Increased Incidence of Sporadic Creutzfeldt-Jakob Disease on the Island of Crete Associated with a High Rate of \textit{PRNP} 129-Methionine Homozygosity in the Local Population

Andreas Plaitakis, MD, Anna K. Viskadouraki, MD, Minas Tzagournissakis, MD, Ioannis Zaganas, MD, Susan Verghese-Nikolakaki, PhD, Vasilis Karagiorgis, Ioannis Panagiotides, MD, Constantine Kilindireas, MD, Eustratios Patsouris, MD, Christine Haberler, MD, Herbert Budka, MD, and Theodoros Sklaviadis, PhD

Since the spring of 1997, when the Neurology Department of the University Hospital of Crete admitted its first patient, nine cases (eight neuropathologically confirmed and one probable) of sporadic Creutzfeldt-Jakob disease (sCJD) have been recorded. This represents an annual incidence five-fold higher than expected based on the island’s population (0.54 million). Molecular analysis of the prion-protein gene (\textit{PRNP}) showed no mutations in any of the seven CJD cases studied. Five patients (ages 64–88 years) were homozygous for methionine-129 of \textit{PRNP} and showed the classic sCJD triad (subacute dementia, myoclonus, periodic electroencephalogram). Brains contained type 1 (unglycosylated 21.5 kDa band) protease-resistant prion protein (PrPres). Two patients (ages 56 and 57 years), both homozygous for valine-129, showed cerebellar ataxia and later dementia not associated with periodic electroencephalogram; brain PrPres was type 2. Genotyping of 205 Cretan controls showed that methionine-129 homozygosity, a susceptibility factor for sCJD, was significantly higher in this population than in other Caucasian populations (57.0% n = 205 vs. 41.5% n = 859, \(p < 0.0001\)). These data are the first to relate a high regional incidence rate for sCJD to the distribution of \textit{PRNP} 129 genotypes in the local population; however, additional factors may be operational.

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Creutzfeldt-Jakob disease (CJD) is characterised clinically by subacute dementia, myoclonus, movement disorders, bulbar dysfunction, and rapid neurological decline, terminating fatally within weeks or months from the onset of the first symptoms. Pathologically, the central nervous system of CJD patients shows spongiform changes and accumulation of an abnormal protease-resistant isoform of prion protein, designated PrPres, that is insoluble and resistant to proteinase K.

The annual incidence of CJD is about one case per million people worldwide. In some populations, however, higher incidence rates have been recorded. As a rule, these high rates relate to the presence of familial CJD cases associated with specific mutations in the prion-protein gene (\textit{PRNP}) that are prevalent in these populations. Also, regional variation in the incidence of sporadic CJD (sCJD) is known to occur in European countries, but the reason(s) for this remain unknown.

Since the spring of 1997, when the Neurology Inpatient Service of the University Hospital of Crete accepted its first patient, nine cases of sCJD (eight definite and one probable) have occurred among natives of this island. This represents an annual incidence five-fold higher than expected on the basis of the island’s population (0.54 million). The clinical and pathological characteristics of these patients and the results of
biochemical, immunocytochemical, and molecular genetic analyses are described below.

Patients and Methods

Clinical Evaluations and Diagnostic Tests
All patients underwent thorough physical and neurological examinations. Brain computed tomographic and magnetic resonance imaging (MRI) scans and lumbar punctures were performed in all cases. Electroencephalograms (EEGs) were obtained upon admission and at various intervals during hospitalization. Complete blood counts, blood chemistries, serum protein electrophoresis and immunoelectrophoresis, serology, antinuclear antibody, erythrocyte sedimentation rate, and measurement of serum levels of vitamin B₁₂ and folate were obtained. Thyroid, parathyroid, and adrenal functions were also evaluated.

