The extensive scanning of the calpain-3 gene broadens the spectrum of LGMD2A phenotypes

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Abbreviations

CK, creatine kinase; HT-DHPLC, high throughput denaturing HPLC; LGMD, Limb girdle muscular dystrophy

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ABSTRACT

Background: Limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of Mendelian disorders, highlighted by weakness of the pelvic and shoulder girdle muscles. Seventeen autosomal loci have been so far identified and genetic tests are mandatory to distinguish among the forms. Mutations at the calpain 3 locus (CAPN3) cause LGMD2A.

Objective: To obtain a non-biased knowledge of the consequences of CAPN3 mutations in the largest genetic study so far performed.

Patients: We studied 530 subjects with different grades of symptoms and 300 controls.

Methods: We set up a non-invasive and cost-effective strategy, based on the high throughput denaturing HPLC analysis of DNA pools.

Results: We identified 141 LGMD2A cases, carrying 82 different CAPN3 mutations (45 novel), along with 18 novel polymorphisms/variants. Females do exhibit a better course than males. In 93.9% of the more severe patient group, we also discovered the defect in the second allele . This proves the sensitivity of our approach. We found CAPN3 mutations in 35.1% of classical LGMD phenotypes. Surprisingly, mutations were also found in 18.4% of atypical patients and in 12.6% of subjects with high serum Creatin Kinase levels.

Conclusions: We set up a non-invasive and cost-effective strategy, based on the high throughput denaturing HPLC analysis of DNA pools, to obtain a non-biased understanding of the consequences of CAPN3 mutations in the largest genetic study ever undertaken. This broadens the spectrum of LGMD2A phenotypes and set the carrier frequency to 1:103.

Key words:

Limb-girdle muscular dystrophy, calpain 3, DHPLC, creatin kinase, DNA pooling

Introduction:

Limb-girdle muscular dystrophies (LGMD) include a broad group of genetically determined progressive disorders with a primary or predominant symmetrical atrophy of the pelvic and/or shoulder girdle musculature, elevated serum creatine kinase and a necrotic regeneration pattern (1). The clinical course is characterized by a great variability, ranging from severe forms with onset in the first decade and rapid progression to milder forms with later onset and a slower course. The most severe forms result in dramatic physical weakness that often leads to other serious complications and, for many, a shortened life span. Other forms can result in relatively minor physical disabilities or develop late in life, allowing affected people to have normal life expectancy and activity levels.

Seventeen LGMD loci have so far been mapped, seven autosomal dominant and ten autosomal recessive (2). The autosomal dominant forms (LGMD1) are generally milder and relatively rare, representing less than 10% of all LGMD (3-8). The autosomal recessive forms (LGMD2) are much more common, having a prevalence of 1:15,000 with a number of geographical differences (9). The products of ten LGMD2 loci have been identified. They are: calpain 3 for LGMD2A (10), dysferlin for LGMD2B (11, 12) alpha-sarcoglycan (adhalin) for LGMD2D (13), beta-sarcoglycan for LGMD2E (14, 15), gamma-sarcoglycan for LGMD2C (16), delta-sarcoglycan for LGMD2F (17, 18), telethonin for LGMD2G (19), TRIM32 for LGMD2H (20), fukutin-related protein for LGMD2I (21) and titin for LGMD2J (22). Linkage analyses indicate that there is further genetic heterogeneity both for LGMD1 as well as for LGMD2.

LGMD2A [MIM 253600] is one of the most prevalent forms of autosomal recessive muscular dystrophy in all countries so far investigated and is caused by loss-of-function mutations in the CAPN3 gene. This spans 52.8kb of genomic sequence at chromosome 15q15.1 and the 3.5-kb transcript is composed of 24 exons encoding a 94kDa proteolytic enzyme, Calpain 3 or p94 (10). This is a skeletal muscle-specific member of the superfamily of the calpain large subunits s, or calcium-activated neutral proteases (EC 3.4.22.17) that are nonlysosomal intracellular cysteine proteases. The

human genome encodes at least fifteen large subunit genes that are supposed to process various intracellular kinases, phosphatases, phospholipases, transcription factors and cytoskeletal proteins to modulate their activities (23). Calpain 3 can be subdivided into four domains. Domain II shows similarity with other cysteine proteases, which share histidine, cysteine, and asparagine residues at their active sites. Domain IV comprises four EF-hand structures that are potential calcium-binding sites. In addition, there are three unique sequences NS, IS1, and IS2. Although it is largely unknown why the absence of this protein leads to muscular dystrophy, it has been suggested that it may play a significant role in intracellular signal transduction systems (24, 25). Baghdiguian et al. (26) claimed that calpain 3 deficiency would cause myonuclear apoptosis and a profound perturbation of the IkBa/NF-kB pathway.

