LGMD2A: genotype–phenotype correlations based on a large mutational survey on the calpain 3 gene

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Summary

We present here the clinical, molecular and biochemical findings from 238 limb-girdle muscular dystrophy type 2A (LGMD2A) patients, representing ~50% (238 out of 484) of the suspected calpainopathy cases referred for the molecular study of the calpain 3 (CAPN3) gene. The mean age at onset of LGMD2A patients was ~14 years, and the first symptoms occurred between 6 and 18 years of age in 71% of patients. The mean age at which the patients became wheelchair bound was 32.2 years, with 84% requiring the use of a wheelchair between the age of 21 and 40 years. There was no correlation between the age at onset and the time at which the patient became wheelchair bound, nor between the sex of the patient and the risk of becoming wheelchair bound. Of the cases where the CAPN3 gene was not affected, 20% were diagnosed as LGMD2I muscular dystrophy, while facioscapulohumeral muscular dystrophy (FSHD) was uncommon in this sample. We identified 105 different mutations in the CAPN3 gene of which 50 have not been described previously. These were distributed throughout the coding region of the gene, although some exons remained free of mutations. The most frequent mutation was 2362AG→TCATCT (exon 22), which was present in 30.7% of the chromosomes analysed (146 chromosomes). Other recurrent mutations described were N50S, 550ΔA, G222R, IVS6-1G→A, A483D, IVS17 + 1G→T, 2069–2070ΔAC, R748Q and R748X, each of which was found in >5 chromosomes. The type of mutation in the CAPN3 gene does not appear to be a risk factor for becoming dependent on a wheelchair at a determined age. However, in the cases with two null mutations, there were significantly fewer patients that were able to walk than in the group of patients with at least one missense mutation. Despite the fact that the results of phenotyping and western blot might be biased due to multiple referral centres, producing a diagnosis on the basis of the classical phenotype is neither sufficiently sensitive (86.7%) nor specific (69.3%), although western blot proved to be even less sensitive (52.5%) yet more specific (87.8%). In this case LGMD2I was a relevant cause of false-positive diagnoses. Considering both the clinical phenotype and the biochemical information together, the probability of correctly diagnosing a calpainopathy is very high (90.8%). However, if one of the analyses is lacking, the probability varies from 78.3 to 73.7% depending on the information available. When both tests are negative, the probability that the sample comes from a patient with LGMD2A was 12.2%. 

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Keywords: calpain 3; limb-girdle muscular dystrophy type 2A; western blot; clinical phenotype; Bayesian analysis; epidemiology

Abbreviations: FSHD = facioscapulohumeral muscular dystrophy; LGMD = limb-girdle muscular dystrophy; CK = creatine kinase


Introduction

Limb-girdle muscular dystrophies (LGMDs) are a group of muscular dystrophies characterized by a predominant involvement of the scapula, pelvic girdle and trunk muscles without affecting the facial muscles. To date, >10 different autosomal recessive LGMDs (LGMD2) have been identified as distinct entities with a similar phenotype (Laval and Bushby, 2004). Although these different entities show some distinctive phenotypic or chronopathological features, there is a clear clinical overlap that makes their differential diagnosis difficult. LGMD type 2A (LGMD2A) has been reported to be the most frequent recessive muscular dystrophy (Dinc¸er et al., 1997; Richard et al., 1997; Topaloglu et al., 1997; Vainzof et al., 1999; Chae et al., 2001; de Paula et al., 2002).

The gene responsible for LGMD2A coding for calpain 3 (CAPN3) was localized by linkage to the chromosomal region 15q15.1–15.3 and subsequently cloned (Richard et al., 1995). To date, >140 different mutations have been described, yet most of them are ‘private’ variants (Chou et al., 1999; Minami et al., 1999; Richard et al., 1999; Chae et al., 2001; Pollit et al., 2001; de Paula et al., 2002; Ginajar et al., 2002; Meznaric-Petrusa et al., 2002; Chrobáková et al., 2004; Fanin et al., 2004; MGC Leiden database: www.dmd.nl).

LGMD2A was the first form of limb-girdle dystrophy identified that is caused by a deficiency of a non-structural protein, the enzyme calpain 3. Calpain 3 (p94) is a calcium-dependent protease that may be implicated in the contractile process since it can bind strongly to titin (Sorimachi et al., 1993). Its localization in the nucleus could suggest an important role in the regulation of transcription factors that indirectly control apoptosis in muscle fibres (Kaplan et al., 1996; Beckmann et al., 1998; Baghdiguian et al., 1999, 2001) although a recent study suggests that this could be a secondary consequence (Kramerova et al., 2004).

Because mutation screening of the CAPN3 gene is relatively labour intensive, expensive and time consuming, it is of great interest for clinicians and geneticists alike to have at their disposal a diagnostic algorithm that takes into account the predictive capacity of the clinical phenotype and of the muscle protein content.

