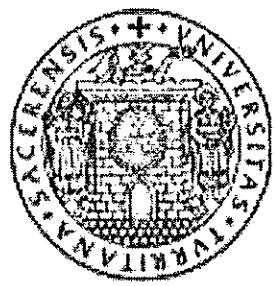

La Paraparesi Spastica Ereditaria in Sardegna: epidemiologia e studi di caratterizzazione genotipo-fenotipo

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Abstract

La paraparesi spastica ereditaria (*hereditary spastic paraparesis*, HSP) rappresenta un gruppo clinicamente e geneticamente eterogeneo di rari disordini neurodegenerativi caratterizzati dalla insorgenza di una progressiva spasticità e debolezza a carico degli arti inferiori. Tale quadro clinico, tipico delle forme pure, si arricchisce di svariati sintomi e segni neurologici e non nelle forme complesse. Il decorso è progressivo causando una diversa compromissione a seconda delle forme e della espressività della malattia. Sono note tutte le modalità di trasmissione ereditaria e, fino ad ora, le diverse forme cliniche sono stati associate ad oltre 70 loci, mentre sono stati mappati circa 60 geni. Tale eterogeneità genetica si ricompone nei meccanismi patogenetici che possono essere raggruppati in poche, principali categorie: alterazioni del traffico intracellulare, dell'omeostasi e del metabolismo lipidico e dell'attività mitocondriale.

La Sardegna a motivo della sua insularità, presenta una popolazione con un profilo demografico e genetico peculiare che dà conto, ad esempio, della elevata incidenza di malattie poligeniche complesse come la sclerosi multipla ed il diabete mellito giovanile. Con la presente ricerca abbiamo voluto ottenere una stima della prevalenza della HSP, malattia monogenica rara, nella popolazione sarda (PubblicazioneI). Studiare la distribuzione delle forme Autosomico Dominanti (AD), Autosomico Recessive (AR), a presentazione sporadica e la presenza di eventuali spot di malattia (Pubblicazioni I, II). Studiare la correlazione tra il genotipo ed il fenotipo (Pubblicazioni II, III, IV). Avere altri spunti di ricerca nella prospettiva della "*proximity to cure*" (Pubblicazione IV).

Si è condotto nella Sardegna nord-occidentale (provincia di Sassari) uno studio di prevalenza "*population based*" tra il Gennaio 2000 ed il 31 Dicembre del 2010. I casi sono stati raccolti secondo un modello semplice a fonti multiple, includendo nello studio i soggetti definitivamente affetti, probabilmente affetti e quelli a presentazione sporadica di malattia, secondo i criteri diagnostici universalmente accettati. Tutti i soggetti che hanno soddisfatto tali criteri e residenti

nell'area in studio al 31 Dicembre del 2010, sono stati considerati casi prevalenti. In questi, previo consenso informato, è stato effettuato un prelievo di sangue venoso per l'analisi genetica. Ogni soggetto che ha partecipato allo studio è stato valutato obiettivamente ed i dati clinici sono stati raccolti in accordo al protocollo SPATAX, sviluppato dal network europeo e mediterraneo per lo studio delle malattie spino-cerebellari, in particolare la severità di malattia è stata quantificata utilizzando la SPRS (*Spastic Paraplegia Rating Scale*). Per ciascun caso indice è stato ricostruito il pedigree da tre fino a dieci generazioni. Si è estesa quindi la valutazione clinico-genetica ad altri nuclei familiari originanti da diverse aree dell'isola.

Al 31 Dicembre 2010, la prevalenza cruda totale 19,9 su 100.000 (95% CI: 18,4- 21,4. L'età media all'esordio era di 36,6 anni. La diagnosi molecolare è stata ottenuta nell'82,1% dei casi. Le forme AD hanno contribuito al carico maggiore di malattia con una prevalenza cruda di 17,5 (24,4 M, 15,7 F; M:F ratio 1,55). IL 76% dei casi prevalenti hanno presentato la stessa ampia delezione multiesonica di *SPAST*, che si estende oltre la 3'-UTR coinvolgendo il gene *SLC30A6*. Infatti in alcune aree della nostra isola l'isolamento geografico e la relativa stabilità delle popolazioni residenti dedite alla pastorizia ed agricoltura, ha favorito lo sviluppo di un vasto pedigree SPG4 i cui "founders" sono originari dell'Anglona. La maggior parte dei pazienti hanno presentato una lieve disabilità, come anche i membri di un'altra famiglia SPG4 originari del sud dell'isola, con una più piccola delezione che si estende oltre la 5'-UTR, interessando il gene *DPY30*. Un giovane di 17 anni, appartenente ad una famiglia del centro dell'isola, ha presentato un quadro clinico di paraparesi spastica ascendente ad esordio infantile (*Infantile Ascending HSP*, IAHSP). L'analisi molecolare ha evidenziato una mutazione omozigote c.3836+1G>T a livello del sito donatore di splicing dell'introne 24 di *ASL2*. La mutazione era nuova, presente in eterozigosi nei genitori e non riscontrata in 500 cromosomi di controllo.