Null mutation of calpain 3 in mice causes abnormal sarcomere formation (27): *in vitro* studies revealed that calpain 3 can bind and cleave titin and that some mutations result in reduced affinity for titin. The spectrum of mutations affecting the CAPN3 gene is highly heterogeneous. The Leiden Muscular Dystrophy web site (http://www.dmd.nl), a specialized mutation database for muscular dystrophies, to date reports 139 pathogenic mutations associated with a LGMD2A phenotype. They distribute over almost all exons of the CAPN3 gene, with nine exons (1, 4, 5, 7, 10, 11, 13, 21 and 22) involved in about 85% of cases. Most mutations (70%) are private variants, although particular mutations were more frequent in some populations. Novel mutations were identified in Japanese LGMD2A patients, which were not found in non-Japanese populations (28).

In a large study based on western blot analysis 53/191 patients with LGMD phenotype (28%) had partial or total Calpain 3 deficiency, while it was observed in 8% patients with of hyperCKemia, 6% with proximal myopathy, and 6% with distal myopathy (25). Recently, Fanin et al. (29) identified 69/208 cases (33.2%) of calpain 3 deficiency by western blot (2004).

The present study was designed bearing in mind two goals: 1) to approach the genetic heterogeneity of a complex disorder such as autosomal recessive LGMDs with a non-invasive cost-effective strategy based on the screening of DNA pools; 2) to obtain a comprehensive non-biased understanding of the

consequences of CAPN3 mutations in myopathic patients, including isolated cases with different degrees of disease.

Materials and Methods:

Selection of patients

DNA samples from 530 LGMD patients and 300 controls were analyzed by HT-DHPLC in the present survey. This number also comprises patients in whom a mutation was afterwards identified in other LGMD genes. At least 92% of DNA samples were from Italian families, recruited at the Universities of Naples, Padua and Rome. Additional patients were from Argentina (three), Croatia (six), Slovenia (five), Germany (six), Greece (one) and Turkey (twenty). Diagnostic criteria follow the European Neuromuscular Centre guidelines at http://www.enmc.org/nmd/diagnostic.cfm. These include limb-girdle onset, progression, autosomal inheritance, no involvement of other systems, elevated serum creatine kinase (CK) , hypodensity at CT scanning, dystrophic changes at muscle biopsy, etc. Since a spectrum of disease severity has been observed in association with most of the genetically defined types, the exclusion of other disorders is a vital part of the general diagnosis of LGMD. Although there is a continuous spectrum of disease severity, we have grouped subjects into five categories: A) severe LGMD characterized by important weakness and wheelchair requirement before age 35; B) intermediate LGMD with weakness and difficulties in deambulation, but ability to walk alone beyond age 35; C) mild with symmetrical scapular winging and hyper-CK-emia; D) isolated hyper-CK-emia; P) presymptomatic, including patients too young to be classified by clinical criteria, but with suggestive laboratory findings (i.e elevated CK>50x, myopathic biopsy, etc.). One hundred twenty-three were classified as A, 142 as B, 158 as C, 87 as D, 20 as P. We included all isolated cases with apparent autosomal recessive inheritance. A preliminary linkage analysis using genetic markers specific for all the previously known limb-girdle dystrophy loci was carried out on suitable families (30).

Mutation discovery strategy

We used the following strategy (fig. 1) structured into six sequential steps:

i. Accurate quantification of genomic DNA, with a fluorometric assay using a reporter dye such as PicoGreen (Molecular Probes).
ii. Dilution of each individual sample of genomic DNA and test of PCR yield.
iii. Pooling of two suitable DNA samples in the same tube.
iv. Set-up of finest PCR conditions, according to DHPLC analysis requirements (i.e. special buffer formulations, primer design to mask known polymorphisms, etc.).
v. Mutation screening of the entire cohort of our patients and normal controls.
vi. Direct sequencing of abnormal migrating fragments only present in patients.

PCR primers for amplification of CAPN3 coding regions were previously published (10); however, some of them produced amplicons showing suboptimal melting profiles once tested by Navigator software. In addition, the PCR yield was often unsuitable for the analysis. Therefore, most amplimers were re-designed. Table 1 reports the twenty-six PCR primer pairs for mutation screening of the coding sequence together with the untranslated regions and the putative promoter. Amplified fragments were 93-296 bp in length. Here, each primer pair was able to amplify an exon together with 25-50 bp of flanking intron sequences on both sides. The mutation studies were performed using a combined procedure based on DHPLC and the assembly of normalized DNA pools to speed up the screening. The melting characteristics of each PCR fragment were predicted using Navigator software. The elution was carried out with a 2-min gradient (Rapid DNA) with a flow of 1.5ml/min, a slope of 5%/min and a drop loading of 3%.