Neuropathology

Complete neuropathological examinations were performed on all 7 patients who came to autopsy (cases 1, 2, 4–8) and brain biopsy in 1 (case 3) (Table 1). For case 9, autopsy was declined. Paraffin-embedded sections from the cerebral hemispheres (frontal, parietal, temporal, and occipital lobes), cerebellum, brain stem, thalamus, and basal ganglia were studied. Serial sections were cut at 5 μm and stained with hematoxylin and eosin for routine histology. Also, van Gieson, periodic acid-Schiff, and Gomori stains were employed. Immunocytochemical studies were carried out according to an optimized pretreatment protocol (formic acid, guanidine thiocyanate, and hydrated autoclaving). Two antibodies against the prion protein (PrP), 3F4 and 6H4, and an antibody to glial fibrillary acidic protein were used. Sections were treated with appropriate secondary antisera and visualised using the avidin-biotin complex and diaminobenzidine.

Purification of PrPres and Western Blot Analysis

Western blots were performed in all 6 sCJD cases in which wet brain tissue was available (Table 2). Prion protein was partially purified from brain tissue of CJD patients and controls as previously described. Tissue samples from the pons, putamen, cerebellum, or occipital lobe were processed separately. Proteins were separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PrPres was detected using the monoclonal antibody 3F4 (1:5,000).

Sequencing of the PRNP

Genomic DNA was obtained from wet brain tissue or from blood leukocytes from 7 patients. A region of PRNP spanning codons 1–242 was amplified by polymerase chain reaction (PCR) using the following primers: 5′-GCT CTA GAG GAT GGC GAA CCT T-3′ (sense) and 5′-GAG AGA GAT CAG GAG G-3′ (antisense). Sequencing was performed using the LI-COR 42000 system (LI-COR, Inc., Lincoln, Nebraska).

Genotyping of Cretan Natives

Genomic DNA was isolated from peripheral blood leukocytes of unrelated control individuals, all natives of Crete. For studying polymorphism at codon 129, a 239 bp segment of the coding region of the PRNP containing a unique NspI restriction site was PCR-amplified using the following primers: 5′-ACCCACAGTCAGTGGAACAAGG-3′ (sense) and 5′-TGTTCTGGTGCTGACTCATC-3′ (antisense). Amplification products were digested by NspI and electrophoresed in 4% agarose.

Statistical Analysis

Overall and age-specific incidence and mortality rates were calculated based on Cretan patients diagnosed with CJD.

