"ANO5 gene analysis in a large cohort of patients with anoctaminopathy: confirmation of male prevalence and high occurrence of the common exon 5 gene mutation."

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Abstract

Limb girdle muscular dystrophy type 2L or anoctaminopathy is a condition mainly characterized by adult onset proximal lower limb muscular weakness and raised CK values, due to recessive ANO5 gene mutations. An exon 5 founder mutation (c.191dupA) has been identified in most of the British and German LGMD2L patients so far reported. We aimed to further investigate the prevalence and spectrum of ANO5 gene mutations and related clinical phenotypes, by screening 205 undiagnosed patients referred to our molecular service with a clinical suspicion of anoctaminopathy. A total of 42 unrelated patients had two ANO5 mutations (21%), whereas 14 carried a single change. We identified 34 pathogenic changes, 15 of which are novel. The c.191dupA mutation represents 61% of mutated alleles and appears to be less prevalent in non-Northern European populations. Retrospective clinical analysis corroborates the

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ANO5 Gene Analysis in a Large Cohort of Patients with Anoctaminopathy: Confirmation of Male Prevalence and High Occurrence of the Common Exon 5 Gene Mutation

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ABSTRACT: Limb girdle muscular dystrophy type 2L or anoctaminopathy is a condition mainly characterized by adult onset proximal lower limb muscular weakness and raised CK values, due to recessive *ANO5* gene mutations. An exon 5 founder mutation (c.191dupA) has been identified in most of the British and German LGMD2L patients so far reported. We aimed to further investigate the prevalence and spectrum of ANO5 gene mutations and related clinical phenotypes, by screening 205 undiagnosed patients referred to our molecular service with a clinical suspicion of anoctaminopathy. A total of 42 unrelated patients had two ANO5 mutations (21%), whereas 14 carried a single change. We identified 34 pathogenic changes, 15 of which are novel. The c.191dupA mutation represents 61% of mutated alleles and appears to be less prevalent in non-Northern European populations. Retrospective clinical analysis corroborates the prevalently proximal lower limb phenotype, the male predominance and absence of major cardiac or respiratory involvement. Identification of cases with isolated hyperCKaemia and very late symptomatic male and female subjects confirms the extension of the phenotypic spectrum of the disease. Anoctaminopathy appears to be one of the most common adult muscular dystrophies in Northern Europe, with a prevalence of about 20%–25% in unselected undiagnosed cases.

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KEY WORDS: ANO5; LGMD2L; gender; muscular dystrophy

Introduction

Limb girdle muscular dystrophy type 2L (LGMD2L) is a condition characterized by proximal weakness affecting primarily the pelvic girdle and leg muscles, with less prominent distal leg weakness and high CK values [Hicks et al., 2011]. LGMD2L, together with a more distal variant also known as nondysferlin Miyoshi muscular dystrophy type 3 (MMD3) as well as some cases of isolated hyperCKaemia and pseudometabolic myopathy, have been shown to be associated with recessive mutations in the *ANO5* gene [MIM #608662; Bolduc et al., 2010; Bouquet et al., 2012; Deschauer et al., 2011; Hicks et al., 2011; Magri et al., 2012; Milone et al., 2012; Little et al., 2013; Neusch et al., 2012; Penttilä et al., 2012; Pénisson-Besnier et al., 2012; Schessl et al., 2012; Wahbi et al., 2012; Witting et al., 2012]. The prevalence of *ANO5* gene related conditions, or so-called anoctaminopathies, is not yet fully established, but preliminary data from Northern England indicate that LGMD2L is the 3rd most common type of LGMD with a minimum prevalence of 0.27/100 000 [Hicks et al., 2011]. To date, 67 variants (35 pathogenic; www.lovd.nl/ANO5) have been detected all over the *ANO5* gene, with one a common mutation (c.191dupA) in exon 5, likely the result of a founder effect of Northern European origin [Hicks et al., 2011], and another in exon 20 (c.2272C>T) more frequent in the Finnish population [Penttilä et al., 2012]. Early clinical and MRI studies indicated wide clinical heterogeneity and a gender difference in expression, with anoctaminopathies appearing to be less frequent and less severe in females [Sarkozy et al., 2012].