Results:

DHPLC strategy and pooling rationale

We analyzed the CAPN3 exons and flanking intronic sequences and the putative promoter using high-throughput denaturing HPLC (HT-DHPLC).(31-36). The high-throughput configuration of the instrument requires only a 2-min gradient run for the analysis. Table 1 shows the sequence of the primers, PCR product sizes, annealing temperatures and the conditions of analysis by DHPLC. DHPLC is able to detect heterozygous mutations in DNA samples in the form of an additional peak of heteroduplex DNA. When a subject carries a homozygous mutation, it is necessary to anneal the sample with a normal control. Instead of normal samples, we pooled equal amounts of DNA from two unrelated patients before PCR. This was sufficient to reduce the number of DHPLC analyses and PCR reactions by at least 66%. In fact, in both cases of a heterozygous or a homozygous mutation, heteroduplex DNA will be generated within a pool. A false negative on DHPLC can only result when a pool is composed of both DNAs that are homozygous for the same mutation.. Nevertheless, this risk is very low, considering the frequency of the homozygous patients. Among the most frequent homozygous mutations: 550delA was found in 12/530 samples, 883-886GATA>CTT and 2212 C>T in 4/530, 139delATC and 2242C>T in 3/530, 802-9G>A and 2184G>A in 2/530 and other twelve mutations only in 1/530. Thus, the probability of forming a false negative pool is 1:1,312 ($a^2 + b^2 + c^2 + ../n^2$, where n is the total number of samples; 214/280,900): too low to affect our analysis of 265 pools.

Patients under study

We studied a randomly collected group of 530 patients and 300 controls. Most patients are isolated cases with apparent autosomal recessive inheritance. We found at least one mutation in 141 patients and only 42 of them were homozygous (29.8%). Eighty-seven of them (61.7%) were isolated cases, while 54 (38.3%) were familial cases.

They were classified as severe LGMD (group A), intermediate LGMD (group B), mild myopathy (C), asymptomatic hyper-CK-aemia (group D), presymptomatic patients (P). We found 49/123 (39.8%) of mutations in group A, 44/142 (31.0%) in group B, 29/158 (18.4%) in group C, 11/87 (12.6%) in group D and 8/20 in group P (40.0%) (Table 2). This strongly suggests that in typical LGMD with wheelchair confinement before or after age 35 (groups A and B), CAPN3 mutations are the most frequent genetic cause, since no other LGMD form involves 35.1% of patients. In addition, a mutation of the second allele was identified in the large majority of groups A and B patients (46/49=93.9% and 38/44 = 86.4%, respectively), but only in 15/29 = 51.7% of group C and 3/11 = 27.3% of group D patients. Since the sensitivity of DHPLC is identical for all samples, a large part of heterozygous patients belonging to group C and D might not have a second mutation. In milder myopathies CAPN3 mutations are no more common than DYSF or FKRP mutations. Mutation detection rate among patients with defects of calpain 3 at WB was 56%.

The level of CK in patients of all groups was 11.1x on average (range 3x to 25x). But there are some young patients who exhibit very high CK levels (up to 100x) and four with normal CK.

In accordance with the observations of Zatz and co-workers (37), we observed a more rapid progression in LGMD2A affected males than in females. Considering patients with both mutations, females in 34.0% of cases were in group A, 48.9% were in group B, whilst in the males 52.6% were in group A and 26.3% belonged to group B. Since the difference between A and B groups depends on the age of wheelchair confinement, females tend to lose their ability to walk later.

Mutations and polymorphisms

Altogether, 82 different mutated alleles have been detected in the CAPN3 gene of which 45 (54.9%) are novel. (Table 3). No putative mutations were encountered in 300 controls. The mutations include 66 single-base-pair substitutions (18 transversions and 48 transitions) which resulted in a change of amino acid in 54 cases (81.8%), in premature stop codons in five cases, and in splicing defects in seven cases. Additional eleven mutations were small deletions, one was an insertion and two indels.

All but five caused a frame-shift, leading to a secondary premature stop codon. We identified twenty-seven novel mutations affecting single amino acids that are conserved. However, this criterion is not fully reliable, since the conserved glutamic acid 107 is polymorphic in humans. Some mutations affect domains that are common to all the other members of the Calpain superfamily: for example, F779 is part of an EF-hand calcium binding motif DFXXF. Patients with the same mutations have a variable phenotypic spectrum. For example, among twelve cases that are homozygous for the 550delA allele, six belong to group A, five to group B and one to group C.

When the mutation of the second allele was not found, we tried to identify it by RT-PCR from muscle RNA, when available. We also preformed long-range PCR analysis. No major genomic deletion in the calpain 3 gene was identified among these patients.