We present here the clinical, molecular and biochemical findings of a large sample of LGMD2A patients from a cohort of LGMD2 patients that underwent molecular diagnosis of the CAPN3 gene at our laboratory.

Patients and methods

Patients

Samples from 484 patients from different geographic origins were analysed by mutation screening of the CAPN3 gene. A neurologist (A.L.M.) revised the clinical information when available to determine if the patients indeed fulfilled the criteria of LGMD2A using the diagnostic criteria proposed at the European Neuromuscular Centre Workshop (Beckmann and Bushby, 1996) and that previously published by Emery (1998). However, to specify a diagnosis of LGMD2A, we used the criteria based on the experience of the Reunion Island and Basque cohorts (Fardeau et al., 1996a, b; Urtasun et al., 1998; Table 1). Sufficient clinical information was available in 314 patients to verify whether patients presented LGMD2, and information was available in 263 patients to determine whether or not their phenotype was suggestive of LGMD2A.

CAPN3 western blot analysis

Muscle biopsies were taken from the quadriceps femoris, biceps brachii or deltoid muscles in each centre after the patients’ informed consent was obtained. Western blots for calpain 3 were performed in the different laboratories; most of them in three laboratories (117 out

<table>
<thead>
<tr>
<th>Table 1 Clinical criteria used to diagnose LGMD2A based on the diagnostic criteria laid down at the European Neuromuscular Centre Workshop*</th>
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<tbody>
<tr>
<td>Major criteria</td>
</tr>
<tr>
<td>*Beckmann and Bushby (1996) and other published studies (Fardeau et al., 1996a, b; Urtasun et al., 1998).</td>
</tr>
</tbody>
</table>

| Patients had to fulfil all the main criteria and at least one of each group of minor criteria; *Beckmann and Bushby (1996) and other published studies (Fardeau et al., 1996a, b; Urtasun et al., 1998). |

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of 146; 86 by F.L. in Paris, 12 by L.M. in Bologna and 19 by H.L. in Munich), and all using the Calp3d/2C4 (anti-exons 1 and 3) and Calp3c/12A2 antibodies (anti-exons 3 and 8; Novocastra) as described by Anderson et al. (1998).

Under normal conditions, three calpain 3 bands of 94, 60 and 30 kDa were detected in western blots. In some cases, the amounts of each protein were diminished to below normal levels but they were clearly present. In others, only trace amounts of the proteins were detected and the bands were scarcely perceptible, or they were absent. We carried out two analyses to determine if the presence or absence of any particular combination of bands in the western blots of calpain 3 had greater diagnostic value. In the first, we considered as normal bands whose intensity was the equivalent of control levels, and abnormal those with a diminished intensity or bands that were absent. In a second more stringent approximation, we considered as abnormal only those at trace levels or that were absent. In both cases, we evaluated the sensitivity and the specificity of each possible combination. To ensure the accuracy of the results obtained, only cases with both characterized mutations in the CAPN3 gene were considered as LGMD2A to establish the predictive value of using the phenotypic criteria and western blotting as diagnostic tools.

**Molecular study**

DNA was extracted from peripheral blood samples obtained after informed consent according to standard procedures at the centre of origin.

When the geographic or ethnic origin of the patient suggested a higher probability of finding previously described mutations in the CAPN3 gene, allele-specific polymerase chain reaction (PCR) was performed with the appropriate primers for each of these mutations (Don et al., 1991; Urtasun et al., 1998). In cases in which the aforementioned mutations were ruled out, only one mutation was detected or the patient was of another geographic or ethnic origin, mutation screening of the CAPN3 gene was performed using the single strand conformation polymorphism (SSCP) technique according to Richard et al. (1995, 1999). All the electrophoretic variants were sequenced in an automated sequencer.

To confirm that the alterations found were mutations and not polymorphisms, a screening was carried out with 100 control Caucasian chromosomes. In order to assess the possible confusion of the clinical diagnosis with other muscular dystrophies, the FKRP gene related to LGMD2I and the facioscapulohumeral muscular dystrophy (FSHD)-linked 4q35 deletion were analysed in two randomly chosen subsets of LGMD2A-negative cases (79 and 42 patients, respectively) by procedures previously described (Lemmers et al., 1998; Brockington et al., 2001).

**Genotype–phenotype correlations**

In order to produce an adequate clinical description, we have taken into account the 238 cases in which mutations were found in the CAPN3 gene (one or two mutations; Fig. 1). To avoid possible bias when interpreting the correlation between genotype and phenotype, we have only considered the cases where two mutations were

![Diagram showing distribution of patients according to clinical characteristics.](https://academic.oup.com/brain/article-abstract/128/4/732/284325)

**Fig. 1** Distribution of patients according to clinical characteristics. Clinical information was only available for 314 of the 484 patients. In 63 cases of the 238 molecularly identified cases clinical information was not available.
identified when calculating the prognostic value of a specific mutation, the age at the onset of the symptoms or the value of the information from western blots and/or the clinical criteria proposed as diagnostic tools. To evaluate the possible correlation between the age at onset and a more severe evolution, a homogeneous sample of patients (also with two mutations identified) was selected according to the period of evolution of the disease. Since the overall mean evolution of the disease until becoming dependent on a wheelchair is 19 years (SD ±6), we analysed a subset of patients in whom the disease had evolved for at least 25 years.

