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Historic, Demographic, and Genetic Evidence for Increased Population Frequencies of CCR5△32 Mutation in Croatian Island Isolates after Lethal 15th Century Epidemics

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Aim To assess the frequency of 32 base pair deletion in *CCR5* (CCR5△32), which has been shown to confer resistance to HIV infection in a homozygous form, in 10 isolated island communities of Dalmatia, Croatia, with different histories of exposure to epidemics during and since the medieval period.

Methods In 2002, DNA analysis of 100 randomly selected individuals from each of the 10 isolated communities of 5 Croatian islands (Susak, Rab, Vis, Lastovo, and Mljet) showed high levels of 3-generational endogamy, indicating limited gene flow. Five of the communities were decimated by epidemics of unknown cause between 1449-1456, while the other 5 villages remained unaffected. Genotyping of the *CCR5* gene was performed using the polymerase chain reaction method with primers flanking the region containing 32-bp deletion.

Results The frequency of CCR5△32 in the 5 villages affected by the epidemic was 6.1-10.0%, and 1.0-3.8% in the 5 unaffected villages. The △32 mutation was found in 71 of 916 alleles among the individuals from the affected villages (7.5%), and in 24 of 968 alleles in unaffected villages (2.5%, $\chi^2 = 27.3$, $P < 10^{-6}$). A previous study in 303 random Croatian blood donors showed the frequency of the CCR5 △32 of 7.1% in the general population. The difference remained significant after correcting for population structure using both STRAT and STRUCTURE software and the genomic control test, to ensure results do not arise from the background genetic differences.

Conclusion Our results and historical evidence, suggest that the mid-15th century epidemic could have acted as a selection pressure for the CCR5△32 mutation.

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C-C chemokine receptor 5 (*CCR5*) has been shown to be a co-receptor for the macrophage-tropic strains of HIV-1 (1,2). In the mid-1990, it was discovered that a mutant variant of this gene with a 32 bp deletion in homozygous form provides almost complete resistance to HIV (1,2), whereas the heterozygous form provides partial resistance and slower progression of AIDS. This mutation is highly prevalent in European populations with an average frequency of 10% but it is almost absent in African, American Indian, and East Asian populations. Within Europe, it shows a strong geographical north-to-south cline, which ranges from a frequency of 0.16 in Mordvinia to 0.04 in Sardinia (3). Slavic populations in the Central Europe are placed in the middle of the European gradient and the estimated frequencies – 10.9% for Poles (4), 10.7% for Czechs (5), 8.7% for Slovenes (6), and 7.1% for Croatians (7) – are similar as those expected based on their geographic location. The analysis of flanking microsatellites in multiple populations showed long-range linkage disequilibrium between selected microsatellite alleles and the 32-bp deletion, which indicates that it traces its origin from a single mutation event, probably having taken place in North-Eastern Europe (8). The high frequency, relatively recent origin, and geographic distribution of the *CCR5-32* deletion allele (CCR5Δ32) together indicate that the deletion has been intensely selected in Europe. Although the allele confers resistance against HIV-1, HIV has not been present in the human population long enough to account for this selective pressure associated with either the heterozygous or homozygous form (8,9).

Controversies regarding the etiology of selection pressure in Europe still remain and there is a prevailing hypothesis that selective rise of this mutation to its current frequency can be attributed to bubonic plague. An alternative hypothesis suggests that viral diseases, such as smallpox or viral heamorraghic fever, were the cause of such a rise.

Lucotte et al (10) compared the frequencies among 40 populations from Europe, the Middle East, and North Africa and confirmed the north-to-south cline. Their research suggested that the *CCR5* 32 bp deletion occurred 1000-1200 years ago and that the Vikings probably disseminated it from north to south. They proposed that it was possibly protective against smallpox in the 8th-10th century.