We found mutations scattered over 21/24 exons of the CAPN3 gene, with no mutation in exons 9 (78bp), 12 (12bp), and 23 (59bp), all of which are among the smallest exons and in which no definite mutation have been described so far. Interestingly, we found ten mutations in exon 1, seven of which are novel (70%). Alternatively spliced exons, such as 15 and 16, show relatively more often out-of-frame mutations.

We identified three novel polymorphisms (498+35G>T, 606T>C, 1524+81C>T) found both in patients and controls and 15 novel variants in addition to others that are already known (Table 4). This may be a major drawback for the analysis, since polymorphisms generate heteroduplexes that may mask true mutations. We therefore designed additional primers that cover these polymorphisms and re-screened samples. We only sequenced the heteroduplexes that are maintained following this second PCR. Otherwise, we have sequenced all the heteroduplex-forming fragments, to avoid missing mutations.

We were unable to demonstrate an association between six synonymous and nonsynonymous variants and LGMD2A: these were found only once in patients and not in controls and therefore could not be classified (Table 4).

Discussion:

Here we present the results of all the sequence variations identified in the CAPN3 gene. Mutations of the CAPN3 gene cause the most prevalent form of limb-girdle muscular dystrophy, LGMD2A. The phenotypic consequences of CAPN3 defects are similar to those due to mutations in many other known and unknown LGMD genes. This immediately indicates that: 1) the direct genetic testing is necessary to discriminate between the forms; 2) in a large fraction of LGMDs a CAPN3 mutation can be found. Unfortunately, the analysis of CAPN3 gene is complex, since it is composed of twenty-four exons and has 218 pathogenic and non-pathogenic alleles, including those here reported. There is, also, no prevalent "hot spot" mutation that can be routinely screened, unlike the cystic fibrosis gene.

We set up a high-throughput strategy for the systematic analysis of the CAPN3 gene using DHPLC. All comparative studies between methods for mutation discovery confirmed the greater efficacy of DHPLC which offers the following three advantages: sensitivity, automation and cost-effectiveness (31-36). In addition, HT-DHPLC adds a fourth benefit, speed. Compared to standard DHPLC, it cuts analysis time and reduces human intervention: two 384-well plates (768 samples) are automatically analyzed in 54 hours. For further enhancement, we analyzed PCR products from DNA pools, since the system is able to distinguish a mutant allele among several wild-type alleles. Sample pooling before PCR cuts the costs of the entire procedure making it the cheapest of all the mutation discovery strategies. In comparison with direct sequencing, standard DHPLC is at least eight times cheaper (32), but together with high-throughput sample pooling the entire procedure becomes up to twenty times cheaper.

With DHPLC, any sequence polymorphism that generates heteroduplex DNA may confound results, by masking pathogenic changes. This problem can be solved by two different ways: 1) sequencing all the heteroduplex-forming fragments, to avoid missing mutations; 2) designing primers that cover or exclude the polymorphic site, when the latter is intronic. We demonstrate that our approach to mutation discovery had an optimal sensitivity based on the following data: in more than 90% of patients with typical severe or intermediate LGMD (groups A and B) we were able to discover the

second allele mutation. DNA-based exon analyses miss deep intronic mutations that activate cryptic splice sites by adding extra sequences. This does not seem to be the case, since RT-PCR, when possible, was unable to detect any defect.

A general problem of human molecular genetics is in the genotype/phenotype correlation for nontruncating mutations. Mutation discovery strategies should discriminate between true pathogenic defects versus rare innocuous variants. Even if the disease is familial and strongly linked to a locus, one must take into account that any sequence variation co-segregates with the true but unidentified mutation. Thus, each variation observed only once should be considered a “putative mutation” only when a complete study of the gene and an adequate number of controls have been made. This implies that heterozygous status should not be classified as autosomal recessive LGMD2A in the absence of the second mutation. The number of controls is a critical issue and depends on the heterogeneity of neutral alleles (38). We consider the standard analysis of 100 normal individuals inadequate. Pooled DHPLC analysis can achieve this goal by increasing the number of analyzed chromosomes at reasonable cost.

Unlike all the other studies on LGMD2A, we included isolated cases, milder and atypical patients. This is helpful to define a wider non-biased phenotypic spectrum associated with defects at the CAPN3 locus.

We identified 82 different mutations. The distribution of mutations along the gene somewhat differs from that reported in the literature, suggesting both population differences and methodological bias. The most frequently mutated exons in our study are 1, 4, 5, 6, 7, 10, 11, 13 and 21 accounting for 87% of all LGMD2A alleles. In Brazilian families, exons 2 and 22 should be included in the amplicons first analyzed. Unexpectedly, we found ten exon 1 variants that constitute 70% of all mutations described worldwide. This may be due to a fault in the screening of this exon by most groups, since our detection rate was enhanced when we split exon 1 in two overlapping fragments (Table 1). The most frequent allele is del 550 A that was found 45 times. Six out of twelve del550 homozygous patients were from

North Eastern Italy. The fraction is much lower than in Croatia (39), but higher than in Southern Italy. This confirms the hypothesis of a common founder (40).