Statistical procedures

We estimated the sensitivity, specificity and predictive values of both the proposed clinical phenotype and the use of western blots as diagnostic tools using a 2 × 2 contingency table. Exact 95% confidence intervals were calculated using a binomial distribution. The sensitivity, specificity and predictive values for the combinations of results from these two tests were also calculated using a multivariate logistic model for those patients where the information from both tests was complete. A multivariate Bayesian model was used that incorporated the dependence between clinical phenotype and western blot in those cases where the information was incomplete (Spiegelhalter, 1985). A correlation between the age of onset and the age at which the patient becomes wheelchair bound was made using a linear regression test. Correlations between the type of mutation and the age of onset or the age at which the patient becomes wheelchair bound were made using an ANOVA (analysis of variance) test with Bonferroni adjustment. All calculations were performed using SYSTAT 9.0. Statistical significance was established when \(P < 0.05\).

Results

Out of the 484 patients in this study, clinical information was available for 314 patients. Of these, 282 presented a LGMD phenotype while 32 did not fulfil the clinical criteria for LGMD2. From the resulting cohort of 282 cases, 181 presented a phenotype strongly suggestive of a calpainopathy, while 82 did not fulfil the selected criteria. In 19 cases, the clinical information available was insufficient to be able to determine whether they may or may not be suffering from a calpainopathy (Fig. 1).

Clinico-epidemiological data

Total cohort

Of the 484 patients analysed, 243 were women and 236 were men (no information regarding sex was available in five cases). The mean age at the time of the study was 34.4 years (SD ±15.7; data from 338 cases). The age at onset was available in 290 patients and the mean was 16.1 years of age (SD ±11.3; range 1–67 years). Information regarding the current status of the disease was available in 194 patients (40.1%). Seventy-five of these patients were wheelchair bound at the time of the study, but information about the age at which they became wheelchair bound was only available in 64 cases. Of these, the mean age at which they became dependent on a wheelchair was 33 years (SD ±11.1), and 50 (78%) were between 21 and 40 years old at the time they became wheelchair bound. Finally, information about the evolution from the onset of symptoms until becoming wheelchair bound was available in 62 cases and occurred within an interval of 4–44 years, with a mean evolution of 19.2 years (SD ±8.3).

Clinical data of LGMD2A

From those 238 LGMD2A patients confirmed by molecular analysis, a LGMD2 phenotype was confirmed in 171 patients. In four cases, the phenotype did not coincide with generally described LGMD, all four of which were women. In three of these cases, the clinical symptoms were compatible with a metabolic myopathy with myalgia that produced no or only slight weakness. In the fourth, the clinical picture was compatible with a congenital myopathy with slow evolution, mainly involving the girdles, and with a dubious precedent of paternal late involvement. Information was not available for 63 patients. Out of the 171 cases considered as having a LGMD2 phenotype, the phenotype of 142 was suggestive of LGMD2A according to the criteria used, while 23 did not present a phenotype suggestive of a calpainopathy according to the clinical criteria used (Table 1). In six cases, insufficient information was available. The principal criteria for excluding these 23 cases was the age at onset in relation to established criteria (early or late) and the metabolic symptoms with myalgia or cramps after exercise but no significant limb weakness (Table 2).

Of the 238 LGMD2A patients, 121 were women (50.8%) and 117 were men (49.2%). The age at onset was known in 175 LGMD2A patients and a mean age of 13.8 years was calculated (SD ±8.1; range 2–49). The first symptoms occurred between 6 and 18 years of age in 125 patients (71%). Out of this LGMD2A cohort, information regarding the current status of the disease was available for 137 patients (57.6%). Fifty-five of these patients were wheelchair bound at the time of the study, but information about the age at which they became dependent on a wheelchair was only available in 50 cases. Of these 50 patients, the mean age at which they became wheelchair bound was 32.2 years (SD ±8.6) and 42 (84%) were between 21 and 40 years old at that time. Information regarding the evolution from the onset of symptoms until becoming wheelchair bound was available in 48 cases, and the mean evolution was 19 years (SD ±6.1), with an interval of 7–36 years of disease evolution.