In contrast, other researchers hypothesized a more recent origin of the mutation and a much stronger selection pressure. *Yersinia pestis* is often suggested as the source of the strong selective pressure, which may explain the cline. This is supported by historical evidence of severe plague epidemics that affectted Europe during medieval times and beyond, especially in the period between 1347 and 1670 (3,11,12). Recently, several research groups have investigated this proposal, with contradictory results. The study of 2900-year-old skeletons from the Bronze Age burials in central Germany revealed that the frequency of the CCR5Δ32 mutation was similar to that in victims from the 14th century pandemic in Lubeck in northern Germany (13,14). Animal studies compared susceptibility to infection and lethal outcome in *CCR5*-deficient and normal mice after infection with *Y. pestis* and showed no difference between these two groups (15). However, caution is needed when interpreting the results of animal studies, since differences in the pathogenesis of *Y. pestis* between mice and men cannot be excluded. Furthermore, in vitro experiments of macrophage uptake of *Y. pestis* showed a 30-fold reduction in homozygous mutant mice (16). Some epidemiological models based on highly complex sets of assumptions supported the role of plague (9), while others supported the role of smallpox (17), and yet others found no evidence of positive selection at all (18). All these studies indicate that the role of the 32-bp deletion in relationship to *Y. pestis* infection is still inconclusive.

Some scientists proposed that highly pathogen filoviruses, closest to those of Ebola and Marburg, could have caused such a high case-fatality and rapid outbreaks due to person-to-person transmition. In this case, the loss of *CCR5* protein on the surface of the cell would represent an advantage because it prevents the entry of the virus into the cell (9).

In this study, we aimed to present further genetic and epidemiological evidence to this ongoing debate. So far, to investigate the hypotheses related to the possible selection pressure on CCR5Δ32 frequencies in Europe, different epidemiological study designs have been deployed but with inconclusive answers. An ideal case-control study should compare the frequencies of this mutation among European sub-populations. Those affected and decimated by epidemics throughout medieval period would be cases and the spared ones would be controls. Carefully documented demographic histories should be essential for classification of populations in terms of exposure. Additionally, both study populations should have the same genetic origin and very low immigration rates since the medieval period to preserve the possible differences in the resulting frequencies of CCR5Δ32 mutation since that period.

To strengthen this study design further, it would be desirable that a valid "negative control" is identified within the affected population (eg, an isolated group of immigrants from the unaffected population who arrived to the affected area after the suspected selection pressure), and that a "positive control" is identified within the unaffected population. Finally, because of the length of the period since the medieval times that is required for the two (or more) investigated sub-populations to be set apart, some genetic differentiation between them should normally be expected. It should, therefore, be demonstrated that any difference observed in CCR5Δ32 mutation frequency between the studied sub-populations is not simply a consequence of genetic differentiation between them and/or population stratification, and that the difference still remains after correction for these processes (19,20).

A persisting difference in CCR5Δ32 frequency would then represent a reasonably strong epidemiological evidence of association, which does not imply causality, between the exposure (disease epidemic in medieval period) and the outcome-current observed frequency of CCR5Δ32 mutation. Only consistent reports from several such studies conducted in favorable sub-populations across Europe would help in reaching a more definite conclusion about the role of medieval epidemics as a selection pressure on the CCR5Δ32 mutation.

Due to the massive migrations in Europe since the medieval period, there are few populations that remained isolated since that period, which are located within the same geographical gradient of CCR5Δ32 frequency, and which are known to have had very differing history of exposure to plague and other epidemics. However, some of the island isolates in Dalmatia, Croatia, may be unique in exhibiting precisely this set of conditions and this may allow us to test the hypothesis that plague and/or other medieval epidemics acted as a positive selective pressure on CCR5Δ32.

MATERIALS AND METHODS

Analysis of historic evidence and the selection of studied population

A careful examination of historic records at the library of the Department for History of Medicine of the Croatian Academy of Sciences and Arts in Zagreb, Croatia, showed that the isolated island populations in Dalmatia could be an exceptionally suitable population for the investigation of the hypothesis that epidemic of lethal infectious disease in the past increased the current frequencies of CCR5Δ32 mutation. Our research initially involved scanning through all historic documents from 1300-1700 period, also known as the period of the most lethal epidemics in the European history, when 3 major outbreaks of "plague" occurred. During this period, Dalmatia was especially severely affected and our particular interest was to document the islands and villages affected by these epidemics and those which were spared. The populations on these islands were of particular interest for this investigation for several reasons: 1) their geographic isolation helped them to avoid the epidemics; 2) when these populations were affected by epidemics, severe consequences were more likely because isolated populations tend to lose heard immunity during the isolation; 3) isolation contributed to the preservation of any differences in CCR5Δ32 frequencies between the affected and unaffected populations, rather than to level them out by gene flow from the large outbred population.