In the present study, we investigated the prevalence and spectrum of *ANO5* gene mutations in the so far largest and clinically most heterogeneous cohort of patients with anoctaminopathy, to better describe the phenotype, gender predominance, and allelic variability of this emerging disease.

Materials and Methods

The study was performed on a total of 205 unrelated individuals of both sexes, referred for *ANO5* gene analysis to the Newcastle upon Tyne Limb-Girdle Referral Centre in view of their phenotypes compatible with anoctaminopathy, ranging from isolated hyperCKaemia, myalgia and raised CK, to MMD3 and LGMD2L. Patients were referred from different clinical centers, but primarily from the UK, Germany, France, Spain, the USA and Belgium. The cohort previously reported by Hicks et al. (2011) was not included in this study. Clinical details for patients with *ANO5* mutations were collected retrospectively. The clinical suspicion of anoctaminopathy was made following clinical examination, muscle assessment and clinical investigations including CK analysis, EMG, muscle MRI and/or muscle biopsy. Possible alternative diagnoses, such as LGMD2B, LGMD2I, and LGMD2A, were excluded as appropriate. DNAs of affected and unaffected relatives were collected and segregation analysis performed where available.

DNA extraction and direct sequencing of the *ANO5* gene (GenBank accession number NM_213599.2) was performed as described elsewhere [Hicks et al., 2011]. Sequence variants were coded reflecting cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Novelty of each sequence variant was checked in the *ANO5* locus specific database (www.lovd.nl/ANO5). To investigate whether novel missense, synonymous or intronic variations not located in splice sites could possibly represent pathogenic sequence variants, we performed in silico analysis using the Alamut mutation analysis software (Interactive Biosoftware, v2.1).

Results

Genetic Analysis

ANO5 gene sequence variants were identified in 90/205 unrelated individuals (44%) and five affected relatives (Table 1, Supp. Table S1). Fifty-one patients were found to carry two or more *ANO5* variants in homozygosity or compound heterozygosity, whereas further 39 patients showed one single *ANO5* change (Tables 1 and 2). A total of 42 *ANO5* gene variants were identified, 27 of which novel (Tables 1 and 2). Twenty variants were missense (47%), four frameshift (9%), five splice site (12%), two stop (5%), one synonymous (2%), and 10 intronic (24%) (Table 2). The pathogenicity of 15 novel variants is supported by their predicted effect on protein product, by their recurrence in multiple unrelated patients, by segregation in affected family members, absence in SNPs databases and in silico analysis. Four missense changes, two of which were previously reported in literature as pathogenic (c.155A>G and c.2387C>T), were considered benign variants based on in silico analysis and/or their frequency in control population (Table 2) [Wahbi et al., 2012]. It was not possible to further comment on the pathogenicity of a missense (c.968C>G) and a synonymous change (c.2256G>A) as well as 10 intronic variants in absence of segregation studies or further cDNA investigations.

Forty-two unrelated patients (N.1-N.42) carried two likely pathogenic *ANO5* gene changes, 19 in homozygosity and 23 in compound heterozygosity (Table 1). Patients N.21B, N.22B, N.28B,

Table 1. Patients with *ANO5* Gene Mutations

Patient	Allele 1	Allele 2	Geographic origin
Patients with two pathogenic variants			
N.01-N.16	c.191dupA	c.191dupA[a]	Great Britain (12); Germany 3); Norway (1)
N.17-N22B	c.191dupA	c.692G>T	Great Britain (7)
N.23	c.191dupA	c.762+1G>A	Great Britain
N.24	c.191dupA	c.1391C>A	USA
N.25	c.191dupA	c.1407+5G>T	Belgium
N.26-N.27	c.191dupA	c.1627dupA	Spain, n.a.
N.28A-N.28B	c.191dupA	c.1643C>T	Germany (2)
N.29	c.191dupA	c.1733T>C	Great Britain
N.30-N.32	c.191dupA	c.1898+1G>A	Great Britain, Poland and USA
N.33	c.191dupA	c.2395C>T	Great Britain
N.34	c.191dupA	c.2417A>G	USA
N.35	c.191dupA	c.2272C>T	Germany
N.36	c.401A>G	c.1898+1G>A	Great Britain
N.37	c.1639C>T	c.1639C>T	n.a.
N.38	c.2030–1G>T	c.2030–1G>T	Sri Lanka
N.39	c.2236–13_2236–4delins ATTCTTCTGGC	c.2236–13_2236–4delins ATTCTTCTGGC	Great Britain
N.40A-N.40C	c.242A>G	c.1097A>G	Germany (3)
N.41	c.400C>T	c.2235+1G>A	France
N.42	c.989dupT	c.2018A>G	Great Britain
Patients with one pathogenic variant			
N.43-N.44	c.191dupA	c.155A>G	Great Britain; n.a.
N.45-N.47	c.191dupA	–	Germany (2); Great Britain
N.48-N.50	c.692G>T	–	Great Britain (3)
N.51	c.762+1G>A	c.155A>G	USA
N.52	c.1925G>T	c.1181–21T>A	Great Britain
N.53	c.1640G>A	–	Great Britain
N.54	c.2417A>G	–	Great Britain
N.55	c.2521C>G	–	Great Britain
N.56	c.2593A>C	–	Pakistan

