Polymorphisms in ARMS2/HTRA1 and Complement Genes and Age-Related Macular Degeneration in India: Findings from the INDEYE Study

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PURPOSE. Association between genetic variants in complement factor H (CFH), factor B (CFB), component 2 (C2), and in the ARMS2/HTRA1 region with age-related macular degeneration (AMD) comes mainly from studies of European ancestry and case-control studies of late-stage disease. We investigated associations of both early and late AMD with these variants in a population-based study of people aged 60 years and older in India.

METHODS. Fundus images were graded using the Wisconsin Age-Related Maculopathy Grading System and participants assigned to one of four mutually exclusive stages based on the worse affected eye (0 = no AMD, 1–3 = early AMD, 4 = late AMD). Multinomial logistic regression was used to derive risk ratios (RR) accounting for sampling method and adjusting for age, sex, and study center.

RESULTS. Of 3569 participants, 53.2% had no signs of AMD, 45.6% had features of early AMD, and 1.2% had late AMD. CFH (rs1061170), C2 (rs547154), or CFB (rs438999) was not associated with early or late AMD. In the ARMS2 locus, rs10490924 was associated with both early (adjusted RR 1.22, 95% confidence interval [CI]: 1.13–1.33, $P < 0.0001$) and late AMD (adjusted RR 1.81, 95% CI: 1.15–2.86; $P = 0.01$); rs2672598 was associated only with early AMD (adjusted RR 1.12, 95% CI: 1.02–1.23; $P = 0.02$); rs10490923 was not associated with early or late AMD.

CONCLUSIONS. Two variants in ARMS2/HTRA1 were associated with increased risk of early AMD, and for one of these, the increased risk was also evident for late AMD. The study provides new insights into the role of these variants in early stages of AMD in India. (*Invest Ophthalmol Vis Sci.* 2012; 53:7492–7497) DOI:10.1167/iovs.12-10073

The late stage of age-related macular degeneration (AMD) is the major cause of serious vision loss in Western populations. Over the past decade, strong genetic associations have been identified in persons with types of late AMD (neovascular AMD and/or geographic atrophy).[1,2] These studies have been largely undertaken in Western settings,[3–16] with few located in countries such as India where there is a burgeoning growth in the older age groups.[17–20] Furthermore, few studies have explored genetic associations with early AMD features and thus the role of the former in the pathogenesis of the condition remains poorly researched.[21–24]

We investigated genetic associations with both early and late AMD using a large population-based study in north and south India: the India Age-related Eye disease Study (INDEYE). We examined variants in a range of genes that have been previously identified primarily in Western studies.[2,8,13–17,19,20,25]

METHODS

Study Participants and Procedures

We used data already collected from a population-based study of people aged 60 years and older: the INDEYE study. The objectives of the INDEYE study were to estimate the prevalence of early and late AMD and of lens opacities, as well as to investigate associations of these conditions with lifestyle factors. The study design, procedures, and results of AMD prevalence in INDEYE have been reported elsewhere.[26] The study took place in two locations: in Haryana state, north India, and Tamil Nadu, south India. Sampled clusters were enumerated to identify people aged 60 and older who were invited to participate in the study. Recruitment into the study was performed between 2005 and 2007. Informed written consent was obtained from all participants before enrollment. Information was read to people who were illiterate in the presence of a local witness, and a thumb impression of the participant signified assent. The study complied with the guidelines in the Declaration of Helsinki, and ethics approval was received from the Research Ethics Committees of the All India Institute of Medical Sciences, Aravind Eye Hospital, London School of Hygiene and Tropical Medicine, Queens University Belfast, and the Indian Council for Medical Research.

Information on household characteristics and sociodemographic variables was collected at enumeration for INDEYE. A structured questionnaire was used to collect data about lifestyle, including tobacco and alcohol use and participants came to the base hospital for

a clinical examination, which included anthropometry, blood pressure, an eye examination, and blood sample collection. Of the 3874 households from which participants were eligible, there were 2898 (74.8%) with a single participant, 948 (24.5%) households with two participants each, 25 (0.6%) households with three participants each, and three (0.1%) households with four participants each. In most households (87.6%) with two or more participants, there was no biological relation between the participants. Only 4.0% of households had related participants and in 8.4% of the households the biological relationships were uncertain.