La prevalenza della HSP riportata in Sardegna è verosimilmente la più elevata tra le popolazioni dell'Europa Occidentale. Nonostante l'elevato tasso di consanguineità della popolazione sarda, solo una minoranza delle forme riscontrate sono recessive, la quasi totalità è a trasmissione autosomica dominante e quindi indipendenti nella loro etiologia da questo fattore. Il fenotipo prevalentemente riscontrato è in relazione con delezioni del gene *SPG4* ed è caratterizzato da età media all'esordio superiore rispetto a quanto precedentemente descritto, lenta progressione, disabilità moderata. Questa evidenza epidemiologica ha delle implicazioni prognostiche per la popolazione generale, sopratutto in relazione alla presenza di una elevata proporzione di casi asintomatici. Una maggiore consapevolezza delle malattie cronico-degenerative come la HSP, dovrebbe portare ad una sorveglianza epidemiologica maggiore ed incoraggiare l'adozione di specifiche misure sanitarie, come il *counseling* genetico dedicato ed un regolare follow-up. Dal punto di vista molecolare, il coinvolgimento dei geni fiancheggianti *SPG4* in diversi nuclei familiari offre lo spunto ad ulteriori ricerche per valutarne l'eventuale ruolo nella modificazione del fenotipo.

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1. Introduzione

1.1 La paraparesi spastica ereditaria (1.1-1.8, Pubblicazione V)

La paraparesi spastica ereditaria *(hereditary spastic paraparesis*, HSP) rappresenta un gruppo clinicamente e geneticamente eterogeneo di rari disordini neurodegenerativi caratterizzati dalla insorgenza di una progressiva spasticità e debolezza a carico degli arti inferiori [1]. Clinicamente, il sintomo iniziale della HSP è la spasticità che inizialmente è causa di impaccio nella deambulazione, quindi nella sua lenta progressione impone l'uso di uno o più sostegni, fino alla sedia a rotelle. Segni neurologici invariabilmente presenti sono il patologico incremento dei riflessi propriocettivi, il riflesso plantare-cutaneo in estensione, la compromissione, in genere lieve, della sensibilità profonda, con particolare interessamento della chinestesia e pallestesia, e debolezza muscolare piramidale. La spasticità e la debolezza sono massime a livello dell'ileo-psoas, tricipite surale, tibiale anteriore [2]. Quando il paziente presenta unicamente queste caratteristiche cliniche la malattia è in forma "pura". Le forme "complesse" sono invece associate con una pletora di segni e sintomi addizionali, fra gli altri: ritardo mentale, neuropatia sensitiva, atassia cerebellare, epilessia, atrofia ottica, retinite pigmentosa, sordità e cataratta.

La variabilità nell'esordio della malattia, nella sua progressione e nel grado di disabilità raggiunto, sia in ambito intra- che inter- familiare, sono caratteristiche ben conosciute nella HSP, in particolare nelle forme pure autosomiche dominanti (AD) [3]. Si ritiene che la variabile penetranza ed espressività che ne sono causa, siano età e sesso dipendenti [4,5].

Il frequente *overlap* nella presentazione clinica esistente tra le varie forme di HSP limita la possibilità di predire il genotipo in base unicamente a quest'ultima. Tuttavia, sia la presentazione clinica che il pattern di ereditarietà rappresentano degli utili indizi nel mirare la diagnosi molecolare [6] anche nei bambini [7].

La continua acquisizione di nuove conoscenze riguardo i geni implicati e le basi molecolari della malattia ha permesso una modernizzazione nei criteri classificativi della malattia, sebbene gli elementi derivanti dall'esame clinico e dalle neuroimmagini rimangano i criteri fondamentali nei provvedimenti terapeutici, che sono allo stato attuale essenzialmente sintomatici.