In the homogeneous subset of patients selected according to the evolution time of the disease (≥25 years of evolution), no correlation was detected between the duration of the illness and the time until the patient became wheelchair bound (Student \(t\) test, \(P = 0.02\)). We also did not detect a correlation between the sex of the patient and a differential risk of being wheelchair bound (Pearson \(\chi^2\) test, \(P = 0.247\)). In the sample of LGMD2A patients, the predictive value of the proposed clinical criteria as a diagnostic tool reached a sensitivity and a specificity of 86.7% (111 out of 128) and 69.3% (88 out of 127), respectively.
Molecular data  
LGMD2A cases  
The 238 LGMD2A patients belonged to 187 different families, yet in 55 of these patients (from 48 different families) we were only able to find one mutation. Out of the 282 cases with a LGMD phenotype, mutations in the \textit{CAPN3} gene were found in 171. In 132 instances, two mutations were characterized, while in 39 only one mutation was detected. Among the 32 patients that did not have a LGMD phenotype, mutations in the \textit{CAPN3} gene were detected in four cases. Of these four patients, mutations were detected in both alleles in two cases, whereas in the other two we were only able to detect one mutation.

We have identified 105 different mutations of which 55 were previously described mutations and 50 were new ones. Moreover, eight unclassified variants were also identified. (Table A in the Supplementary material). In 93 cases, homozygous mutations were identified, whereas 90 patients were compound heterozygotes and in 55 patients (23%) only one of the two mutations could be detected. The mutation found most frequently was 2362AG→TCATCT (exon 22), which was present in 30.7% (146 chromosomes) of the chromosomes analysed, mainly in those from the Basque Country. Indeed, 51 patients were homozygous for the 2362AG→TCATCT mutation, most of them from the Basque country, while 27 were heterozygous. Sixteen polymorphisms were also characterized, nine of which were new while seven had been described previously (Table B in the Supplementary material).

Other muscular dystrophies

Of the 79 cases that were analysed at the molecular level for the \textit{FKRP} gene, in 20 a phenotype suggestive of LGMD2A was observed but not associated with mutations in the \textit{CAPN3} gene. Of these, mutations were detected in the \textit{FKRP} gene in 19 of the \textit{FKRP} cases analysed (24%) of which 12 were from Central Europe, five from Spain and two from the USA. The predominant L276I mutation was observed in 10 cases, 47.4% of the chromosomes analysed (18 out of 38).

In 42 patients without mutations in the \textit{CAPN3} gene, molecular analysis of the FSHD gene identified three incidences (7.1%), each from a different family, where a 28 kb fragment of the D4Z4 region was found in the 4q35 chromosome. Of the patients with a phenotype suggestive of LGMD2A, molecular studies of FSHD were performed in five cases, but mutations were not found in any of them.

### Table 2  Cases of LGMD2A whose phenotype was not suggestive of a calpainopathy

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Age at onset</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td>2362AG→TCATCT/not found</td>
<td>Very early onset, 2 years</td>
<td>Never able to get up from a chair</td>
</tr>
<tr>
<td>IVS6-2 A→G/R355W</td>
<td>Late onset, 40 years</td>
<td>Hypertrophy; scapulo-humeral involvement</td>
</tr>
<tr>
<td>IVS6-1 G→A/not found</td>
<td>Early onset, during childhood</td>
<td>Slow evolution; still walking 25 years after onset</td>
</tr>
<tr>
<td>G567W/not found</td>
<td>Very early onset, 2 years</td>
<td>Muscular aches</td>
</tr>
<tr>
<td>S260F/IVS4 + 4A→G</td>
<td></td>
<td>Light facial involvement; pelvis–scapular involvement; calf hypertrophy</td>
</tr>
<tr>
<td>L484 M/not found</td>
<td>Late onset, 40 years</td>
<td>Still walking 25 years after onset</td>
</tr>
<tr>
<td>A420D/IVS20-16 A→G</td>
<td>Late onset, 31 years</td>
<td>Despite the late age of onset, the weakness is compatible with LGMD2A</td>
</tr>
<tr>
<td>A420D/IVS20-16 A→G</td>
<td>Late onset, 36 years</td>
<td>Insufficient period of evolution to evaluate the progression of the disease</td>
</tr>
<tr>
<td>2362AG→TCATCT/2362AG→TCATCT</td>
<td>Early onset, 2 years</td>
<td>Insufficient period of evolution to evaluate the progression of the disease; weakness compatible with LGMD2A</td>
</tr>
<tr>
<td>A160G/IVS7 + 3 A→G</td>
<td>Very early onset</td>
<td>Hearing problems; Raquis deformity</td>
</tr>
<tr>
<td>N505/N505S</td>
<td>Early onset, 5 years</td>
<td>Mother affected</td>
</tr>
<tr>
<td>N505/N505S</td>
<td>Early onset, 5 years</td>
<td>Mother affected</td>
</tr>
<tr>
<td>G445R/not found</td>
<td>Late onset, 20 years</td>
<td>Muscular ache</td>
</tr>
<tr>
<td>140TCA/not found</td>
<td>Late onset, 45 years</td>
<td>Slightly ‘winged’ scapulae from childhood; very slow evolution; still walking 35 years after the onset of the disease</td>
</tr>
<tr>
<td>P82L/not found</td>
<td>Late onset, 25 years</td>
<td>Still walking 25 years after onset, force 4.</td>
</tr>
<tr>
<td>G234E/C328C (unclassified variant)</td>
<td>Late onset, 40–45 years</td>
<td>Calf hypertrophy; vestibular balance disorder</td>
</tr>
<tr>
<td>R748X/not found</td>
<td>Onset 16 years</td>
<td>Rigid spine; elbow contractures</td>
</tr>
<tr>
<td>2069–2070ΔAC/2362AG→TCATCT</td>
<td>Early onset, 4 years</td>
<td>Clinical evolution after early onset; weakness compatible with LGMD2A</td>
</tr>
<tr>
<td>R748Q/not found</td>
<td>Early onset, 5 years</td>
<td>Calf hypertrophy; walking and gait disorder</td>
</tr>
<tr>
<td>Y401C/2362AG→TCATCT</td>
<td>Early onset</td>
<td>Very benign evolution; reduction of the muscle mass in the calves after reaching 25 years of age with a rapid evolution thereafter</td>
</tr>
<tr>
<td>550ΔA/550ΔA</td>
<td>Early onset, 5 years</td>
<td>Insufficient evolution to determine phenotype</td>
</tr>
<tr>
<td>R440Q/not found</td>
<td>Early onset, 3 years</td>
<td>Insufficient evolution to determine phenotype</td>
</tr>
<tr>
<td>D753N/not found</td>
<td>Late onset, 40 years</td>
<td>Very benign evolution</td>
</tr>
</tbody>
</table>
Table 3  Distribution of patients still walking or wheelchair bound with respect to the type of mutation

