0001). Therefore we reanalysed the association between B/B homozygosity and ARMD both in separately age matched males (53 patients, 138 controls) and females (114 patients, 211 controls). For age matching, among

Table 1 CST3 genotype counts and frequencies in patients with ARMD and control subjects

<table>
<thead>
<tr>
<th>CST3 genotype counts and frequencies (%)</th>
<th>No:</th>
<th>A/A</th>
<th>A/B</th>
<th>B/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>167</td>
<td>103 (61.7)</td>
<td>53 (31.7)</td>
<td>11 (6.6)</td>
</tr>
<tr>
<td>Female</td>
<td>114</td>
<td>69 (60.5)</td>
<td>40 (35.1)</td>
<td>5 (4.4)</td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>34 (64.2)</td>
<td>13 (24.5)</td>
<td>6 (11.3)</td>
</tr>
<tr>
<td>All controls</td>
<td>344</td>
<td>240 (68.3)</td>
<td>108 (31.9)</td>
<td>16 (4.6)</td>
</tr>
<tr>
<td>All controls age matched</td>
<td>175</td>
<td>115 (65.7)</td>
<td>57 (32.6)</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td>Age matched, female</td>
<td>211</td>
<td>150 (71.1)</td>
<td>56 (26.5)</td>
<td>5 (2.4)</td>
</tr>
<tr>
<td>Age matched, male</td>
<td>138</td>
<td>89 (64.5)</td>
<td>46 (33.3)</td>
<td>3 (2.2)</td>
</tr>
<tr>
<td>Controls Hamburg (HH)</td>
<td>235</td>
<td>175 (66.0)</td>
<td>55 (39.9)</td>
<td>5 (2.1)</td>
</tr>
</tbody>
</table>

Influence of sex and age matching of controls on odds ratio (OR) for exudative ARMD in Hamburg cases and controls only (167 patients; 114 female, 53 male) in association with CST3 genotype B/B

<table>
<thead>
<tr>
<th>OR 95% CI</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>All controls age matched (n=349)</td>
<td>3.01 1.19 to 7.62 0.023</td>
</tr>
<tr>
<td>All controls from Hamburg (n=235)</td>
<td>4.04 1.11 to 14.76 0.028</td>
</tr>
<tr>
<td>Age matched controls HH (n=175)</td>
<td>3.24 1.11 to 9.52 0.036</td>
</tr>
<tr>
<td>Females HH, controls age matched</td>
<td>1.85 0.43 to 7.92 0.485</td>
</tr>
<tr>
<td>Males HH, controls age matched†</td>
<td></td>
</tr>
</tbody>
</table>

HH = Hamburg.
*Two sided Fisher’s exact test; †OR cannot be calculated due to 0 age matched males from Hamburg carrying the B/B genotype.

χ² = (X/Xobs − X/Xexp)²/X/Xexp

where X/Xobs is the observed genotype count of the respective CST3 genotypes (A/A, A/B, or B/B) and X/Xexp is the respective genotype count expected under HWE which is calculated based on the frequencies (F_A and F_B) of the observed allelic variants (here: A and B) at a given locus: FA +FB = 1, therefore (FA)² = B/B exp = 1; B/Bobs = 12; FA+FB = 1; FAFB = A/B exp. Note: (FA)² = A/A exp, (F_B)² = B/B exp, 2F_AF_B = A/B exp.

The strongest difference between patients and controls was observed in the B/B homozygotes with 6.6% and 2.3%, respectively, suggesting an odds ratio (OR) for ARMD in the B/B homozygotes in the patients. In accordance with this there were slightly less (B/Bobs = 12) than expected under HWE (B/B exp = 17.3) B/B homozygotes in the controls (χ² = 1.62) and more than expected (B/B exp = 11 v B/Bobs = 8.4; χ² = 0.81) B/B homozygotes in the patients (Σχ² = 2.43, ns).
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
In both males and females there was a significant effect of CST3 B/B on disease free survival analysed by Kaplan-Meier analysis. In males with genotypes A/A or A/B the mean disease free survival time was 86 years (SE 2; 95% CI: 82; 89) which was 74 years (SE 4; 95% CI: 67; 81) in B/B homozygotes (log rank p = 0.006). In females with genotypes A/A or A/B the mean disease free survival time was 85 years (SE 1; 95% CI: 83; 86) and 76 years (SE 2; 95% CI: 72; 70) in B/B homozygotes (log rank p = 0.041). 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) in B/B homozygotes (log rank p = 0.0006). The graphic plot of the hazard function of the pooled survival analysis is shown in Figure 1. The log rank statistics were very similar (p = 0.0005) if a mean disease onset of 3 years before clinical presentation was assumed (data not shown). The oldest control subject homozygous B/B was 75 years, and 137 of 517 controls (26.5 %) were older than 75. The oldest patient homozygous B/B was 85, whereas only 10 of 167 patients (6 %) were older than 85. A composite image of the late phase fluorescein angiographies of both eyes of all 11 patients with CST3 B/B is shown in Figure 2.

**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 onsets 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-
glutaminyl substitution at position 68 seen in the Icelandic form of hereditary cerebral haemorrhage with amyloidosis (HCHWA-I). The resulting aberrant cystatin C in HCHWA-I 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-I 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 capthepsins and their inhibitors in ARMD.

ACKNOWLEDGMENTS

The authors are grateful to Dr FG Holz, Department of Ophthalmology, 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.

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