I meccanismi patofisiologici responsabili della HSP sono diversi e in gran parte non perfettamente conosciuti. Gli studi sui modelli cellulari ed animali della malattia [10-16] propongono come responsabili delle degenerazione delle lunghe fibre del tratto cortico-spinale la compromissione dei meccanismi di scambio intracellulare, della produzione energetica mitocondriale, del metabolismo lipidico e del trasporto assonale. In conseguenza si ottiene la disfunzione del tratto piramidale ed i segni clinici osservati [17].

Nelle forme complesse, come evidenziato alla Risonanza Magnetica (RM) [18], sono coinvolte, oltre al tratto piramidale, altre strutture dell'encefalo come il cervelletto, la corteccia cerebrale, la sostanza bianca.

Ciò è chiaramente illustrato dalla forma di HSP associata all'assottigliamento del corpo calloso *(HSP associated with thinning of the corpus callosum,* HSP-TCC) e ritardo mentale. Originariamente descritta nei paesi del Mediterraneo, la HSP-TCC ha in realtà una distribuzione ubiquitaria [19,20] e dà conto del 30-35% delle forme autosomiche recessive (AR) [21].

1.2 Etereogeneità genetica e fenotipica

La variabilità clinica osservata nella HSP è forse resa ancora più complessa dalla notevole etereogenità genetica. Sono state descritte tutte le modalità di trasmissione (AD, AR, X-linked) che sono associate ad una molteplicità di geni o loci. L'ereditarietà matrilineare è stata postulata come ulteriore meccanismo di trasmissione. Infatti è stata recentemente descritta una famiglia in cui cinque componenti hanno presentato un progressivo disturbo della deambulazione ad esordio

tardivo, nei quali è stata identificata una mutazione omoplasmica m.9176T > C (p.L217P) del DNA mitocondriale (mtDNA) che sintetizza per la ATPasi6 [22].

Inoltre, i casi sporadici rappresentano una evenienza abbastanza frequente nella pratica clinica e spesso è difficile stabilire se siano il risultato di una mutazione de novo oppure possano trovare spiegazione in una trasmissione autosomica dominante a bassa penetranza o in una autosomica recessiva non riconosciuta.

Attualmente, in accordo all'ultima classificazione, sono stai identificati almeno 70 loci differenti e mappati circa 50 geni *SPG* (**Sp**astic **g**ait) [23,24].

Nell'ambito delle forme ad ereditarietà mendeliana, quelle autosomiche dominanti (ADHSP) costituiscono nei paesi occidentali il 70-80% del totale [25], e sono pressoché esclusivamente pure. Le forme autosomiche recessive (ARHSP) globalmente meno frequenti, sono invece comuni nelle popolazioni ad elevato tasso di consanguineità, per la maggior parte sono complesse ed associate ad un esordio più precoce [26].

1.3 Le forme X-linked

Sul cromosoma X sono stati identificati cinque loci. SPG1 è una forma rara risultato della mutazione del gene *L1CAM* che codifica per la molecola di adesione delle cellule neurali coinvolta nella migrazione e differenziazione dei neuroni. Solo gli individui di sesso maschile portatori della mutazione sono clinicamente affetti, presentando un fenotipo complesso con ritardo mentale, afasia, andatura a forbice e pollici addotti (sindrome MASA). Altre rare manifestazioni fenotipiche sono: l'idrocefalo dovuto alla stenosi dell'acquedotto e l'agenesia del corpo calloso. Le forme pure, congenite di malattia sono eccezionali.

SPG2 è causata dalla mutazione del gene PLP1, che codifica per la proteina proteolipidica della mielina. SPG2 è una variante allelica della malattia di Pelizaeus-Merzbacher (PMD), una

leucodistrofia ipomielinizzante. La PMD è causata nella maggior parte dei casi da una duplicazione del gene, mentre le mutazioni puntiformi (missenso, nonsenso) sembrano essere responsabili per la sua forma allelica SPG2 [27]. SPG16, SPG22 e SPG34, i cui geni sono ancora sconosciuti, sono forme complicate da ritardo mentale.

1.4 Le forme Autosomiche Dominanti (ADHSP)

Attualmente sono noti meno di 20 loci AD e sono stati mappati 11 geni.