Table 1. Demographic and Clinical Features of Creutzfeldt-Jakob Disease Cases

<table>
<thead>
<tr>
<th>Number</th>
<th>Age (yrs)/ Gender</th>
<th>Occupation</th>
<th>Presenting Symptoms</th>
<th>Neurological Features</th>
<th>Hospital Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56/M</td>
<td>Electrician</td>
<td>Severe gait ataxia, photophobia, nervousness for 2–3 months</td>
<td>Profound cerebellar-oculomotor deficits, mild cognitive decline</td>
<td>Rapid neurological decline, death in 2 months</td>
</tr>
<tr>
<td>2</td>
<td>57/M</td>
<td>Post office employee</td>
<td>Gait disturbances, mild memory loss for 2 months</td>
<td>Cerebellar and extrapyramidal syndrome, mild cognitive decline</td>
<td>Rapid neurological decline, death in 2 months</td>
</tr>
<tr>
<td>3</td>
<td>76/M</td>
<td>Farmer</td>
<td>Visual illusions, mental changes for 1 month</td>
<td>Profound dementia, mutism, myoclonus, head scratching</td>
<td>Rapid decline, coma in 2 months, death in 7 months</td>
</tr>
<tr>
<td>4</td>
<td>68/F</td>
<td>Farmer</td>
<td>Depression, emotional lability for 2 months</td>
<td>Profound dementia, visual hallucinations, myoclonus, ataxia</td>
<td>Rapid neurological decline, death in 2.5 months</td>
</tr>
<tr>
<td>5</td>
<td>88/M</td>
<td>Farmer</td>
<td>Mental changes for 1 month</td>
<td>Profound dementia, visual hallucinations, myoclonus, ataxia</td>
<td>Rapid neurological decline, death in 5 weeks</td>
</tr>
<tr>
<td>6</td>
<td>81/F</td>
<td>Housewife</td>
<td>Insomnia, irritability for 1 year, memory loss for 2 months</td>
<td>Profound amnesia, myoclonus, akinetic mutism</td>
<td>Rapid neurological decline, death in 2 weeks</td>
</tr>
<tr>
<td>7</td>
<td>64/M</td>
<td>Restaurant owner</td>
<td>Dizziness, unsteadiness, mental changes for 1 month</td>
<td>Profound dementia, movement disorder, myoclonus, seizures</td>
<td>Rapid neurological decline, death in 4 months</td>
</tr>
<tr>
<td>8</td>
<td>65/M</td>
<td>Farmer</td>
<td>Dizziness, gait disturbances, mental changes for 1.5 months</td>
<td>Profound dementia, myoclonus, akinetic mutism</td>
<td>Rapid neurological decline, death in 2 months</td>
</tr>
<tr>
<td>9</td>
<td>65/F</td>
<td>Farmer</td>
<td>Distorted vision, hallucinations, mental changes for 2 months</td>
<td>Profound dementia, myoclonus, hypertonus, visual hallucinations</td>
<td>Rapid neurological decline, death in 2 weeks</td>
</tr>
</tbody>
</table>
from the spring of 1997 to the fall of 2000 (3.5 years). These were compared to CJD rates reported for France, Germany, Italy, the Netherlands, and the United Kingdom for the years 1993–1995.9 Age-adjusted rates were then calculated according to the method of direct standardisation, to reflect the age distribution of all of the above populations in 1996. Data on the age distribution of the above European populations were taken from the U.S. Census Bureau (www.census.gov) and those for the population of Crete from the National Statistical Service of Greece (1991 census). For incidence rates, 95% confidence intervals were calculated using the Poisson distribution.

The overall distribution of the 3 codon 129 genotypes (Met/Met, Met/Val, and Val/Val) and the percentage of a particular genotype in the Cretan population were compared to those of 5 other Caucasian populations using the \( \chi^2 \) test. Also, the \( \chi^2 \) test was used for evaluating whether the distribution of the 2 alleles (Met\(^{129}\), Val\(^{129}\)) in the local population followed the Hardy-Weinberg equilibrium. All tests were 2-sided.

### Results

#### Clinical Findings

From the spring of 1997 to the fall of 2000, 9 previously healthy individuals, all natives of Crete and living on the island, died of sCJD. The demographic characteristics, presenting symptoms, neurological features, and clinical course of these patients are summarized in Table 1. Seven patients (Cases 3–9, Table 1), 64 to 88 years of age, showed the classic triad of subacute dementia, myoclonus, and periodic sharp-wave changes on EEG (Table 2), whereas 2 patients (Cases 1 and 2), who were affected at a younger age (56 and 57 years), presented with prominent cerebellar ataxia and later dementia. All patients experienced a swift clinical decline, characterized by rapidly advancing dementia, increasing ataxia, bulbar dysfunction, and global neurological deterioration. Generalized seizures, akinetic mutism, stupor, and coma supervened, culminating in death within 2 weeks to 7 months from admission. None of our CJD cases had a known iatrogenic exposure, such as previous neurosurgical procedures (including dura mater grafting) and growth hormone therapy.

#### Diagnostic Tests

EEG recordings detected periodic discharges and/or pseudoperiodic sharp-wave complexes in all 7 patients (Cases 3–9) who showed the classic CJD triad. In contrast, the 2 patients who presented with ataxia (Cases 1 and 2) showed nonspecific abnormalities (slowing) on EEG. While routine cerebrospinal fluid studies (cytology, chemistry, and serology) were normal in all patients, protein 14-3-3 was positive in 6 cases (Table 2). MRI scans of the brain either were normal or revealed some degree of diffuse or focal cortical atrophy (Cases 1, 3, 5, and 6). A symmetrical, nonenhancing, high-intensity T2 signal was noted in the basal ganglia and, to a lesser degree, in the thalamus in Cases 1, 3, 4, 6, and 9.