A thorough analysis of the 12 most informative historic sources (21-32) showed that disasterous epidemics of an infectious disease struck the islands of Rab and Susak in the years 1449 and 1456. Between 60 and 95% of the inhabitants of these two islands died or were forced to leave (21-32). In contrast to this, on the islands of Vis, Lastovo, and Mljet there was no evidence of any major epidemics during last 1000 years (21,22,29,30). In addition, the village of Barbat on the affected island of Rab was founded by the settlers from southern Dalmatia in the 18th century, ie, after the epidemic, so it could serve as a "negative control" (24).
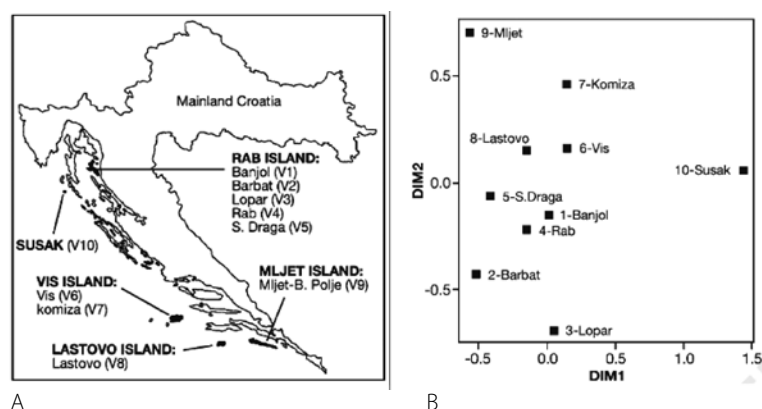
Figure 1.

Figure 1. (A) Geographic location of the 10 studied villages on islands of the Eastern Adriatic, Dalmatia, Croatia (20). **(B)** Representation, in two-dimensional space, of genetic distances between villages, based on allele frequencies at 26 autosomal short tandem repeat (STR) markers – modified from ref. 20).

We included 10 villages from these 5 islands into the study. The selected villages were included in the research program ("10 001 Dalmatians") on genetic regulation of biological quantitative traits distribution in isolated human populations (19,20,33-35). Ten villages were selected based on historic evidence that suggested that 5 of them were exposed to a disasterous epidemics in the years 1449 and 1456, while the other 5 had no history of such exposure in their written history that dates back to 9th century. The geographic locations of the villages are shown in Figure 1A. The reported case fatality ratios in the 5 villages affected by the epidemics in mid-15th century was more than 90% in Rab and 60-70% in the villages of Supetarska Draga, Banjol, Lopar, and Susak. In contrast, the villages Vis, Komiža, Lastovo, Mljet, and Barbat represent the unaffected "control population."

Sample selection and genotyping

In all 10 selected settlements, a random sample of 100 examinees was identified and then recruited as a part of a larger research program (20,33-35). The island of Susak was an exception, with 72 volunteering individuals recruited from a total population of 188 (20). Methods of sampling of the examinees, field work activities, and procedures of obtaining the materials, their storage, and transport to the UK Medical Research Council Human Genetics Unit in Edinburgh have been described in detail elswhere (20,33,34).

Genotyping of the *CCR5* polymorphism was performed at the Medical Research Council Human Genetics Unit using the polymerase chain reaction (PCR) method with the following primers flanking the region containing 32-bp deletion: forward primer ACCAGATCTCAAAAAGAAGGTCT, and reverse primer CATGATGGTGAAGATAAGCCTCACA.

The PCR products were analyzed by 2% agarose gel electrophoresis. The normal allele was detected as a 225-bp fragment and the *CCR5* 32-bp deletion allele was detected as a 193-bp fragment. These are standard methods to detect CCR5Δ32 mutation in humans and they have been described in greater detail elsewhere (3,8,18).

All available DNA samples from the 10 villages were genotyped. The success rate of genotyping was 100% for Banjol, Barbat, and Komiža villages; 98% for Vis and Lopar; 97% for Susak; 94% for Rab and Mljet; and 92% for Lastovo.