We then compared mutation discovery results with availble western blot data. Seventy patients with reduced or absent calpain 3 WB were analyzed and we found a causative mutation in 56% of cases. This, together with the data by Fanin et al. (29) implies that WB analysis alone could give both to false positive and false negative results. Conclusive diagnosis of LGMD2A is thus molecular. We show that reliability only derives from very large patient-to-control studies that are feasible using high-throughput mutation analysis techniques.

Accordingly, the milder the phenotype, the higher the percentage of patients with one single CAPN3 mutation. Considering A and B patients in the current definition of LGMD with autosomal recessive inheritance, 93/265 (35.1%) of the total can be classified as LGMD2A. Mutations were also found in patients belonging to group C (18.4%) in half of which the second mutation was not found. The results of the screening indicate that 12.6% of patients with no weakness and increased CK levels (group D) do have CAPN3 mutations.

At the end of this study there were patients from groups C and D with a single pathogenic change. We think that these patients may either be true heterozygous for a pathogenic CAPN3 allele or they may carry a yet unrecognized hypomorphic allele variation. In some of these patients, we found polymorphisms or variants, also identified in normal controls: One should assume that they are neutral changes. However, the combination of a polymorphism in one copy of the CAPN3 gene with a LGMD2A mutation in the other copy could even be responsible for very mild phenotypes with low penetrance, similar to the effect of the 5T allele in cystic fibrosis (41-42).

The more rapid progression in affected males than females has no explanation. Should sex steroids modulate the calpain 3 mechanism, this could have implications for treatment.

When the involvement of CAPN3 gene in disease was reported for the first time (10), six different mutations were found within La Réunion island patients, although a single founder was expected. A digenic inheritance model and several alternative hypotheses were proposed by Beckmann to explain

the “so called” “La Réunion paradox”. Considering the number of patients recruited by all centers and the population of origin, we estimate that autosomal recessive LGMDs have a cumulative prevalence of 1:15,000, and 35.1% of cases are LGMD2A. Thus, the prevalence of typical LGMD2A should be 1:42,700 and the carrier frequency 1:103. Additional mutations are carried by atypical patients or subjects with high CK, most of which are not recruited for genetic studies. This recalculation of the carrier frequency helps to explain the paradox. La Reunion now has a population of 720,900 people: we expect to find at least 7,000 disease carriers . During the years 1707-1804 the population of the island increased 88 fold. This implies that multiple waves of immigration occurred and many different founders were introduced.

To our knowledge, this is the largest molecular study of LGMD2A on a highly heterogeneous population of patients. CAPN3 mutations account for about one third of classical LGMD cases and the other nine genes account for about an additional third of cases, the remaining third will be due to yet unknown genetic causes. The question arises as to whether there is a multitude of genes each accounting for a limited number of these cases, or there are a few major disease genes that cause all these forms. The former may be more realistic and similar to the widespread genetic heterogeneity observed in other human diseases (i.e., retinitis pigmentosa or mental retardation) (43-46).

Data access:

We make available materials used for the present investigation upon justified request to the corresponding author. Mutation information is being submitted to Leiden public database

Acknowledgments and affiliations:

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Tables:

Table 1 – Primers, PCR product sizes, annealing temperatures and HT-DHPLC analysis conditions for all the exons of CAPN3 gene.