[a]Patient N.05 and N.13 also with variant c.364–67T>C with uncertain pathogenicity. *ANO5* gene, GenBank accession number NM_213599.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

N.40B-C carried the pathogenic variants found in their affected relative (i.e., N.21A, N.22A, N.28A and N.40A). In 14 patients (N.43-N.56) only one single pathogenic variant was identified. cDNA analysis or genomic dosage analysis was not performed to rule out a possible 2nd deep intronic change or deletion/duplication of the part/entire *ANO5* gene. Seven novel pathogenic variants were recurrent (Tables 1 and 2). The common c.191dupA exon 5 mutation was found in homozygosity in 16 patients, in compound heterozygosity with another identified mutation in 19, and in heterozygosity without a second detectable mutation in further five individuals. Geographic origin of patients, indicated in Table 1, confirms that the common variant is present also in populations other than the British and German populations. Additionally, two already reported (c.692G>T, c.1927dupA) and two novel (c.1898+1G>A, c.2417A>G) mutations were also identified in multiple patients, and further three novel changes were found in homozygosity in three individuals (Tables 1 and 2). Parental DNA screening or *ANO5* genomic dosage analysis was not performed yet for the homozygous patients, and hemizygosity of parts or the entire *ANO5* locus cannot be ruled out.

Phenotypic Analysis

Among patients with two likely pathogenic changes (total 47 patients, including 42 unrelated subjects and five affected relatives), 39 were male (83%) and eight were female (17%). Clinical information was available for 44 patients (34 males, and seven females) (Table 3). Age at onset ranged between teens and late 70s, with an average age at onset of 35 years. At time of genetic diagnosis, 3/7 females and 3/4 males were completely asymptomatic or very mildly symptomatic. Average CK values were 4,000 IU/l, ranging from around 200 IU/l up to about 30,000 IU/l. Detailed information about last muscle assessment was available for 38/47 individuals. A predominantly proximal involvement was observed in 22/38 (57%) patients, whereas 4/38 (10%) and 3/38 (8%) patients showed a more distal or proximal plus distal involvement, respectively. No or very mild clinical symptoms (myalgia and/or raised CK) were reported in nine patients with two *ANO5* mutations (25%).

Symptoms at onset and overall clinical presentation in males were similar to what has been previously described for patients with *ANO5* gene mutations (Table 3; Hicks et al., 2011]. Although age at onset was similar on average (34 years), in this cohort we observed a wider range, with patients showing first symptoms in their teens to patients only symptomatic in late adulthood, with one patient (Pt. N.38) showing first symptoms of proximal lower limb weakness and wasting at the age of 77 years only. Moreover, the phenotype was extremely mild in three individuals, only showing raised CK values (Pt. N.15 and N.36) or some mild proximal leg weakness after a disease course of 32 years (Pt. N40B). Patient N.21B was diagnosed as part of family screening and showed no symptoms of the disease. CK values were not available at the time of study.

Five female individuals (N.03, N.21A, N.27, N.28B, 40C), with an age at last assessment ranging from 14 to 68 years, showed raised CK values only or asymmetric calf atrophy, whereas female patients N.02 and N.14, aged 68 and 42 years, respectively, showed a more pronounced LGMD type weakness. Female patient N.08 (not included in Table 3 in view of lack of detailed clinical information) was diagnosed as part of a family screening of a previously reported LGMD2L patient. At the age of 44 years she does not report any clinical symptoms, but no further clinical information was available at time of the study.