Our earlier study of the same 10 villages using 26 microsatellite polymorphic markers from ABI Prism linkage panel 11 and 19 (on chromosomes 7, 8, 12, and 13), spaced at least 5 cM apart, showed that the 10 chosen villages were extremely differentiated (Figure 1) (20). This was favorable because it was unlikely that genetic drift would by chance always increase the frequency of CCR5Δ32 in one cluster of populations and decrease it in another cluster. STRUCTURE software (*pritch.bsd.uchicago.edu/software*) was used to separate 945 individuals with successful genotyping according to their likely village of origin. Each of the clusters of 5 villages that we defined contained 3 populations that were genetically relatively similar to each other (Rab, S. Draga, and Banjol among the affected; and Vis, Komiža, and Lastovo among the unaffected), and 2 highly differentiated outliers (Susak and Lopar among the affected; and Barbat and Mljet among the unaffected) (20). This confirmed that the individuals from Barbat village from Rab island were indeed of different genetic origin than the rest of the villages on the Rab island, as the historic records indicated (20,24). The information based on these 26 microsatellite markers was clearly sufficient to detect sub-structure and differentiate between the 10 villages, so it was used in this study to correct for population stratification and by the genomic control method.

Statistical analyses

Differences in allele frequency of CCR5Δ32 between the 2 groups of 5 settlements and between each group and a published sample from the Croatian general population was determined by χ^2 test (36). The initial difference between the 2 clusters of 5 villages in the frequency of CCR5Δ32 was corrected for population stratification using 26 microsatellite loci already used in the study of Vitart et al (20). Correction for stratification and the association test in the presence of the population structure was performed as proposed by Pritchard et al (37,38), using the STRAT software. According to this method, the association at each locus was first tested by χ^2 test, ignoring the presence of population structure (rare alleles with frequency <10% were pooled), to determine whether a population structure exists. Under the null-hypothesis (that the two clusters of populations do not differ significantly in allele frequencies), the sum of the statistics for all of the markers has a χ^2 distribution with degrees of freedom from all of the markers. In effect, this statistic tests for an overall difference in allele frequencies between the case and control subjects. The STRUCTURE program developed by Pritchard et al (39) was used to estimate the ancestry of the individuals in the sample using 26 microsatellite markers. The association using STRAT (*pritch.bsd.uchicago.edu/software/ STRAT.html*) was performed conditional on the ancestry of the individuals in the sample. We then employed

the "genomic control" procedure by estimating a multiplicative factor that inflates the standard χ^2 distribution and is proportional to the degree of stratification. It was estimated using the same 26 microsatellite polymorphisms studied by Vitart et al (20). At the end, χ^2 test statistic of the *CCR5* polymorphism association with "exposure to epidemics" was corrected with this factor to account for background population genetic differences.

RESULTS

Characteristics of epidemics based on historic records
It is known that an epidemic of unknown cause struck Venice and Verona in the 1420s and inflicted a detectable increase in mortality. This happened some 70 years following the major epidemic of "*pestilenza*" in Venice in 1348 , which claimed the lives of nearly half of the city's population (Figure 2). After that major wave in 1348, the population continued to be challenged intermittently by new epidemics (Figure 2), but those waves inflicted considerably smaller mortality. However, after the wave in the 1420s the populations of Venice and Verona reached their lowest levels in the 15th century – 14 000 in Verona and 85 000 in Venice. It is not certain whether the occasional challenges after 1348 were caused by the same infectious agent (believed to be *Y. pestis*), or other causative agents. While the epidemic caused by *Y. pestis* was prevalent in the Mediterranean basin, it is believed that the epidemic that struck Verona and Venice in 1420s had arrived from the Northern Europe through the "route of Amber" that stretched through the Alps (27,40).