<table>
<thead>
<tr>
<th></th>
<th>Still walking</th>
<th>Wheelchair bound</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Missense–missense</td>
<td>9</td>
<td>64.3</td>
<td>5</td>
</tr>
<tr>
<td>Missense–null</td>
<td>20</td>
<td>69</td>
<td>9</td>
</tr>
<tr>
<td>Missense–?</td>
<td>8</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>Null–null</td>
<td>29</td>
<td>48.3</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>58.4</td>
<td>47</td>
</tr>
</tbody>
</table>

Global comparison, Pearson $\chi^2$ test $P = 0.114$; null–null and null–missense comparison, Pearson $\chi^2$ test $P = 0.067$, Fisher exact test $P = 0.074$; null–null and missense–missense comparison. Pearson $\chi^2$ test $P = 0.282$, Fisher exact test $P = 0.377$; missense–missense and null–missense comparison, Pearson $\chi^2$ test $P = 0.759$, Fisher exact test $P = 1$. ? = mutation not found.

**Phenotype–genotype correlation**

In patients with at least one missense mutation, the mean age of onset was later than in patients with two null mutations (15.67 ± 8.82 versus 11.36 ± 4.24; $P = 0.001$). The risk of patients that had at least one missense mutation (null–missense and missense–missense) becoming wheelchair bound at a determined age was not significantly different from that for those with two stop codon mutations (Table 3). Indeed, by analysing cases in which the disease had evolved for >25 years, we found that when two null mutations were present, more patients were reliant on a wheelchair than in the group of patients with at least one missense mutation (Pearson $\chi^2$ test, $P = 0.047$; Fisher exact test, $P = 0.085$).

**Western blot analysis**

Information from western blots was available from 146 patients for different structural or enzymatic muscle proteins. To analyse the predictive value of the western blots, 27 cases were ignored due to the failure to detect two mutations, and a further eight were disregarded due to the lack of information regarding the quantity of any particular protein band (Table 4).

A total deficit of calpain 3 (absence of the 94, 60 and 30 kDa bands in the blot) was observed in 27 cases, and 21 of these were confirmed as LGMD2A cases in the molecular analysis due to the detection of both mutations. Normal protein levels for the three bands were observed in 15 cases, of which three had been confirmed as LGMD2A cases by the detection of both mutations in the CAPN3 gene (F397L/R748X, E553K/R572W, 1743ΔTG/S606L; Table C in the Supplementary material). This signified that as a diagnostic tool, western blotting has a sensitivity of 52.5% and a specificity of 87.8% (Table 4). If we considered the presence of protein to be the detection of any band in the western blot, the sensitivity was slightly reduced (42.8%) and the specificity was marginally improved (Table 4). An independent analysis of the sensitivity and specificity was also carried out where each of the bands in the western blots was considered independently (Table 4). In these analyses, the sensitivity and specificity varied depending on the way in which the results were treated. If we considered all bands whose intensity differed from that of the controls as an abnormal result, we obtained a sensitivity of >80% (and in some combinations of >90%), although the specificity was very low (<40%; Figure A in the Supplementary material). However, the specificity reached 70–80% if we considered only bands at trace levels or that were absent as abnormal, but at the cost of reducing the sensitivity to barely greater than 60% in the different combinations (Fig. B in the Supplementary material). In either case, the presence of two abnormal bands (whichever they were) indicated a positive result in this test, suggesting that we were dealing with a case of LGMD2A. Indeed, the absence of the 60 kDa band signified a sensitivity of 60% with a specificity of 85% (Figs A and B in the Supplementary material). The observed highest sensitivity and specificity corresponded to the absence of the 30 and 60 kDa bands, respectively (Table 4).