Grading of AMD

Full details of the fundus grading and prevalence have been provided elsewhere.[26] Two 35° stereo fundus images (TRC 50 EX; Topcon Corporation, Tokyo, Japan) were obtained from each eye and graded using the Wisconsin Age-Related Maculopathy Grading System (WARMGS)[27] at a single reading center (Department of Ophthalmology and Vision Science, Queens University Belfast). Features of AMD were classified into one of five mutually exclusive severity stages of the Rotterdam staging system: stage 1, soft distinct drusen ($\geq$63 μm) only *or* pigmentary irregularities only; stage 2, soft indistinct ($\geq$125 μm) or reticular drusen only *or* soft distinct drusen ($\geq$63 μm) with pigmentary irregularities; stage 3, soft indistinct ($\geq$125 μm) or reticular drusen with pigmentary irregularities; stage 4, either choroidal neovascularization (CNV; presence of any of the following: serous or hemorrhagic retinal or retinal pigment epithelial detachment, subretinal neovascular membrane, and periretinal fibrous scar) or geographic atrophy (GA; well-demarcated area of retinal pigment atrophy with visible choroidal vessels).

Genotyping

We chose single-nucleotide polymorphisms (SNPs) that were previously published showing associations for late AMD in India and Europe. For complement factor H (CFH), we chose rs1061170 and for component 2 (C2) we chose rs547154, as this had been the most significant signal for late AMD in a previous Indian case-control study.[17] A previous European study had characterized a potentially nonsynonymous coding variant R151Q near complement factor B (CFB) in the SKIV2L gene (rs438999).[28] In the HtrA serine peptidase 1 (HTRA1)/age-related macular susceptibility 2 (ARMS2) region, we selected rs10490923, rs10490924, and rs2672598, as these SNPs captured common haplotypes in this region previously associated with late AMD in an Indian case-control study.[19]

Genomic DNA was extracted from peripheral blood leukocytes using Quiagen kits (Qiagen GmbH, Hilden, Germany). DNA concentration was standardized for working DNA using an Eppendorff automatic pipetting machine (Eppendorf AG, Hamburg, Germany). DNA was plated into 384 wells, and the three SNPs were genotyped using TaqMan assays in an ABI 7900 real-time PCR (Applied Biosystems, Foster City, CA). The genotyping procedure was performed according to the manufacturer's protocol. Clustering of genotypes was inspected for separation between genotypes and accuracy of genotype calls. Clustering algorithms for genotype assignment used three quality-control steps: (1) all individual calls with a significant number of outlier SNPs were checked for DNA concentration and plated DNA amended accordingly, (2) setting an SNP pass value by assigning a pass or fail status for each SNP and genotyping plate, and (3) genotyping was repeated for calls that did not pass in the first run (5% of calls). All steps for sample preparation and genotyping were automated and tracked with a bar-coding system that fed data directly into a database, which then provided genotyping results for individual samples.

Statistical Analysis

All analyses were done in STATA 11 (StataCorp 2007, College Station, TX). Early AMD was defined as stages 1, 2, and 3 and late AMD as stage

4. People with no sign of early or late AMD were classified as "no AMD." The numbers for analysis were as follows: no AMD features (n = 1899), early AMD (n = 1628), and late AMD (n = 42). Power calculations showed that for variants with minor allele frequencies (MAFs) of 0.1, 0.2, and 0.3, the study had 77%, 96%, and 99% power, respectively, to detect allelic associations of 1.2 and larger with early AMD at P = 0.05.

Multinomial logistic regression was used to investigate associations with early or late AMD with people with no AMD as the baseline comparison group. Multinomial logistic regression was expressed as relative risk ratios (RRR). All analyses used robust SEs to take account of the cluster-sampling design.

We undertook unadjusted associations between AMD and SNP genotypes, then we adjusted for age, sex, and study location. Age was included as a continuous variable. We fitted models that allowed for separate effects of the heterozygous genotypes and risk/rare homozygous genotypes, as well as those that allowed for additive genotype effects.