A total of seven unrelated patients were confirmed with anoctaminopathy, but did not carry the common c.191dupA mutation on either allele. Age at onset was 44 years on average. Four patients showed a milder phenotype, with a very late onset (77 years), normal power or only mild proximal weakness at last assessment. Muscle wasting was present in half of them, whereas upper limb function was normal in all.

Clinical information was available for 11/14 unrelated patients with one single pathogenic variant (data not shown). Five individuals showed a phenotype compatible with a diagnosis of LGMD2L, whereas three subjects only showed raised CK values with or without calf hypertrophy. In one individual (Pt N.50) this variant was also found in further two family members also affected by isolated hyperCKaemia.

Discussion

In this study, we report on the clinical analysis and molecular screening of the *ANO5* gene in the largest cohort of anoctaminopathy patients identified so far. Our results confirm the high frequency of the common c.191dupA mutation which represents 51/84 (61%) of mutated alleles in the cohort of patients with two mutations (Tables 1 and 2). Occurrence of this Northern European founder mutation in patients of different geographic origin indicates that it has likely spread worldwide, although at lower frequencies compared with the British and German populations. This is

Table 2. List of Detected *ANO5* Gene Changes

Nucleotidic change	Amino acidic change	Exon intron	Protein domain	Zygosity	Reported	Interpretation?
c.139–59dupT		IVS3	CTD	1 HzPt	Novel	Benign in silico
c.155A>G	p.Asn52Ser	4	CTD	3 CHzPt + 2 HzPt	Reported	Benign in silico
c.191dupA		5	CTD	16 HomoZpt+ 19 CHzPt + 5 HzPt	Reported	Frameshift mutation
c.242A>G	p.Asp81Gly	5	CTD	1 CHzPt	Reported	Pathogenic missense change
c.364–8delT		IVS7	CTD	5 HomoZpt+ 8 HzPt	Reported	Unconfirmed pathogenicity
c.364–67T>C		IVS7	CTD	2 HzPt	Novel	Unconfirmed pathogenicity
c.400C>T	p.His134Tyr	7	CTD	1 CHzPt	Novel	Pathogenic in silico
c.401A>G	p.His134Arg	7	CTD	1 CHzPt	Novel	Pathogenic in silico
c.692G>T	p.Gly231Val	8	CTD	5 CHzPt+ 3 HzPt	Reported	Pathogenic missense change
c.746C>G	Ala249Gly	8	CTD	1 HzPt	Novel	Benign in silico
c.762+1G>A		IVS8	CTD	1 CHzPt+1 HzPt	Novel	Putative splice mutation
c.878+78delT		IVS9	CTD	1 HzPt	Novel	Benign in silico
c.879–41A>T		IVS9	CTD	1 HzPt	Novel	Unconfirmed pathogenicity
c.968C>G	p.Ala323Gly	10	CTD	1 HzPt	Novel	Unconfirmed pathogenicity
c.989dupT		10	CTD	1 CHzPt	Novel	Frameshift mutation
c.1097A>G	p.Asn366Ser	11	ETD 1	1CHzPt	Reported	Pathogenic in silico
c.1119+35G>A		IVS11	ETD 1	1 CHzPt	Novel	Unconfirmed pathogenicity
c.1120–24A>T		IVS12	ETD1	2 HzPt	Novel	Unconfirmed pathogenicity
c.1181–21T>A		IVS13	TMD2	1 HzPt	Novel	Unconfirmed pathogenicity
c.1181–48T>A		IVS13	TMD2	1 CHzPt +8 HzPt	Novel	Unconfirmed pathogenicity
c.1391C>A	Ala464Asp	14	TMD3	1 CHzPt	Novel	Pathogenic in silico
c.1407+5G>T		IVS14	TMD3	1 CHzPt	Reported	Putative splice mutation
c.1627dupA		15	CTD3	2 CHzPt	Reported	Frameshift mutation
c.1639C>T	p.Arg547STOP	16	CTD3	1 HomoZpt	Novel	Stop mutation
c.1640G>A	p.Arg547Gln	16	CTD3	1 HzPt	Novel	Pathogenic in silico
c.1643C>T	pThr548Ile	16	CTD3	1 CHzPt	Reported	Pathogenic in silico
c.1733T>C	Phe578Ser	16	CTD3	1 CHzPt	Reported	Pathogenic missense change
c.1898+1G>A		IVS17	ETD3	4 CHzPt	Reported	Putative splice mutation
c.1925G>T	Arg642Leu	18	ETD3	1 HzPt	Novel	Pathogenic in silico
c.2018A>G	Tyr673Cys	18	ETD3	1 CHzPt	Reported	Pathogenic missense change
c.2030–1G>T		IVS18		1 HomoZpt	Novel	Putative splice mutation
c.2235+1G>A		IVS19	TMD7	1 CHzPt	Novel	Putative splice mutation
c.2236–13_2236–4delinsATTCTTCTGGC		20	TMD7	1 HomoZpt	Novel	Frameshift mutation
c.2256G>A		20	TMD7	1 HzPt	Novel	Unconfirmed pathogenicity
c.2272C>T	Arg758Cys	20	TMD7	1 CHzPt	Reported	Pathogenic missense change
c.2387C>T	p.Ser796Leu	20	TMD7	1 HzPt	Reported	Benign in silico
c.2395C>T	p.Arg799STOP	20		1 CHzPt	Novel	Stop mutation
c.2417A>G	p.Tyr806Cys	21	ETD4	1 CHzPt+ 1 HzPt	Novel	Pathogenic in silico
c.2521–13A>G		IVS21	TMD8	1 HzPt	Reported	Unconfirmed pathogenicity
c.2521C>G	p.His841Asp	22	TMD8	1 HzPt	Novel	Pathogenic in silico
c.2593G>T	p.Ile865Leu	22	TMD8	1 HzPt	Novel	Pathogenic in silico
c.2698A>C	p.Met900Leu	22	CTD5	1 HzPt	Novel	Benign in silico