In eight out of 25 patients (32%) where both mutations were characterized and normal or reduced protein levels were observed, at least one mutation was localized in the muscle-specific regions (NS, IS1 and IS2) of the CAPN3 gene. Conversely, in nine out of 11 patients with mutations detected in the muscle-specific regions, different levels of protein were detectable in western blots (Table C in the Supplementary material).

Information from western blots was available for 12 of the 19 LGMD2I cases characterized. In three cases, completely normal amounts of protein were observed, whereas in four others, all bands were diminished (except one for which no
LGMD correspond to LGMD2A. If we exclude the Basque 60% of the patients whose phenotype approximates to sions. However, despite these limitations, we estimate that were sent for molecular analysis. Thus, the nature of the LGMD due to a deficit in patients at multiple centres suspected of suffering from a retrospective analysis of data derived from Discussion

The fact that we were unable to find mutations in the CAPN3 gene for nearly half of the cases sent for molecular study could be interpreted in different ways. In the first place, the phenotype of >10% of cases was not consistent with a diagnosis of LGMD despite the fact that the patients were referred for a molecular study of the CAPN3 gene. Moreover, among those whose phenotype was in accordance with this diagnosis, some 45% (82 out of 181) presented characteristics clearly different from those considered for the classical type 2A phenotype, even though one-third of these (23 out of 82) actually had mutations in the CAPN3 gene. Conversely, we were unable to find mutations in the CAPN3 gene in nearly 25% of the cases with a phenotype suggestive of LGMD2A. The existence of phenotypes other than that classically considered as LGMD2A may account for up to 12% of the patients diagnosed with LGMD2A using molecular techniques (Pollit et al., 2001). This was generally the case with patients that suffered a pseudometabolic myopathy with myalgia and exercise-related cramps, or a contractual phenotype. It should be emphasized that the clinical criteria used, based on the classic phenotype described by Erb (1884) and the studies carried out in the Reunion Island and the Basque Country, are fairly sensitive but not sufficiently specific. Indeed, this should be taken into account when only the clinical information is available prior to carrying out any molecular studies.

We should also highlight that due to the technical constraints, the number of mutations found may be an underestimate since the sensitivity of SSCP is between 80 and 90% (Hayashi, 1992). This is the most plausible reason to explain why only one mutation was detected in 22% of the patients here, or in up to 43% of cases in a recently published study (Fanin et al., 2004). These cases could present a real diagnostic problem, especially when no alterations are detected in western blots and if they occur in regions with a high prevalence of carriers. In such instances, only the complete sequencing of the gene, the study of the cDNA from muscle RNA (Chrobakova et al., 2004), of the protein in leukocytes (De Tullio et al., 2003), or eventually an assay of enzymatic activity (Ono et al., 2004) would enable us to improve the diagnosis.

According to our data, LGMD21 represents an important alternative diagnosis that must be taken into account. Here, LGMD21 represented 20% of the subgroup of patients with a LGMD2A phenotype but with no mutations in the CAPN3 gene, confirming the estimations made in the UK (Poppe et al., 2002) and Central European populations (Brockington et al., 2001). In contrast, the number of cases of FSHD in our cohort appears to be mainly anecdotic, although it should not be disregarded. In 25% of the cases of LGMD21 in our cohort, calpain 3 levels were totally normal whereas in 75% the levels were diminished or even highly diminished in the muscles of these patients, despite them not suffering from population to avoid the bias introduced due to a founder effect, the percentage is slightly greater than 50%, and it decreases to 37.7% if we consider only those cases in which the two mutations could be detected.

Bayesian analysis of the clinical phenotype and western blots as diagnostic tools

To assess the relevance of these two diagnostic tools, we determined the specificity and sensitivity for the patients upon whom a complete genetic screen had been performed and for those on whom both analyses had been performed. Only 77 patients fulfilled the latter criteria and, by logistic regression, we were not able to demonstrate the superiority of one test against the other.

To avoid the limitations of such a small series and given the absence of both types of information in all patients, we performed a Bayesian multiple analysis with dependence. If the phenotype and the western blot suggested a calpainopathy, the probability of this being true was very high (90.8%). However, if one of the analyses was missing, the probability varied from 78.3 to 73.7% depending on the information available (Table 5).