#### Neurohistology and Immunocytochemistry

Examination of brain tissue, obtained at autopsy in 7 cases, showed typical CJD histopathology (Fig 1). Prominent spongiform changes, loss of nerve cells, and proliferation of astrocytes were observed mainly in the cerebral and cerebellar cortex, thalamus, and basal ganglia. A predilection for the deeper layers was found in the cerebral cortex. In the frontal, parietal, and occipital lobes, the small vacuoles appeared to coalesce, forming larger, multilobulated vacuoles. The white matter showed no significant changes. In no case did we observe inflammatory changes, senile plaques, and/or Kuru-like plaques. Anti-PrP immunocytochemistry revealed moderate to high amounts of synaptic PrP deposits (Fig 1B) in all tissue blocks examined. In case 1, small, unicentric, plaque-like deposits were observed in the granular layer of the cerebellum. For case 3, only brain material obtained by biopsy was available. Although neurohistological examination was hampered...
by severe artificial damage of the sample, we observed reactive astrocytes and positive PrP immunoreactivity.

**Western Blot**
Western blot analysis revealed PrPres in all tested brain regions (occipital lobe, cerebellum, pons, and putamen) of CJD patients (Fig 2A). In 4 Met\textsuperscript{129}Met cases studied (Table 2), the unglycosylated 21.5 kDa band was similar to type 1 (Fig 2B), as defined by Parchi et al.\textsuperscript{10} In contrast, in the 2 Val\textsuperscript{129}Val cases (Table 2), the unglycosylated 21.5 kDa band was similar to type 2 (Fig 2B), as defined by Parchi et al.\textsuperscript{10}

**PRNP Analysis**
Sequencing of the entire coding region of the \textit{PRNP} did not detect mutations in any of the 7 patients (cases 1–6, 9) studied. Cases 1 and 2 were homozygous for Val\textsuperscript{129}, whereas cases 3–6 and 9 were homozygous for Met\textsuperscript{129} (Table 2).

**Genotyping of the Local Population**
Genotyping of 205 control individuals from the local population (all natives of Crete) showed that 117 (57\%) were homozygous for Met\textsuperscript{129}, 77 (37.5\%) were heterozygous (Met/Val), and 11 (5.5\%) were homozygous for Val\textsuperscript{129} (Table 3). Codon 129 genotype rates for 205 Cretan natives gave an allele frequency of 0.76: 0.24 Met:Val. This distribution follows the Hardy-Weinberg equilibrium ($\chi^2 = 0.07, p > 0.95$) and is significantly different from that of other Caucasian populations (Table 3).

**Incidence Rates**
The overall incidence and mortality rates for the 3.5-year period was 4.76 cases per million per year. This is about 5 times greater than that reported for the United States (0.9 cases per million)\textsuperscript{11} and for several European countries,\textsuperscript{9} including Finland.\textsuperscript{4} Age-specific incidence rates for age groups 50–59, 60–69, 70–79, and 80+ were substantially higher than those reported for...
other European countries (Table 4). Also, the age-adjusted incidence for Crete was 4.84 cases per million, which is more than 5 times higher than that calculated for Germany (0.49 cases/million), Italy (0.63 cases/million), the United Kingdom (0.85 cases/million), the Netherlands (0.95 cases/million), and France (0.99 cases/million). We observed a relatively consistent mortality rate with time between the spring of 1997 and the fall of 2000 (Table 5).