Exon	*Primers 5'-3'*		*Product size (bp)*	*PCR temperature, •C*	*DHPLC temperature, •C*
	Forward	*Reverse*			
Prom	ttcagtacctcccgttcacc	gatgcttgagccaggaaaac	296	60	58.5
1A	ctttccttgaaggtagctgtat	**cggctgatgatggctgaatagatgc**	210	60	64.5
1B	**gaggctgggggtggaaacccaagtg**	gaggtgctgagtgagaggac	279	60	59
2	actccgtctcaaaaaaatacct	attgtccctttacctcctgg	239	60	58.5
3	**ctcacgatctgtgccctgtgtc**	**cgctggccctcttaactacactctc**	185	60	61.4
4	**ggacacatttcctaacagtaatttgagt**	**ctagccccacctcacattctaaag**	227	60	61.7
5	**gtgaccccaaattggtcttcat**	**ggtaaaaggcttcctgacacttg**	262	60	60.7
6	**ccctgtgttgttccctacattct**	**ggagggatggtcagttctgata**	220	58	59.5
7	aatgggttctctggttactgc	**cttcatgaggttaacccaggag**	169	60	63
8	**acagaagattccctttccagaga**	**atcttcaatactctcatggctcagt**	239	60	63.5
9	**cctgcttccttaattcctccatttt**	**ctcttccccacccttacccttct**	159	60	60.3
10	cctcctcacctgctcccata	tttttcggcttagaccctcc	252	60	64
11	**gtgaatgcgtgcttcctttctg**	**aaagtatgtttcctgggctggag**	242	60	62.7
12	**attcacatctgaagcatcttcctt**	**gtagtgctgcacacaactgctg**	93	60	56.3
13	tgtggcaggacaggacgttc	ttcaacctctggagtgggcc	337	60	63
14	**gggttctctagaggctggttct**	**gacttttcgcacagaagatctaaac**	191	60	62
15	**tcacttattctgcatttactgtttcc**	**agtacacacacttcatgctctctacc**	94	60	52.4
16	**cagactgtaatcctcccttccttcctg**	**tgtagcgtgctggccaccccac**	205	60	62.1
17	acctctgacccctgtgaacc	**gcgtcctgggcttggaggtac**	146	58	60
18	**tcctgaaccatgaccctcctct**	**ctgctttgagacaacgggactc**	154	60	60

Exon	*Primers 5'-3'*		*Product size (bp)*	*PCR temperature, •C*	*DHPLC temperature, •C*
	Forward	*Reverse*			
19	tcctcactcttctccatccc	accctgtatgttgccttgg	159	60	62.5
20	**gtgtgctgtgtagccctgacctc**	**cttgactcccacgcttcatttttct**	134	60	60.3
21	**gaatggggttgatttggagattcag**	**cacatccctgccaccccttc**	170	60	61.4
22	**ctccacgtccacctctaacatgg**	**agagggcagcccccctctccca**	179	60	61.4
23	**tctggcctgtgcattctttcac**	**ccctccaccttgagttttaatg**	137	60	58.5
24	**gggtcactcttttctgatctaca**	**agggtgaaactgaaatcctgag**	131	60	60

Note: the new primers are indicated in bold.

Table 2 – Distribution of the mutations by sex and groups.

		Group A	***Group B***	***Group C***	***Group D***	***Group P***	***Total***
Male	*HO*[a]	11	6	3	0	1	21
	CHE[b]	19	9	5	3	0	36
	HE[c]	3	2	12	7	3	27
	total mutated	***33***	***17***	***20***	***10***	***4***	***84***
	total studied	81	64	138	71	10	364
Female	*HO*[a]	9	9	3	0	0	21
	CHE[b]	7	14	4	0	1	26
	HE[c]	0	4	2	1	2	9
	total mutated	***16***	***27***	***9***	***1***	***3***	***57***
	total studied	42	78	20	16	10	166
TOTAL	*HO*[a]	20	15	6	0	1	42
	CHE[b]	26	23	9	3	1	62
	HE[c]	3	6	14	8	6	37
	total mutated	***49***	***44***	***29***	***11***	***8***	***141***
	total studied	123	142	158	87	20	530

Note: [a]homozygous, [b]compound heterozygous , [c]heterozygous.

Table 3 - Causative mutations identified in CAPN3 gene.

Exon	*Nucleotide change (cDNA, NM_000070)*	*Aminoacid change (protein, NP_000061)*	*HE[a]*	*CHE[b]*	*HO[c]*	*HO Families*
1	77C>T	P26L	1	-	-	
	139_141del	**I47del**	-	-	3	(2)
	143G>A	**S48N**	1	-	-	
	146G>A	**R49H**	-	2	-	
	229G>A	D77N	2	3	-	
	245C>T	P82L	2	2	1	(1)
	247_251del	**D83_E84delfs+2X5**	-	2	-	
	259C>G	**L87V**	1	-	-	
	304C>T	**P102S**	-	1	-	
	308C>T	**P103L**	1	0	0	
2	319G>A	E107K	1	-	-	
	328C>T	R110X	2	2	-	
3	**398C>T**	**A133V**	-	2	-	
	479C>G	A160G	1	-	-	
	498+1G>A	**splicing**	-	1	-	
4	533T>C	I178T	-	-	1	(1)
	548C>G	P183R	-	1	-	
	550delA	T184fs+1X35	2	19	12	(10)
	590G>A	**R197H**	-	2	-	
	598_612del	F200_L204del	1	6	-	
	620A>C	**K207T**	-	1	-	
5	**633G>T**	**K211N**	1	-	-	