<table>
<thead>
<tr>
<th>Phenotype Western blot</th>
<th>Two mutations detected</th>
<th>No mutations detected</th>
<th>Observed probability</th>
<th>Estimated probability to be a true LGMD2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>13</td>
<td>3</td>
<td>81.2% 90.8%</td>
</tr>
<tr>
<td>Unknown</td>
<td>+</td>
<td>5</td>
<td>2</td>
<td>71.4% 78.3%</td>
</tr>
<tr>
<td>+</td>
<td>Unknown</td>
<td>90</td>
<td>21</td>
<td>81.1% 73.7%</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>15</td>
<td>8</td>
<td>65.2% 65.5%</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>3</td>
<td>1</td>
<td>75% 42.1%</td>
</tr>
<tr>
<td>Unknown</td>
<td>–</td>
<td>13</td>
<td>14</td>
<td>48.1% 40.1%</td>
</tr>
<tr>
<td>–</td>
<td>Unknown</td>
<td>7</td>
<td>60</td>
<td>10.4% 17.1%</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>7</td>
<td>27</td>
<td>20.6% 12.2%</td>
</tr>
</tbody>
</table>

Positive phenotype = suggestive of calpain phenotype; negative phenotype = non-suggestive of calpain phenotype; positive western blot = total absence of calpain 3; negative western blot = presence of calpain 3.

Table 5

The existence of phenotypes other than that classically considered as LGMD2A may account for up to 12% of the patients diagnosed with LGMD2A using molecular techniques (Pollit et al., 2001). This was generally the case with patients that suffered a pseudometabolic myopathy with myalgia and exercise-related cramps, or a contractual phenotype. It should be emphasized that the clinical criteria used, based on the classic phenotype described by Erb (1884) and the studies carried out in the Reunion Island and the Basque Country, are fairly sensitive but not sufficiently specific. Indeed, this should be taken into account when only the clinical information is available prior to carrying out any molecular studies.

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LGMD2A. However, in no case was a complete absence of the protein observed. This reduction in the quantity of protein has also been observed in other types of dystrophies in which the results of western blot for the CAPN3 protein may contrast with the results of molecular studies of the CAPN3 gene (Anderson et al., 1998; Minami et al., 1999; Chae et al., 2001; Fanin et al., 2001, 2003; Pogue et al., 2001; Pollit et al., 2001).

From the clinical point of view, we should emphasize that although 71% of patients begin to show symptoms between 6 and 18 years of age, the chronological limits have widened notably with respect to the age of the first appearance of the symptoms. Indeed, in 5.7% (10 out of 175) of our subjects and 13% of those in the cohort analysed by Fanin et al., onset was not observed until after reaching 30 years of age. The analysis of the subsequent evolution of the disease supports an earlier proposal that age of onset is not a predictive factor for the severity of the progression of the disease (Pollit et al., 2001), contradicting some other studies (Fanin et al., 2004).

This could be interpreted in accordance with the hypothesis that calpain 3-mediated cleavage produces an adaptive response of muscle cells to external and/or internal stimuli (Taveau et al., 2003). Thus, calpain 3 deficiency could be compensated for in the first few years by the development of alternative mechanisms, and the deficit will begin to be noted in a more or less gradual form in terms of function of the type of mutation present in the CAPN3 gene from the moment that these mechanisms fail (Spencer et al., 2002). In fact, in our set of patients, those with null–null mutations do indeed present a greater risk of being wheelchair bound after 25 years of disease evolution than patients with other more benign types of mutations. However, the precise moment of needing a wheelchair appears to be independent of the type of mutation. This implies the existence of additional factors, genetic and/or environmental, that might regulate the expression of the phenotype, even within the same family (Penisson-Besnier et al., 1998).

In general, our data offer fairly solid support for the notion that western blot analysis is less accurate than one might have thought (Chae et al., 2001; Pollit et al., 2001; Fanin et al., 2001, 2003, 2004; Pogue et al., 2001). This variability affects patients with the same mutations (Anderson et al., 1998; Minami et al., 1999), but could be due to technical artefacts introduced when processing the biopsy sample that might lead to protein degradation (Fanin et al., 2003). Nevertheless, the fact that we are dealing with a non-standard procedure in terms of the time of processing, and that this has been carried out in various different laboratories, could have introduced certain undetected errors in our study. Despite these possible technical artefacts or selection bias due to the contribution of different centres, other dystrophies whose deficient proteins are structurally or functionally related to CAPN3 may also provoke secondary deficits in CAPN3 in muscle, as shown previously (Anderson et al., 2000; Udd et al., 2000; Fanin et al., 2001; Haravuori et al., 2001; Pogue et al., 2001; Voit et al., 2001; Figarella-Branger et al., 2002; Hackman et al., 2002). In the six cases (22%) where the protein was absent in western blots but no mutation in the CAPN3 gene was detected, the final diagnosis remains to be resolved. In four of these cases, mutations in the FKRP gene and the deletion of FSHD have been ruled out.