Quarantine practices, first introduced in Dubrovnik Republic in 1377, probably encouraged health autorities in Dalmatian cities to use isolation measures, which managed to delay the spread of infectious epidemics to busy Dalmatian towns and communes, such as Rab. However, although the two waves that occurred in mid-15th century did not inflict mortality on the scale of the 1348 epidemic in Venice, on the islands of Susak and Rab they caused the deadliest epidemic ever recorded on Dalmatian islands. In the town of Rab, more than 90% of population cumulatively died in the two waves, while villages Banjol, S. Draga, Lopar, and the island of Susak lost 60-70% of inhabitants (20-32). An unusual characteristic of both epidemics was that they occurred during summer months (July-September), while the bubonic plague typically occurred during autumn and winter months (September-February). This poses the question whether *Y. pestis* was the cause of these two epidemic waves (20-32,41).
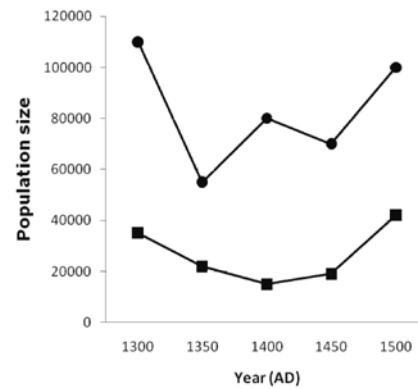
Figure 2.

Figure 2. Estimated population size of Italian cities Venice (circles) and Verona (squares), from 1300-1500, and the impact of recurring epidemics of *pestilentia* on population size throughout these periods (extracted and modified from ref. 40).
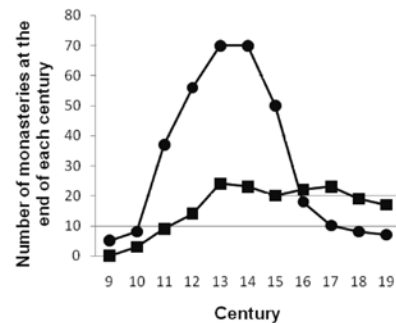
Figure 3.

Figure 3. The number of male-run (circles) and female-run (squares) Benedictine monasteries in Croatia from the 9th to 20th century (extracted and modified from ref. 42).

The scale and importance of these two epidemics to the local community is reflected in the graph presented in Figure 3. The graph shows the rise and fall of the Benedictine monasteries in Croatia. The Benedictine monasteries were especially frequent in northern parts of Croatian coast and their number increased from only 5 in the 9th century to more than 70 by the end of 14th century. The number of Benedictine monasteries and churches in Croatia was large and they were the ones usually offering help to poor and diseased in the society. During the epidemics in 1449 and 1456, these monasteries were the places where the diseased sought refuge and treatment. Figure 3 shows how

their number declined sharply after those years, first to 50 by the end of the 15th century, and then with further epidemics in the region the number decreased to only 15 by the end of the 16th century. It is likely that the epidemics in 1449 and 1456 substantially contributed to the fall of Benedictines in Croatia (42).

Possible effect of epidemics on CCR5Δ32 mutation frequency

Table 1 presents the frequencies of CCR5Δ32 mutation in all 10 selected villages, along with the number of examinees recruited and the number of successful genotypes for each village population. In the populations of the 5 affected villages, the frequencies of CCR5Δ32 ranged from 6.1% to 10.0%. In comparison, in the populations of the 5 unaffected villages, including the "negative control" village of Barbat, the frequencies were lower, with the range from 1.0% to 3.8% (Table 1). When compared with the estimated frequency for the general Croatian population of 7.1% (7), the frequencies in the 5 unaffected populations were all exceptionally low.

Differences in the frequencies of CCR5Δ32 between populations of the 5 affected villages, 5 unaffected populations, and a sample from Croatian general population is shown in Table 2. When pooled together, the difference between the populations of the 5 affected villages and the general population was not significant ($P = 0.636$). However, the difference between the frequency of CCR5Δ32 in the pool of the populations of the 5 unaffected vs the general population was highly significant ($P = 1.0 \times 10^{-5}$). The difference between the pooled populations of 5 affected vs unaffected was even more significant ($P = 1.7 \times 10^{-7}$). The latter difference remained significant after correcting for population sub-structure in both samples using STRAT and STRUCTURE software ($P = 2.3 \times 10^{-5}$) and after correction of this outcome using genomic control test ($P = 0.043$).