La più comune ADHSP è causata da mutazioni del gene *SPG4* (*SPAST*/SPG4) che codifica per la spastina, una proteina appartenente alla famiglia delle AAA-proteins (*ATPase associated with diverse cellular activities*). Lo spettro mutazionale di *SPAST* è ampio, includendo mutazioni missenso, nonsenso, delezioni e duplicazioni. Le mutazioni identificate finora danno conto del 40-50% di tutte le ADHSP [28,29]. Sono responsabili di una forma pura di paraparesi spastica di variabile severità, con una distribuzione bimodale dell'età d'esordio con i picchi attorno ai 9 e 30-35 anni. Nonostante il fenotipo essenzialmente pura, in circa la metà delle SPG4 HSP presenta alterazioni della propriocezione, disfunzioni sfinteriche, sintomi riconducibili ad una lieve compromissione del sistema nervoso periferico. Nel 10% circa dei casi è stato riscontrato tremore delle mani. Sono stati descritti occasionalmente anche fenotipi più complessi per la presenza di declino cognitivo, neuropatia periferica, TCC [30].

La seconda forma in ordine di frequenza tra le ADHSP è la SPG3A, dovuta a mutazioni del gene *ATL1*/SPG3A che codifica per l'atlastina1 e che dà conto di circa il 10% dei casi [31,32]. Le forme associate a mutazioni di questo gene sono per la maggior parte pure ed usualmente ad esordio precoce, prima dei 10 anni [33].

KIAA0196/SPG8 che codifica per la strumpellina, *SPG31* che codifica per la proteina mitocondriale REEP1 e *KIF5A/ SPG10* che sintetizza la kinesina5A, sono altri geni mutati in alcune famiglie con

forme pure e che hanno delle frequenze dell'8%, 5% e 3% rispettivamente [34]. I loci/geni ADHSP sono meno frequenti.

Talvolta sono presenti delle caratteristiche cliniche peculiari, come è il caso di *BSCL2/SPG17* che è associato ad una forma complessa di malattia, conosciuta anche come sindrome di Sylver, caratterizzata da una prominente amiotrofia distale degli arti superiori [35]. *RTN2*, il gene mutato in SPG12, codifica per la proteina reticulon2 che interagisce con la spastina ed è coinvolto nella formazione del reticolo endoplasmico [36]. I pazienti usualmente presentano una paraparesi in forma pura, rapidamente progressiva tale da richiedere la sedia a rotelle poco dopo l'esordio. SPG9, SPG29 e SPG38 sono implicate in forme complesse della malattia. Il locus SPG9 è stato identificato in due famiglie nelle quali gli affetti manifestavano cataratta, reflusso gastro-esofageo, amiotrofia ed anormalità scheletriche attorno ai 30 anni [37].

1.5 Le Forme Autosomiche Recessive (ARHSP)

La varietà delle caratteristiche cliniche associate alle ARHSP correla bene con la crescente lista dei loci e geni implicati in queste forme: sono stati clonati 33 geni e più della metà di questi negli ultimi due anni. SPG5A è dovuta a mutazioni del gene *CYP7B1* che codifica per l'enzima oxysterol 7-alpha-hydroxylase 1 [38] e dà conto di circa il 10% delle ARHSP [39], essendo così probabilmente la seconda forma più frequente. Il fenotipo SPG5 è generalmente puro. In un singolo paziente sono stati descritti lievi segni cerebellari ed alterazioni della sostanza bianca alla RM encefalo simili a quelle della sclerosi multipla [40]. Estensivi studi elettrofisiologici in tre famiglie hanno evidenziato velocità di conduzione nervose e quadro elettromiografico nella norma, mentre i potenziali evocati somato-sensoriali, motori e visivi sono risultati alterati. Di rilievo il fatto che l'atrofia ottica sia un evento non raro nella SPG5 [41]. L'accumulo del 27-idrossicolesterolo nel

sangue dei pazienti SPG5 potrebbe avere un impatto importante come biomarker nella pratica clinica.

SPG7 è una rara ARHSP dovuta a mutazioni del gene *SPG7* che codifica per la paraplegina, subunità di una AAA-protein localizzata nella membrana mitocondriale interna. SPG7 costituisce circa il 4-7% delle ARHSP [42], ma sono state individuate un vasto numero di polimorfismi (varianti missenso) con un ruolo ancora incerto, potrebbero infatti essere fattori di suscettibilità o mutazioni patogenetiche [42,43]. SPG7, inizialmente descritta come una forma pura della malattia, si manifesta sia unicamente con una progressiva paraparesi spastica sia variabilmente associata con atrofia cerebellare, atassia, atrofia ottica, neuropatia, deficit intellettivo [44]. Mutazioni di *SPG7* sono inoltre causa di circa il 10% delle forme sporadiche di malattia del motoneurone superiore senza coinvolgimento bulbare [45] e di atassia spastica con atrofia ottica [46].