Exon	*Nucleotide change (cDNA, NM_000070)*	*Aminoacid change (protein, NP_000061)*	*HE*[a]	*CHE*[b]	*HO*[c]	*HO Families*
	649G>A	E217K	-	1	-	
	664G>A	G222R	-	1	-	
	674C>G	**T225R**	-	1	-	
	689A>G	**D230G**	-	-	1	(1)
	697G>C	G233R	-	1	-	
	701G>A	G234E	-	1	-	
	739G>A	**D247N**	1	-	-	
	755T>C	**M252T**	2	-	-	
	755T>A	**M252K**	-	1	-	
	759_761del	K254del	-	1	-	
	801+1G>A	splicing	-	2	-	
6	**802-9G>A**	**splicing**	-	6	2	(1)
	848T>C	**M283T**	-	-	1	(1)
	883_886delGATAinsCTT	**D295delfs+1X55**	-	-	4	(1)
7	956C>T	P319L	-	1	-	
	964T>C	**Y322H**	1	-	-	
	967G>T	**E323X**	-	1	1	(1)
	1000C>T	**H334Y**	-	1	-	
	1001_1002insA	**H334fs-2X8**	-	-	1	(1)
8	**1058T>C**	**L353P**	1	-	-	
	1061T>C	**V354A**	-	1	-	
	1061T>G	V354G	-	1	-	
10	1291G>A	V431M	1	-	-	

Exon	*Nucleotide change (cDNA, NM_000070)*	*Aminoacid change (protein, NP_000061)*	*HE*[a]	*CHE*[b]	*HO*[c]	*HO Families*
	1303G>A	**E435K**	1	1	-	
	1309C>T	R437C	-	2	1	(1)
	1342C>T	R448C	1	-	1	(1)
	1343G>A	R448H	-	2	-	
11	**1401_1403del**	**E467del**	1	3	-	
	1448C>A	**A483D**	-	1	-	
	1465C>T	R489W	-	2	-	
	1468C>T	R490W	-	1	-	
	1469G>A	R490Q	-	1	1	(1)
	1477C>G	**R493G**	-	1	-	
13	1611C>A	Y537X	-	1	-	
	1621C>T	R541W	-	6	1	(1)
	1622G>A	R541Q	-	7	-	
	1706T>C	**F569S**	1	-	-	
	1714C>T	R572W	-	1	-	
	1715G>C	**R572P**	1	-	-	
	1745+1G>A	**splicing**	-	-	1	(1)
14	1746-20C>G	splicing	2	3	-	
15	**1792_1795del**	**K598_T599delfs+1X62**	1	3	-	
16	1817C>T	S606L	-	1	-	
	1865delAG	E622fs+2X7	-	2	-	
	1868_1877del	**E623_G626delfs+1X34**	-	1	-	
17	**1917_1922del**	**Q640_P641del**	1	-	-	

Exon	*Nucleotide change (cDNA, NM_000070)*	*Aminoacid change (protein, NP_000061)*	*HE*[a]	*CHE*[b]	*HO*[c]	*HO Families*
	1984G>T	**A662S**	1	-	-	
	1992+1G>T	splicing	-	3	-	
18	**2020_2022del**	**K674del**	-	1	-	
19	2105C>T	A702V	1	-	1	(1)
20	**2184G>A**	**Q728Q (splicing)**	1	-	2	(1)
21	**2204_2205del**	**T736fs+2X27**	-	1	-	
	2212C>T	**Q738X**	-	-	4	(1)
	2242C>T	R748X	2	9	3	(3)
	2243G>A	R748Q	1	-	-	
	2257delGinsAA	**D753fs-2X10**	-	1	-	
	2257G>A	D753N	1	3	-	
22	**2330T>C**	**I777T**	-	1	-	
	2335T>A	**F779I**	1	-	-	
24	**2440-9_-6del**	**splicing**	-	1	-	

Note: The new mutations are indicated in bold ([a]heterozygous, [b]compound heterozygous , [c]homozygous).