DISCUSSION

We tested several hypotheses. The first stated that if the epidemics in mid-15th century that struck the islands of Susak and Rab and spared the islands of Vis, Lastovo, and Mljet acted as selection pressure on CCR5Δ32 mutation, If this was correct, then we would expect the present-day frequency of this mutation in each one of the 5 affected villages to be higher than in the each one of the 5 unaffected villages. Furthermore, the reference value for CCR5Δ32 frequency in Croatian general population should lie between the values for affected and unaffected villages. The pattern of frequencies presented in Table 1 shows that the frequencies were generally considerably higher in the affected than in the unaffected villages, and that the frequency in general population indeed lied between the two pooled values, although it was much closer to the value found in affected villages. However, not all affected villages had the frequency greater than the estimate for general population (7.1%), and the overall difference be-

TABLE 1. Number of examinees in each of the 10 selected villages (recruits), number of individuals that were successfully genotyped for CCR5Δ32 (genotyped), number of alleles genotyped in each village (alleles), number of alleles with CCR5Δ32 mutation in each village (CCR5Δ32) and estimated frequency of CCR5Δ32 mutation in each of the 10 village populations

Villages	Number of:				Percentage of
	recruits	genotyped	alleles	CCR5Δ32	CCR5Δ32 carriers (95% CI)
Affected:					
Rab	100	94	188	12	6.4 (2.90-9.90)
Banjol	100	100	200	20	10.0 (5.84-14.16)
Supetarska Draga	100	96	192	13	6.8 (3.24-10.36)
Lopar	100	98	196	12	6.1 (2.75-9.45)
Susak	72	70	140	14	10.0 (5.03-14.97)
all	472	458	916	71	7.8 (6.06-9.54)
Unaffected:					
Vis	100	98	196	5	2.6 (0.37-4.83)
Komiža	100	100	200	3	1.5 (0-3.18)
Lastovo	100	92	184	7	3.8 (1.04-6.56)
Mljet	100	94	188	3	1.6 (0-3.39)
Barbat	100	100	200	6	3.0 (0.64-5.36)
all	500	484	968	24	2.5 (1.52-3.48)

TABLE 2. Comparison of the difference in population frequency of CCR5Δ32 between the 5 affected villages, the 5 unaffected villages, and a sample from general population of Croatia.

	N1 (alleles)	f1 (Δ32) (95% CI)	N2* (alleles)	f2* (Δ32) (95% CI)	$P^†$
Affected vs general population	916	7.8% (6.06-9.54)	606	7.1% (5.06-9.14)	0.636
Unaffected vs general population	968	2.5% (1.52-3.48)	606	7.1% (5.06-9.14)	1.0×10^{-5}
Affected vs unaffected‡	916	7.8% (6.06-9.54)	968	2.5% (1.52-3.48)	1.7×10^{-7}
Affected vs unaffected§	916	7.8% (6.06-9.54)	968	2.5% (1.52-3.48)	2.3×10^{-5}
Affected vs unaffectedII	916	7.8% (6.06-9.54)	968	2.5% (1.52-3.48)	0.043

*Information from ref. 7.
†χ^2 test.
‡Unadjusted value.
§Adjusted for population structure using STRAT and, STRUCTURE softwares (39).
IIAdjusted using genomic control after the first correction (39).

tween populations from the 5 affected villages put together and the sample from the general population was not significant. This could still mean that these frequencies were higher in the affected villages after the epidemics in the 15th century, but that a gene flow existed between the general population and the 5 affected villages during the past several centuries, which levelled out the frequencies to some extent. Another explanation is that both the general population and the affected populations were exposed to recurring epidemics that did act as selection pressure over an undefined period of time and increased the frequency of Δ32 mutation in both populations to the level that is observed today.

One way to further explain the difference in allelic frequencies between the villages would be to test the second hypothesis, which states that a "gradient" of CCR5Δ32 frequency should be expected in the villages, such that the less isolated affected and unaffected villages (with lesser endogamy) have more similar frequencies to the general population because of gene flow, while the most isolated affected and unaffected villages demonstrate larger differences in CCR5Δ32 frequencies. The most isolated villages in both groups should therefore have the highest or the lowest frequencies. In the group of affected villages, the most isolated are Lopar and Susak, with endogamy levels of 98.4% and 85.7% and CCR5Δ32 frequencies of 6.1% and 10.0%, respectively. Rab, Banjol, and S. Draga are less isolated and have endogamy levels of 39.4%, 41.9%, and 46.9% and frequencies of CCR5Δ32 mutations of 6.4%, 10.0%, and 6.8%, respectively. In comparison, in the group of unaffected villages, the most isolated are Mljet, Komiža, Barbat, and Vis, with endogamy levels of 93.5%, 91.3%, 90.3%, and 87.7% and frequencies of 1.6%, 1.5%, 3.0%, and 2.6%, respectively. Lastovo is less isolated and has endogamy levels of 71.7% and a frequency of CCR5Δ32 mutation of 3.8%. Although not perfectly consistent with predic-