Sensitivity Analyses

In sensitivity analyses, caste and socioeconomic status (based on housing characteristics) were investigated for confounding effects because these could potentially lead to nonrandom mating in the population, whereas tobacco use, diabetes status, and body mass index were included to investigate if they improved the precision of the association. Results did not change on further adjustment (data not shown), and therefore the age-, sex-, and study location–adjusted analyses are shown in the main text.

Most (87%) of the households with multiple participants (constituting 25% of all households) had no biological relations and the analysis using robust SEs allowed for clustering in households and villages. Sensitivity analyses were carried out excluding all participants with possible relations in the same household (see Supplementary Material, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10073/-/DCSupplemental).

Exclusion of the nine AMD stage 3 cases did not change results (data not shown).

Because tobacco smoking in particular has been found to have modifying effects for ARMS2/HTRA1 rs10490924 in some studies on European populations,[10,29] we tested for an interaction with tobacco use (current, ex, never) using design-adjusted Wald-tests (allowing for the clustered sampling design). In view of different genotype frequencies by location for rs1061170 and rs10490924, we investigated in sensitivity analyses whether results differed by study location.

RESULTS

Full details of distribution of the AMD severity stages in the INDEYE sample have been previously reported.[26] The demographic characteristics of participants with a blood sample and at least one genotype are shown in Table 1. In our population, the use of any type of tobacco (current or past) was 61%; 42% had ever smoked, 13% only chewed tobacco, 6% had both chewed and smoked tobacco, and 39% had never used tobacco. Most smokers (83%) smoked beedies (hand-rolled tobacco) and only 7% smoked imported Western cigarettes.

The Supplementary Table 1 (see Supplementary Material and Supplementary Table 1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10073/-/DCSupplemental) shows the SNP genotype distributions by AMD stages and associated genes. Hardy-Weinberg equilibrium was maintained for all SNPs in the control group. We checked our observed genotype frequencies against published literature and genetic databases. When investigating genotype frequencies by study location, we found a slightly higher MAF for Y402H, and a slightly lower MAF for rs10490924 in north India than in south India (see

TABLE 1. Characteristics of Participants by AMD Stage for Those Who Have at Least One of the Genotyped SNPs

	Persons	No AMD*	Early AMD*	Late AMD*
	3569	1899	1628	42
Study center				
South India	1734	874 (46.0)	837 (51.4)	23 (54.8)
North India	1835	1025 (54.0)	791 (48.6)	19 (45.2)
Age category				
60 to 69	2528	1369 (72.1)	1140 (70.0)	19 (45.2)
≥70	1041	530 (27.9)	488 (30.0)	23 (54.8)
Sex				
Male	1753	897 (47.2)	835 (51.3)	21 (50.0)
Female	1816	1002 (52.8)	793 (48.7)	21 (50.0)

* Column percentages.

Supplementary Material and Supplementary Table 2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10073/-/DCSupplemental). Both genotype frequencies of the CFH Y402H rs1061170 were similar to those previously reported.[30] The MAF of rs438999 (near CFB) and rs547154 (complement C2) were both 0.18, which was more than twice the MAF observed for central European populations (0.08 and 0.06 respectively) but in accord with previous observations made in Texas Gujarati Indians (MAF = 0.16 for both SNPs).[31,32] The observed MAF of 0.14 for rs10490923 in our study (ARMS2) was similar to central Europeans (MAF = 0.13),[33] but MAFs for rs10490924 (ARMS2) and rs2672598 (HTRA1) were higher (0.32 and 0.53) when compared with 0.27 and 0.43 in Europeans, respectively.[34,35] The MAF of 0.32 for rs10490924 in our study was similar to that observed in Gujarati Indians (MAF = 0.36)[34] and to that reported by Kaur et al.[19] in a population from southern India. There was no published population-based MAF for Indians for rs2672598.

Preliminary analysis showed that the associations between stage 1 and stage 2 AMD with the variants was similar (there were too few cases of stage 3 for separate analysis) and we therefore merged the three early-AMD stages into a single group because of the small numbers in stages 2 ($n = 240$) and 3 ($n = 9$) relative to stage 1 ($n = 1379$). Table 2 shows crude and adjusted associations for complement pathway SNPs that have been associated with AMD in European studies,[8,14-16] and with late AMD in an Indian case-control study.[17,20] There was no evidence for an association between rs1061170 and early stages of AMD irrespective of type of genetic model. Analyses found no evidence of effect modification by study location (P value for interaction = 0.138). For late-stage AMD, the point estimates suggest a per-allele increase in risk of 1.50, 95% confidence interval (CI) 0.96 to 2.34 in the age-, sex-, and location-adjusted analyses, $P = 0.08$. For the SNPs in C2 (rs547154) and CFB (rs438999), there was no evidence of association with early or late AMD.