ANO5 gene, GenBank accession number NM_213599.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
CTD: cytoplasmic topological domain; ETD: extracellular topological domain; TMD: transmembrane domain; CHzPt: compound heterozygous patient, HzPt: heterozygous patient; HomoZpt: homozygous patient; IVS: intron.

corroborated by the observation that the mutation has not been observed in homozygosity in other populations, with the exception of two unrelated French patients [Wahbi et al., 2012]. However, our result could have been biased by the predominance of British and German patients in our cohort.

We identified 27 novel variants in the *ANO5* gene, 15 of which are likely pathogenic (Tables 1 and 2). Mutations are spread across the gene, indicating the absence of additional mutation hot spots, although some mutations appear to be recurrent (Tables 1 and 2). The frequency of the second most common variant (c.692G>T) suggests that it could represent an additional common change in our population (Table 1). Interestingly, the common Finnish mutation appears to be rare in our cohort, with a single occurrence in a German patient in compound heterozygosity with the c.191dupA mutation (Pt N.35). Mutations leading to loss of protein function (frameshift, splice and stop mutations) were the most common changes in the "2-mutation" group of patients (66/84 alleles, 79%) although the relative frequency of missense changes (18/84 alleles, 21%) could imply further pathogenetic mechanisms.

We have identified several exonic changes that appear to be benign variants or to have uncertain pathogenicity. Among these, the nonpathogenic variant c.155A>G (p.Asn52Ser) in exon 4 (dbSNP rs143777403), found with allelic frequency of 0.0001–0.001 in North American and African American control populations, was identified in five patients from our cohort. The c.2387C>T (p.Ser796Leu) in exon 22, also a benign variant by in silico analysis, was found in another individual. Interestingly, these two missense variants were described as pathogenic by Wahbi and coauthors (2012) in three unrelated subjects, one of which was compound heterozygous for this and the c.155A>G variant. These findings point toward caution when novel missense changes are found (especially if in compound heterozygosity), and no further data corroborating the pathogenicity of the change is available. We identified three patients who were homozygous for a novel variant and 14 patients with one