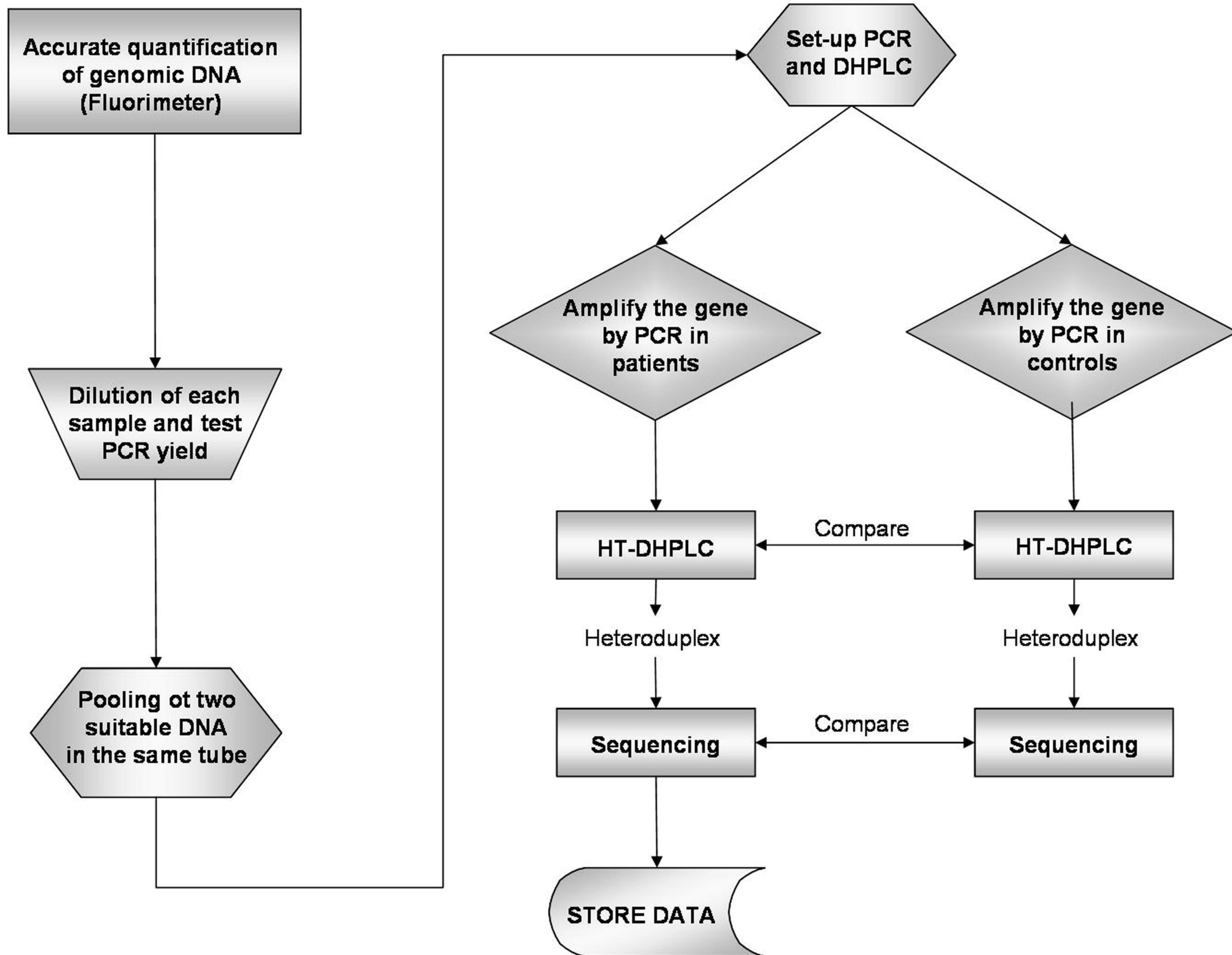
Table 4 – Polymorphic variants identified in CAPN3 gene.

Exon	*Nucleotide change (cDNA, NM_000070)*	*Aminoacid change (protein, NP_000061)*	*References*	*Frequency*
1	96T/C	T32T	47	0.12
2	**163G/A ***	**G55R ***		
	292G/A *	**V98I ***		
	318C/T	C106C	47	0.04
3	**477C/T ***	**Y159Y ***		
	495C/T	F165F	47	0.01
4	498+35G/T			
	499-17G/A			
	606T/C	**S202S**		0.06
5	706G/A	A236T	47	0.10
	798T/C	**I276I**		
6	**945+5G/A***			
	945+14C/T			
	945+56C/T			
	945+91C/T			
7	984C/T	C328C	47	
10	**1290A/G**	**T430T**		
11	**1524G/A***	**E508E**		
	1524+81C/T			
13	1542C/T+	H514H		
	1668C/T	I556I	47	<0.01
14	**1746-64C/T**			0.02
16	**1914+30G/A**			

Exon	***Nucleotide change (cDNA, NM_000070)***	***Aminoacid change (protein, NP_000061)***	***References***	***Frequency***
18	**1993-5delCTC ***			
19	**2115+46G/A**			
22	**2292C/T**	**D764D**		
	2380+12delA		47	0.10

Note: the new variants identified are reported in bold. An asterisk indicates variations of unclear pathological significance, found only once in patients.

Figure 1 - Flow-chart shows the general design of the strategy.


Accurate quantification of genomic DNA (Fluorimeter)
Dilution of each sample and test PCR yield
Pooling ot two suitable DNA in the same tube
Set-up PCR and DHPLC
Amplify the gene by PCR in patients
Amplify the gene by PCR in controls
HT-DHPLC
Compare
HT-DHPLC
Heteroduplex
Heteroduplex
Sequencing
Compare
Sequencing
STORE DATA