Table 3 shows association results for SNPs in the HTRA/ARMS2 region. There was no evidence of association for rs10490923 (ARMS2) with early or late AMD. For rs10490924 (ARMS2) and rs2672598 (HTRA1), associations were observed with early stages of AMD. Only rs10490924 was associated with late AMD. In an adjusted allelic analysis of rs10490924 (ARMS2), having one T allele or more increased the relative risk of early AMD by 22% (RRR 1.22, 95% CI: 1.13-1.33), and the relative risk of late AMD by 80% (RRR 1.81, 95% CI: 1.15-2.86). Analyses found no evidence of effect modification by study location (P value for interaction = 0.916). For rs2672598 (HTRA1), having one G allele or more increased the risk of

TABLE 2. Crude and Adjusted RRRs with their 95% CIs and *P* Values for the Associations between AMD and SNPs within or near Genes Involved in the Complement Pathway

	CT versus TT	CC versus TT	Additive Model
CFH			
rs1061170			
Early AMD RRR (95% CI) *P* value			
Unadjusted	0.88 (0.76-1.01) *P* = 0.07	0.93 (0.73-1.18) *P* = 0.53	0.93 (0.84-1.03) *P* = 0.17
Age, sex, and location adjusted	0.88 (0.77-1.02) *P* = 0.09	0.93 (0.73-1.18) *P* = 0.5	0.94 (0.84-1.04) *P* = 0.20
Late AMD RRR (95% CI) *P* value			
Unadjusted	1.75 (0.84-3.66) *P* = 0.14	2.15 (0.75-6.16) *P* = 0.15	1.50 (0.97-2.34) *P* = 0.07
Age, sex, and location adjusted	1.70 (0.81-3.58) *P* = 0.16	2.15 (0.76-6.07) *P* = 0.15	1.50 (0.96-2.34) *P* = 0.08

	AC versus CC	AA versus CC	Additive Model
Complement C2			
rs547154			
Early AMD RRR (95% CI) *P* value			
Unadjusted	0.93 (0.79-1.10) *P* = 0.41	1.30 (0.94-1.79) *P* = 0.12	1.00 (0.89-1.12) *P* = 0.96
Age, sex, and location adjusted	0.93 (0.79-1.10) *P* = 0.42	1.27 (0.92-1.76) *P* = 0.14	1.00 (0.89-1.12) *P* = 0.99
Late AMD RRR (95% CI) *P* value			
Unadjusted	1.12 (0.60-2.09) *P* = 0.72	1.82 (0.44-7.61) *P* = 0.41	1.21 (0.69-2.11) *P* = 0.51
Age, sex, and location adjusted	1.13 (0.60-2.10) *P* = 0.41	1.87 (0.44-7.97) *P* = 0.40	1.22 (0.69-2.16) *P* = 0.51

	AG versus AA	GG versus AA	Additive Model
Near CFB, in exon of SKIV2L (DEAD box protein, putative RNA helicase involved in antiviral activity)			
rs438999			
Early AMD stage 2 RRR (95% CI) *P* value			
Unadjusted	0.94 (0.80-1.11) *P* = 0.48	1.24 (0.88-1.75) *P* = 0.23	1.00 (0.89-1.12) *P* = 0.99
Age, sex, and location adjusted	0.94 (0.80-1.11) *P* = 0.49	1.23 (0.87-1.73) *P* = 0.24	1.00 (0.89-1.12) *P* = 0.99
Late AMD RRR (95% CI) *P* value			
Unadjusted	1.13 (0.59-2.14) *P* = 0.72	0.91 (0.12-6.85) *P* = 0.92	1.07 (0.60-1.90) *P* = 0.83
Age, sex, and location adjusted	1.14 (0.59-2.18) *P* = 0.70	0.93 (0.12-7.16) *P* = 0.95	1.08 (0.60-1.95) *P* = 0.80