tions, these data imply that it is likely that immigration into villages and gene flow did act to level out the differences in CCR5Δ32 mutation frequency between the general population and the villages, while genetic drift probably contributed further. Overall, given all the possible scenarios, we believe that the results support the hypothesis that there is a true difference between the affected and unaffected group of villages in CCR5Δ32 frequency.

An additional important argument is found in the data from the village Barbat. If the epidemics acted as a selection pressure on CCR5Δ32, then the frequency of CCR5Δ32 mutation in this village would be expected to be smaller than in the general Croatian population and other villages on the island of Rab, because the settlers arrived from Southern Dalmatia in the 18th century. This is precisely what was found. Also, the difference between the frequency of the CCR5Δ32 in the 5 affected villages and the 5 unaffected villages, when pooled together, was highly significant and remained so after correction for population structure and genomic control for 26 other unlinked genomic polymorphisms (Table 2). Taken together, this evidence supports the association between the 15th century epidemics and the frequency of CCR5Δ32 mutation in the 10 selected villages, although it does not in itself imply direct causality.

Another way to check for plausibility of our conclusion is to assume, based on the historic evidence, that the islanders of Vis, Lastovo, and Mljet were indeed never exposed to any major lethal infectious epidemics in the 1000-year history of their islands. The frequencies observed there could be representative of the baseline frequencies present in the European population since the peopling of Europe. We can also assume that the lethal epidemics in Rab spared the carriers of the mutation (either the heterozygotes or homozygotes). In order to do a rough calculation, we can suppose that the "baseline" frequency of CCR5Δ32

mutation is about 2.5% (an overestimate given the worldwide frequencies) and both homo- and heterozygote carriers were spared (which is uncertain and probably unlikely). With the average case fatality ratio of around 75% (which is probably an overestimate), roughly only a quarter of the mutation-free population would survive, resulting in relative increase of the mutation in the population. Even under such scenario, most favorable for the enrichment of the frequency of CCR5Δ32 mutation in the surviving population, it would "only" increase 4-fold, ie, to 10.0%. This is in line with our findings, when some gene flow and genetic drift over the past 600 years are accounted for. Therefore, repeated episodes of highly lethal epidemics over much longer periods of time were probably required to bring the frequency of CCR5Δ32 to the levels present in some northern territories of Europe, ie, close to 20%. The repeated waves of the epidemics of unknown cause during the entire 15th century, which occurred outside the three major waves of plague, could therefore perhaps represent an explanation for the rise of CCR5Δ32 mutation in Europe.

In conclusion, according to historic records, the epidemics in 1449 and 1456 were the most disasterous ever to hit the Croatian coastal region, with a cumulative mortality more than 70%. In this article, we gathered strong circumstantial evidence that these epidemics were not caused by bubonic plague, but rather by a viral disease with an unusually long incubation period. The hypothesis of a viral disease (possibly a hemorrhagic fever) is supported by the following historic evidence: peak incidence in summer months, long incubation period (about 1 month), apparent transmission from person to person, very high case fatality ratio, and its likely arrival from northern Europe/Russia, where several regions are commonly known for occasional outbreaks of rare and highly lethal zoonoses. In addition, a viral disease is more likely to interact with CCR5dΔ32 than a bacterial disease. The disease arrived from the northern Europe to Venice through the "route of amber" and it had inflicted recurring lethal attacks throughout the whole 15th century. In Croatia, it caused the fall of Benedictines in the region, who treated the diseased.

Genetic-epidemiological evidence presented in this study points to the conclusion that these recurring epidemics could have acted as a selection pressure upon an already existing and widespread (but rare) CCR5Δ32 mutation and resulted in the unusually high frequencies that are observed across Europe today, but more studies undertaken in similar settings are needed to confirm this conclusion.

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