**Discussion**

During the past three and a half years, the annual incidence of sCJD on Crete was 4.76 cases per million. This is about five times greater than annual mortality rates recorded in recent years in several European countries as well as the United States. Although the population of Crete has a high life expectancy, this does not appear to be a major factor since age-specific and age-adjusted incidence rates were four- to ten-fold greater than reported elsewhere. To our knowledge, CJD had not been previously recorded on Crete, but a Neurology Department did not exist on the island until 1997. Hence, it is unclear if the epidemiology of the disease has changed recently in this region. Because the reporting period is relatively short, long-term data are needed to see whether the incidence rates recorded here

Table 3. Polymorphism at Codon 129 of the Prion-Protein Gene (PRNP) in Healthy Individuals

<table>
<thead>
<tr>
<th>Population</th>
<th>Number Tested</th>
<th>Met&lt;sup&gt;129&lt;/sup&gt;Met</th>
<th>Met&lt;sup&gt;129&lt;/sup&gt;Val</th>
<th>Val&lt;sup&gt;129&lt;/sup&gt;Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cretan natives</td>
<td>205</td>
<td>117 (57%)</td>
<td>77 (37.5%)</td>
<td>11 (5.5%)</td>
</tr>
<tr>
<td>Other Caucasians (combined data &lt; 0.0001)</td>
<td>859</td>
<td>357 (41.5%) &lt; 0.0001</td>
<td>409 (47.5%) &lt; 0.02</td>
<td>93 (11%) &lt; 0.03</td>
</tr>
<tr>
<td>Austrian&lt;sup&gt;a&lt;/sup&gt; (&lt; 0.008)</td>
<td>300</td>
<td>129 (43%) &lt; 0.003</td>
<td>146 (49%) &lt; 0.02</td>
<td>25 (8%) ns</td>
</tr>
<tr>
<td>French&lt;sup&gt;b&lt;/sup&gt; (&lt; 0.003)</td>
<td>161</td>
<td>63 (39%) &lt; 0.001</td>
<td>82 (51.1%) &lt; 0.02</td>
<td>16 (9.9%) ns</td>
</tr>
<tr>
<td>Italian&lt;sup&gt;c&lt;/sup&gt; (&lt; 0.003)</td>
<td>206</td>
<td>93 (45%) &lt; 0.03</td>
<td>83 (40%) ns</td>
<td>30 (15%) &lt; 0.02</td>
</tr>
<tr>
<td>U.K.&lt;sup&gt;d&lt;/sup&gt; (&lt; 0.002)</td>
<td>106</td>
<td>39 (37%) &lt; 0.002</td>
<td>54 (51%) &lt; 0.04</td>
<td>13 (12%) ns</td>
</tr>
<tr>
<td>U.S.A.&lt;sup&gt;e&lt;/sup&gt; (&lt; 0.02)</td>
<td>86</td>
<td>33 (38%) &lt; 0.006</td>
<td>44 (51%) &lt; 0.05</td>
<td>9 (11%) ns</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values in parentheses refer to comparisons with Cretan natives with respect to the overall distribution of the 3 codon 129 genotypes (Met<sup>129</sup>Met, Met<sup>129</sup>Val, and Val<sup>129</sup>Val). p values without parentheses refer to comparisons with Cretan natives with respect to the percentage of the particular genotype.

<sup>b</sup>Data from Zimmerman et al. 20
<sup>c</sup>Data from Laplanche et al. 15 and Deslys et al. 16
<sup>d</sup>Data from Medori et al. 21 and Salvatore et al. 18
<sup>e</sup>Data from Collinge et al. 22
<sup>f</sup>Data from Brown et al. 23
The increased incidence of CJD on Crete is not due to familial cases of the disease in the island’s population. None of our cases had a positive family history, and analysis of the coding region of the PRNP failed to detect mutations associated with familial CJD. We cannot exclude the possibility of DNA changes outside of the coding region of the PRNP studied here, but no such changes predisposing to CJD are currently known.