TABLE 3. Crude and Adjusted RRRs with their 95% CIs and P Values for the Associations between AMD and SNPs within or near HTRA1 and ARMS2

	AG versus GG	AA versus GG	Additive Model
ARMS 2			
rs10490923			
Early AMD RRR (95% CI) P value			
Unadjusted	1.00 (0.86–1.16) P = 0.97	1.01 (0.63–1.60) P = 0.98	1.00 (0.87–1.15) P = 0.99
Age, sex, and location adjusted	1.00 (0.87–1.16) P = 0.96	1.02 (0.65–1.62) P = 0.92	1.01 (0.88–1.15) P = 0.93
Late AMD RRR (95% CI) P value			
Unadjusted	0.99 (0.45–2.19) P = 0.98	1.03 (0.15–6.88) P = 0.98	1.00 (0.55–1.82) P = 0.99
Age, sex, and location adjusted	0.97 (0.44–2.15) P = 0.94	1.03 (0.17–6.40) P = 0.98	0.98 (0.54–1.81) P = 0.96

	GT versus GG	TT versus GG	Additive Model
rs10490924			
Early AMD RRR (95% CI) P value			
Unadjusted	1.17 (1.01–1.34) P = 0.03	1.61 (1.32–1.96) P < 0.0001	1.26 (1.13–1.35) P < 0.0001
Age, sex, and location adjusted	1.16 (1.01–1.32) P = 0.04	1.57 (1.29–1.91) P < 0.0001	1.22 (1.13–1.33) P < 0.0001
Late AMD RRR (95% CI) P value			
Unadjusted	1.33 (0.63–2.83) P = 0.46	3.44 (1.51–7.85) P = 0.003	1.79 (1.13–2.86) P = 0.02
Age, sex, and location adjusted	1.40 (0.66–2.96) P = 0.38	3.50 (1.53–8.00) P = 0.003	1.81 (1.15–2.86) P = 0.01

	AG versus AA	GG versus AA	Additive Model
HTRA1			
rs2672598			
Early AMD RRR (95% CI) P value			
Unadjusted	1.07 (0.90–1.27) P = 0.45	1.25 (1.03–1.52) P = 0.03	1.12 (1.02–1.24) P = 0.02
Age, sex, and location adjusted	1.06 (0.89–1.25) P = 0.52	1.24 (1.02–1.51) P = 0.03	1.12 (1.02–1.23) P = 0.02
Late AMD RRR (95% CI) P value			
Unadjusted	2.42 (0.94–6.23) P = 0.06	2.40 (0.79–7.32) P = 0.12	1.37 (0.90–2.10) P = 0.15
Age, sex, and location adjusted	2.42 (0.97–6.06) P = 0.06	2.51 (0.82–7.63) P = 0.11	1.41 (0.91–2.17) P = 0.12

early AMD by 12% (adjusted allelic RRR 1.12, 95% CI: 1.02–1.23), but there was no evidence of an association with late AMD for this SNP.

We found no modifying effects of tobacco use with ARMS2/HTRA1, either for any tobacco use, or specifically for tobacco smoking or for tobacco chewing. The sensitivity analyses excluding study participants with multiple relations in the same household were of similar magnitude and direction (see Supplementary Material and Supplementary Tables 3, 4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10073/-/DCSupplemental). The association for rs2672598 (HTRA1) was not detectable in this subsample with 26% reduced sample size.

DISCUSSION

For our study of genetic risk factors of early AMD, we restricted our analysis to well-characterized SNPs that have been extensively studied in European and Indian case-control studies of late AMD.[13–17,19,20,25,36,37] Variants in CFH, C2, and CFB shown to be associated with AMD in Western populations did not appear to increase the risk of early forms of AMD in India. Two variants in the ARMS2/HTRA1 region were associated with increased risk of early AMD. The point estimate of the association of the Y402H SNP in CFH with late AMD was consistent with previously published European studies.[13–16,38] However, the present study had limited power to confirm previously reported associations with late AMD, which may explain the lack of association signals with late AMD for all except one of the candidates studied.