Table 3. Phenotypic Analysis of Patients with 2 Pathogenic *ANO5* Variants

Patient	Age (years)	Sex	Onset		CK (IU/L)	Pattern of muscle involvement at last assessment									Other features
			Age	Symptoms		Ambulant	UL prox	LL prox	LL Distal	Walk on toes	Walk on heels	Muscle atrophy	scapular winging	AS	
N.01	50	m	38	Thigh weakness	241–2,603	Yes	–	+++	–	Diff	Diff	Thighs	–	+	–
N.02	68	f	64	Proximal LL weakness, thigh wasting	n.a.	Yes	+/–	+++	–	Diff	Diff	Thighs and calves (AS)	–	+	
N.03	20	f	–	–	3,300–8,800	Yes	–	–	–	Able	Able	–	–	–	–
N.04	63	m	50s	Walking difficulties	2,122	Yes	+/–	+++	+	n.a.	n.a.	–	–	–	Calf hypertrophy, KH
N.05	44	m	20s	Thigh and calf wasting (AS)	4,060	Yes	–	+++	+/–	Able	Able	Calves, thighs (AS)	–	+	Calf hypertrophy (as child), TA contractures, ptosis
N.06	35	m	20s	Calf swelling, back pain, leg weakness	1,642–3,080	Yes	+/–	++	++ (AS)	Unable	Unable	–	–	+	Muscle twitching, TA contractures
N.07	56	m	40s	LL weakness and wasting	1,517	Yes	++	+++	+	Unable	Unable	n.a.	–		
N.09	40	m	20s	Reduces sport fitness and muscle wasting	4,000	Yes	+/–	++	–	Able	Able	Biceps/pectoral. quads (AS)	–	+	
N.10	64	m	46	Raised CK and myalgia	2,200–3,300	Yes	+/–	+	–	Able	Able	Quads	–	–	Sleep apnoea, CTS
N.11	43	m	20s	n.a.	5,400	Yes	no	–	+	Diff	Diff	Calves (AS)	–	+	Thigh hypertrophy
N.12	16	m	16	Myalgia, raised CK	2,000–30,000	Yes	+/–	–	–	Able	Able	–	–	–	Neck flexion weakness
N.13	70	m	38	Difficulty running and calf asymmetry	1,800	Yes	+/–	+ (AS)	+	Unable	Unable	Biceps and calves (AS)	–	+	Calf hypetrophy (AS)
N.14	42	f	30s	Prox LL weakness	2,500–3,000	Yes	–	+	–			Vasti	+/–	n.a.	Calf hypertrophy, CTS
N.15	67	m	50s	Raised CK and myalgia	2,100	Yes	–	–	–	Able	Able	–	–	n.a.	Calf hypertrophy
N.16	44	m	40	Myalgia	1,500–2,500	Yes	+/–	–	–	Able	Able	–	+	–	
N.17	47	m	20s	Myalgia	1,600–6,000	Yes	–	+++	–	Able	Unable	Thighs, hamstrings especially, calf (AS)	–	+	Thigh hypertrophy, CTS
N.18	65	m	30s	Walking difficulties	3,661	no 60yrs	–	++++	–	Unable	Unable	Quads	–	–	Calf hypertrophy
N.19	40	m	34	LL weakness	5,000	Yes	–	+++	+/–	Able	Able	Hamstrings, buttocks, calves	–	–	
N.20	65	m	40s	Walking difficulties	2,000	Yes	–	+	–	n.a.	n.a.	n.a.	–	n.a.	
N.21A	14	f	–	–	1,200	Yes	–	–	–	Able	Able	–	–	–	–
N.21B	17	m	–	–	n.a.	–	–	–	–	Able	Able	–	–	–	–
N.22A	72	m	50s	Walking difficulties	900	Yes	+ (AS)	+++ (AS)	n.a.	Diff	Diff	+	+	+	
N.22B	74	m	40s	UL weakness	900	Yes	+	+ (AS)	n.a.	Diff	Diff	Medial gastrocnemius (AS)	+	+	Calf, paraspinal muscle hypertrophy
N.23	41	m	38	LL weakness and wasting	209–6,301	Yes	+/–	+++	–	Unable	Diff	Thighs and calves (AS)	+ (AS)	+	KH (AS)
N.24	55	m	28	Weakness in gastrocnemius muscle	2,500–4,500	Yes	n.a.	n.a.	n.a.	N.a.	N.a.	n.a.	n.a.	n.a.	
N.25	42	m	23	Wasting and weakness left calf	5,300–12,500	Yes	–	–	++	Unable	Able	calves	–	+	
N.27	41	f	34	hyperCKaemia	2,503	Yes	–	–	–	Able	Able	Gastrocnemius medialis (AS)	–	+	
N.28A	56	m	40	Asymmetric thigh weakness	1,500–6,100	Yes	+/–	++	–	Diff	Diff.	Thighs (AS)	–	+	
N.28B	53	f	–	–	2,280	Yes	–	–	–	Able	Able	–	–	–	

(*Continued*)

Table 3. Continued.