In a similar manner to the clinical phenotype, false-negative results were also detected in western blots. While some authors have calculated these to represent some 20% (Fanin et al., 2004), in our study these reached nearly 48% when we consider the absence of all the bands as a pathological result. It is possible that the underestimate of Fanin et al. (2004) results from the less exhaustive screening for mutations than that applied here and because they considered a decrease in any of the bands as pathological. It appears that the mutations in these cases were situated preferentially in domain III of the protein, a region in which mutations can disrupt the interactions between domains II and III. These mutations may alter the autocatalytic activity of the protein and, as a consequence, justify the presence of the protein in western blots (Jia et al., 2001; Fanin et al., 2003).

In patients with a clinical and radiological phenotype of LGMD2A but with normal protein levels in the western blots, it appears to be most appropriate to commence such screens in the IS1, IS2 and flanking regions. Indeed, one of the mutations found in our cohort from a false-negative case in western blots, S606L, has also been isolated in another similar case (Talim et al., 2001). However, this approach should not be adopted in cases where the mutations are of the stop codon type, provoking the failure to produce protein. The combinations of the bands considered in the western blots should be made according to need, or alternatively to detect the maximum number of the cases or to optimize a molecular study. In both cases, the simultaneous absence of the 94 and 60 kDa bands, of the 60 and 30 kDa bands or the isolated absence of the 60 kDa band are the most reliable indicators in terms of specificity and sensitivity (Figs A and B in the Supplementary material).

From the molecular point of view, our study confirms that apart from the ethnic or geographic groupings, no single mutation predominates. Except for 2362AG→TCATCT and a few other mutations, the mutations detected were new and non-recurrent ‘private’ mutations. The prevalence of carriers of the 2362AG→TCATCT mutation in some areas of the Basque Country justifies a systematic screening of this mutation in the Basque population, as has been proposed for other recessive diseases with a very prevalent mutation such as cystic fibrosis (Dankert-Roelse and te Meerman, 1997; Vries et al., 1997; Gilbert, 2001; Bobadilla et al., 2002). While the 550ΔA mutation previously was associated with patients from the East and South-East of Europe, and more recently Italy (Topaloglu et al., 1997; Pogoda et al., 2000; Canki-Klain et al., 2004; Fanin et al., 2004), it was found here in German and French patients, confirming a wider European distribution. On the other hand, in the CAPN3 gene, the mutations N50S, A483D and IVS12-1 G→A have been found in the Maghreb, where a clear predominance
of limb-girdle dystrophies due to mutations in the α, γ-sarcoglycan and FKRP genes exists. We also identified the A483D mutation in a patient of Italian origin (A483D/R748X). Of the other frequently identified mutations, the G222R mutation has only been found in the Spanish population to date (all patients except one are Basque), whereas the R748Q mutation that is found in several Spanish patients had also previously been described in a Turkish population (Dinc¸er et al., 1997).

The distribution of mutations throughout the CAPN3 gene does not aid in identifying clear hot-spots, other than the relative accumulation in exons 10 and 11 encountered in the relative studies that might reflect the large number of CpG islands in this region (Fanin et al., 2004). Indeed, the CG→TG or CG→CA transitions could represent a mutational mechanism due to the spontaneous deamination of the 5-methylcytosine (Cooper and Youssoufian, 1998). While the absence of mutations in exons 12, 14, 18, 23 and 24 described in other studies is confirmed, there is insufficient evidence to suggest ignoring these exons in the analysis of suspicious cases. The clinical–molecular correlation is complicated when interpreting cases of double heterozygosity due to the different functional impact of each mutation on the activity of CAPN3. Hence other factors must also regulate the phenotype since variability is observed even between patients homozygous for a given mutation. Indeed, the differences in terms of the interval of onset (14–52 years; Penisson-Besnier et al., 1998) and the evolution in patients of a similar age (~60 years), still walking as well as wheelchair bound, in patients with the R461C mutation is notable (Chae et al., 2001). Such variability is even seen between siblings, for example in two 2362AG → TCATCT homozygous patients in our series the age of onset differed by 2 years and each had a very different evolution. One of the brothers became wheelchair bound 12 years after the onset of symptoms, whereas the other sibling was able to walk with support 17 years after the onset (Gardner–Medwin–Walton scale stage VI). Other siblings also showed a 10 year difference in becoming wheelchair bound.

This study also set out to obtain useful information to understand the functional role of CAPN3 in the genesis of this condition, to offer the clinician clues to help them reach a diagnosis in the easiest and most accurate manner, and to be able to better predict the evolution of the illness. In this sense, the Bayesian analysis demonstrated that the probability that a patient truly suffers LGMD2A is 90% when no protein can be detected in western blots and the phenotype coincides with the characteristic LGMD2A phenotype. The phenotype is a more accurate predictor than western blots when we compare patients from whom one of the two tests is indicative and the other is not.

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References

Supplementary material
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Genotype–phenotype correlations of LGMD2A based on the calpain 3 gene