We observed two distinct phenotypes of CJD. The first was seen in seven patients, 64–88 years of age, who showed the classic sCJD triad of rapidly advancing dementia, myoclonic jerks, and periodic sharp waves on EEG. Five of these were tested and found to be homozygous for Met129 of PRNP. Brains showed accumulation of type 1 PrPres (unglycosylated 21.5 kDa band), as defined by Parchi et al. The second phenotype was seen in two patients, 55 and 57 years of age, who showed cerebellar ataxia and later dementia in the absence of periodic EEG changes. Both were homozygous for Val129 and their PrPres (unglycosylated band 21.5 kDa) was type 2. These findings are in accordance with those of recent reports showing that codon 129 polymorphism influences the clinical expression of sCJD.

Genotyping of Cretan controls revealed that Met129 homozygosity was significantly higher in these individuals (57%, n = 205; p < 0.0001) than in other Caucasian controls (41.5%, n = 859). However, Met/Val129 heterozygosity was lower on Crete (37.5%, n = 205; p < 0.02) than elsewhere (47.5%, n = 859). Also, significant differences were found when Cretan controls were compared to each of five different control populations for which genotypic data were available (Table 3).

Palmer et al. originally reported that 90% of patients with sCJD and only 49% of controls were homozygous at codon 129 of PRNP. They suggested that, while the heterozygous state confers relative resistance to sCJD, the presence of Met129 homozygosity increases the risk for sCJD. Additional studies in other Caucasian populations have corroborated these findings. Laplanche et al. estimated that the Met129Met genotype confers a 3.4-fold higher risk for developing sCJD than other genotypes. Also, Windl et al. determined that in the United Kingdom the relative risk for Met/Met:Val/Val:Met/Val is 14:4:1. Hence, the present data, showing that Met129Met is increased by about 40% in the population of Crete, suggest that these genotypic characteristics are responsible, at least in part, for the excessive number of sCJD cases recorded on this island.

The CJD patients described here came from unrelated Cretan families whose origin was traced back to the nineteenth century. None came from families that migrated to Crete from Asia Minor in 1922 or that moved to Crete from elsewhere. These observations provide additional evidence that the CJD cases relate to a genetic susceptibility of the native population rather than to environmental factors. However, the high rate of Met129Met cannot account for the occurrence of two Val129 homozygous CJD cases since Cretan controls showed low rates of Val129 homozygosity (5.5% vs. 11%). It is thus possible that additional factors are operational in this population.

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We thank Dr T. Manousis for help and Irene Skoula for excellent technical assistance.

Table 4. Age-Specific Incidence Rates

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Crete</th>
<th>France</th>
<th>Germany</th>
<th>Italy</th>
<th>Netherlands</th>
<th>U.K.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;39</td>
<td>0</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>40–49</td>
<td>2.76</td>
<td>0.27</td>
<td>0.10</td>
<td>0.23</td>
<td>0.63</td>
<td>0.26</td>
</tr>
<tr>
<td>50–59</td>
<td>9.40</td>
<td>2.03</td>
<td>0.90</td>
<td>1.33</td>
<td>1.54</td>
<td>1.16</td>
</tr>
<tr>
<td>60–69</td>
<td>20.81</td>
<td>3.30</td>
<td>1.69</td>
<td>2.44</td>
<td>2.56</td>
<td>3.26</td>
</tr>
<tr>
<td>70–79</td>
<td>7.60</td>
<td>4.48</td>
<td>2.44</td>
<td>2.14</td>
<td>4.21</td>
<td>3.42</td>
</tr>
<tr>
<td>&gt;80</td>
<td>28.66</td>
<td>1.39</td>
<td>0.55</td>
<td>0.85</td>
<td>2.33</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Age-specific incidence rates are expressed as numbers of cases per million per year. Data for France, Germany, Italy, The Netherlands, and The United Kingdom are from Will et al.9

Table 5. Sporadic Creutzfeldt-Jakob Disease Cases per Year

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of Cases by Clinical Onset</th>
<th>Number of Cases Deceased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1998</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1999</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2000</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

References

Plaitakis et al: Sporadic CJD Incidence and Methionine Homozygosity 233