Patient	Age (years)	Sex	Onset		CK (IU/L)	Pattern of muscle involvement at last assessment									Other features
			Age	Symptoms		Ambulant	UL prox	LL prox	LL Distal	Walk on toes	Walk on heels	Muscle atrophy	scapular winging	AS	
N.29	64	m	30s	Difficulties with climbing stairs	829	Yes	+/–	+++	+++	Unable	Unable		–	+	Calf hypertrophy (AS), IHD Anaemia, FVC 70%
N.30	61	m	30	Weakness in LL muscles	5,956	Yes	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
N.31	28	m	20	Difficulties running	7,868	Yes	–	+++ (AS)	–	Able	Able	UL, hamstrings, buttocks, quads	–	+	Sensory axonal polyneuropathy
N.32	52	m	20s	hyperCKaemia and calf wasting	>3,000	Yes	+/– (AS)	+++	–	Unable	Able	Vastus medialis, calf (AS)	+/–	+	TA contractures
N.33	32	m	20s	LGMD type weakness	n.a.	Restricted	–	+/–	+	n.a.	n.a.	Biceps, medial quads, calf (AS)	n.a.	+	
N.34	32	m	22	Calf wasting, unable to stand on toes	10,000	Yes	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
N.35	29	m	23	Calf muscles stiffness when walking	1,700–2,900	Yes	–	–	+/–	Diff	Unable (AS)	Calves (AS)	–	+	
N.36	54	m	32	Proximal LL weakness	4,029	Yes	–	+/–	–	Able	Able	–	–	–	
N.38	77	m	77	Proximal LL weakness and raised CK	4,400	Yes	n.a.	+ (AS)	–	Able	Able	LL (AS)	–	+	
N.39	59	m	38	Left LL weakness	3,000	Yes	–	+/–		Unable	Unable	Thighs (AS)	–	+	Hypertension, KH (AS)
N.40A	32	m	21	Decreased endurance, inability standing on right tiptoes and raised CK	4,200–22,000	Yes	–	++	+	Unable	Able	Adductors, quadriceps, and calves	–	+	KH
N.40B	13	m	–	–	1,970	Yes	–	–	–	Able	Able	–	–	–	
N.40C	23	f	21	Myalgia	861	Yes	–	–	–	Able	Able	–	–	–	
N.41	56	m	35	Difficulties running	1,168–3,612	Yes	–	++	++	Unable	Able	Thighs and calves	–	–	
N.42	44	m	39	Distal weakness and calf wasting	2,666–4,200	Yes	–	+	++	Unable	Able	UL (distally), thighs, calves	–	–	Hand weakness, back twitching

Pt, patient number; CK, creatine kinase; UL, upper limbs; LL, lower limbs; AS, asymmetry; m, male patient; f, female patient; diff, able with difficulties; n.a, data not available; TA, Achilles tendons; KH, knee hyperextension; IHD, ischemic heart disease; FVC, forced vital capacity; CTS: carpal tunnel syndrome; +, ++, +++: grade of weakness (from milder to more severe).

heterozygous change only. One of the limitations of this study is the lack of cDNA studies to exclude second deep intronic changes or gene dosage analysis, but deletion/duplication analysis is currently in progress in some of these individuals.