Our study found a high prevalence of early AMD, in particular stage 1 AMD, which reached nearly 40% prevalence in both study centers.[26] The European EUREYE study used the

same grading system as the INDEYE study and found similarly that 36.48% (95% CI, 32.66%–40.30%) of the study population aged 65 or older was graded as early AMD stage 1.[39] Thus, the prevalence of early stage 1 AMD in India is comparable to Europe. As shown previously,[26] the INDEYE study population had a low frequency of persons with more advanced features of both early AMD (stages 2 or 3) and late AMD.

Studies in Europe have reported associations between early AMD and CFH Y402H rs1061170 and ARMS2 (rs10490924).[40–42] Of the candidate SNPs tested, we found associations only with HTRA1/ARMS2 region and early AMD. Our study had high power to detect associations with early AMD. For example, given that the MAF for CFH Y402H was 0.3 in the INDEYE study, there was 99% power to detect an association of an odds ratio (OR) of 1.2 with early AMD if it was present. The Munster study in Germany had reported an allelic OR of 1.9 (99% CI: 1.2–2.9) for the association of CFH Y402H with stage 1 AMD.[41]

In an Indian case-control study, Y402H has been shown to be a risk factor for late AMD with an 11-fold OR reported for the homozygous C genotype.[20] Furthermore, an ecological study of late AMD suggested that the allele frequencies for Y402H in India are as expected, given the observed Indian prevalence of late AMD by age.[43] There is to date no other study of early AMD in India. The absence of an association with CFH Y402H rs1061170 may suggest alternative molecular pathways for the development of early AMD features in India when compared with Europe.

Different polymorphisms in the HTRA region have been associated with slightly different phenotypes of advanced AMD: for example, rs1040924 has been associated with earlier age of onset of AMD, whereas rs2672598 was associated with differences of retinal pigment epithelium pigmentation.[44] These findings would fit with our observations for early

AMD. Also, the association found for rs10490924 (ARMS2) in our study was within the confidence range of the Munster study of early AMD.[41] The previous Indian case-control study of the HTRA region studied cases with late AMD and found no association for rs10490923, for rs10490924 an OR of 8.24 (4.54–14.94; comparing TT to GG), and for rs2672598 an OR of 5.14 (2.86–9.24, comparing GG versus AA).[19] These associations are broadly consistent with our study findings; as outlined previously we had too few cases of AMD to detect associations with late AMD. In contrast to previous studies that described marked effect modification for rs10490924 by smoking,[10] we did not find an interaction between tobacco use and the risk alleles at the ARMS2 locus. The type of smoking habit in India varies from Western populations. In India, men tend to smoke beedies (made from home-grown tobacco). In southern India, chewing tobacco is more prevalent than in the north, especially among women, and in the northern states of India, huqqa smoking is more common among both sexes. These differences in exposure to tobacco-derived products may have contributed to the lack of association for the other ARMS2/HTRA1 variants studied here. The low use of smoking Western-style cigarettes meant we were unable to investigate this type of tobacco use.

The strengths of our study were the random sampling used to identify a representative population sample, the uniform criteria (WARMGS) used to grade fundus images, and the availability of robust demographic information. The associations between ARMS2 and early AMD have not previously been reported in the Indian population. We found MAFs for our candidate SNPs that were comparable with published frequencies in reference databases. However, we did note differences that may be due to random sampling variation or because of the use of hospital-based controls with their inherent biases in the study by Kaur et al.[17]

One limitation of our study was the exclusion of 27% of study participants who had ungradable fundus images owing to cataract. However, genotype and allele frequencies in this group were similar to the group with no signs of AMD (data not shown). Some of our participants shared the same households (25% of all households) but there were no biological relationships in 87.6% of the households that had multiple participants. Our analysis used robust SEs to allow for the clustering in households and villages. In sensitivity analyses, excluding all participants for whom there may be biological relations in the same household found broadly similar results. Hence, it is unlikely that there is residual confounding by consanguinity in our analyses. As this study was aimed at undertaking confirmatory genetic analyses, adjustment for multiple testing was not carried out.

In conclusion, we have found associations of SNPs within the ARMS2 and HTRA1 region with early AMD in India, but no association of early AMD with CFH Y402H. These findings suggest that sequence of pathways by which AMD develops in India may differ from those present in European populations.

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