We have previously described the clinical phenotype of anoctaminopathies as an adult onset disease, characterized by slowly progressive muscle weakness mainly affecting the pelvic girdle and the lower limbs [Hicks et al., 2011]. Clinical analysis of the present cohort of patients extends our initial findings to a large group of patients not restricted to the common founder mutation (Table 3). Being our center the only currently offering *ANO5* screening in the UK, the prevalence of proximal disease is unlikely to be caused by the referral bias previously suggested [Hicks et al., 2011]. No major significant cardiac or respiratory disease was observed in this cohort, suggesting that these are not major features of the condition. We provide further evidence for male predominance in anoctaminopathies, females being overall less frequently and less severely affected than males [Hicks et al., 2011, Penttilä et al., 2012]. Phenotypic analysis of the here reported affected females reveals a milder clinical picture, with five patients showing only raised CK values or calf hypertrophy. The oldest female individual, homozygous for the common exon 5 mutation, showed a late disease onset (64 years), and at the age of 68 years she was still ambulant and she was able to stand on tiptoes and heels with minor difficulties. These findings suggest that gender differences could be in part due to the milder phenotype of affected females that is therefore less likely to be ascertained. In fact, two asymptomatic females were identified after a molecular diagnosis was reached in their brothers. Recruitment bias could also in part be responsible for gender difference, as patients seen in specialized centers usually show a more severe phenotype, and therefore females with milder phenotypes might not come as frequently to our attention. In this study, we also identified four male subjects showing a very mild phenotype, ranging from isolated hyperCKaemia to mild proximal leg weakness with onset in late adulthood (Table 3). In particular, one subject only showed symptoms in his late 70s, confirming that indeed age at onset and severity of anoctaminopathies can be extremely variable also in males (Hicks et al., 2011; Penttilä et al., 2012; Schessl et al., 2012].

No genotype–phenotype correlations were noted in anoctaminopathy so far. Nevertheless, a clinical comparison between patients carrying the common exon 5 mutation and those carrying different changes revealed some interesting findings (Tables 1 and 3). Pattern of muscle involvement and female/male ratio appear similar, although the phenotype is somehow milder, with onset at later age in patients carrying mutations other than the c.191dupA. Unfortunately the relatively low number of patients with different *ANO5* changes does not allow any statistically significant conclusions. Conversely, phenotypic analysis of heterozygous patients did not evidence major differences compared with patients with two mutations, although three heterozygous patients presented raised CK values and mild calf hypertrophy only. Although we cannot exclude a second non-yet identified change in these subjects, family analysis of one individual (Pt. N.50) showed that raised CK values segregated with the heterozygous mutation in two other family members (data not shown). An association between isolated hyperCKaemia and *ANO5* heterozygous carrier status was suggested by Milone and coworkers, who identified an otherwise asymptomatic carrier of the c.191dupA mutation (Milone et al., 2012). Likewise, isolated hyperCKaemia or very mild phenotypes were also found in a few male and female patients carrying two pathogenic *ANO5* mutations [Hicks et al., 2011; Penttilä et al., 2012; Schessl et al., 2012; Wahbi et al., 2012]. Systematic analysis of *ANO5* gene in wider cohort of subjects with isolated hyperCKaemia, as well as CK measurements in otherwise healthy heterozygous family members could help to clarify these findings.

Our results show that *ANO5* gene mutations are responsible for about ¼ of cases of undiagnosed muscular dystrophy with adult onset and raised CK, screened at our service over the last two years (present series and Hicks et al., 2011]. This value is similar to what reported in the Finnish population [Penttilä et al., 2012] and indicates anoctaminopathy as one of the most common form of LGMD in our population, possible reflection of the founder effects observed in Northern Europe [Hicks et al., 2011]. Indeed, observed incidences in the UK and Finland are higher than in Italy [Magri et al., 2012] where only about 2% of undiagnosed LGMD patients were shown to carry *ANO5* mutations. Based on its relative frequency, screening of the *ANO5* gene now represents an early step in the diagnostic work-up of these patients. However, in view of the increasing genetic heterogeneity and prevalence of families without the common mutation (14%), analysis should not be limited to founder mutations only [Penttilä et al., 2012]. More systematic studies on isolated hyperCKaemia patients will also help in giving better prevalence data for anoctaminopathies in general.

In conclusion, we confirmed that *ANO5* gene mutations are responsible for about ¼ of cases of undiagnosed muscular dystrophy in our population, being the exon 5 change the most prevalent. We expanded the allelic heterogeneity of the disease and recognized a broader clinical spectrum of the disease, ranging from isolated hyperCKaemia to full blown LGMD2L, with clear male predominance both in terms of overall prevalence and severity of the disease.

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