KIAA1840/SPG11 codifica per la spatacsina ed è il gene più frequentemente mutato nelle ARHSP (25% dei casi) [21,47]. Il fenotipo è relativamente omogeneo. L'esordio è precoce, prima dei 25 anni, con paraparesi spastica e coinvolgimento cognitivo, spesso diagnosticato come ritardo mentale, che progredisce verso una severa disabilità nell'arco di 10-20 anni. Alcuni pazienti presentano inoltre un coinvolgimento degli arti superiori, disartria pseudo-bulbare, parkinsonismo, segni cerebellari ed atrofia muscolare. Gli studi di imaging mostrano l'assottigliamento del corpo calloso (TCC) con un grado variabile di atrofia corticale che peggiora con il tempo. Studi recenti hanno specificato che l'assottigliamento del corpo calloso non evolve con la progressione della malattia e la RM dell'encefalo rileva in circa il 60% dei casi delle iperintensità della sostanza bianca con un pattern specifico [47] anche nelle fasi precoci della malattia [48].

Gli studi con la PET hanno evidenziato un grado variabile di ipometabolismo del glucosio nelle regioni corticali e talamiche; l'elettroneurografia in genere mostra una neuropatia prevalentemente assonale motoria o sensori-motoria [49]. Un quadro clinico e radiologico molto simile è legato a

mutazioni del gene *ZFYVE26*/SPG15 che codifica per la spastizina, proteina che interagisce con la spatacsina. Si ritiene che la SPG15 dia conto del 5% delle ARHSP [50,51]. Il fenotipo SPG15 è stato inizialmente associato alla sindrome di Kjellin, una forma complessa di HSP ad esordio prima della terza decade con degenerazione maculare, segni cerebellari, coinvolgimento cognitivo ed assottigliamento del corpo calloso. I segni cerebellari e la degenerazione maculare non sono stati trovati in modo consistente negli studi successivi, per converso il fenotipo della sindrome si è ampliato per il riscontro di parkinsonismo e neuropatia assonale con atrofia muscolare distale. Collettivamente, SPG11 e SPG15 sono le due forme più frequentemente responsabili delle AR-HSP TCC, dando conto del 60% e 12% dei casi rispettivamente [47,51]. Meno frequentemente, TCC e compromissione delle funzioni cognitive sono riscontarti in pazienti con SPG21, SPG32, SPG7 e SPG4 [52]. Gli altri loci ARHSP sono meno comuni essendo stati riscontrati in poche famiglie con caratteristiche cliniche ancora più complesse [53].

1.6 I casi sporadici

L'identificazione di una eventuale mutazione patogenetica nei casi sporadici di HSP rappresenta un rilevante obiettivo nella pratica clinica, non sempre raggiungibile. Nell'ambito della diagnostica differenziale devono essere prese in considerazione un elevato numero di patologie (come le leucodistrofie, la sclerosi multipla, neoplasie del sistema nervoso centrale (SNC), l'infezione da *human T-lymphotrophic virus*, la distonia L-Dopa responsiva, la sclerosi laterale primaria, l'atassia di Friedreich) che andrebbero escluse con gli studi di imaging (RM) e le analisi biochimiche [8]. Inoltre bisogna tenere presente che l'insorgenza di spasticità a carico degli arti inferiori in un paziente con storia familiare negativa, può aversi all'esordio della sclerosi laterale amiotrofica (SLA), della malattia di Machado-Joseph (SCA3), nella malattia di Alzheimer ad esordio precoce da mutazione della presenilina 1, nelle anormalità strutturali dell'encefalo e del midollo e nelle

malattie mitocondriali. Gli esami da eseguire nell'ambito di una appropriata diagnostica differenziale sono molteplici, in particolare nei bambini con un progressiva compromissione della deambulazione e spasticità [7]. Tra questi, vi sono anche le indagini molecolari per valutare la presenza di mutazioni nei geni HSP noti. In particolare, circa il 15-20% dei pazienti con presentazione sporadica di malattia sono portatori di una mutazione in *SPAST*, sebbene in questi casi il fenotipo sembri meno severo rispetto alle forme familiari [54]. I casi apparentemente sporadici di ADHSP possono esssere il risultato di una penetranza incompleta [54], mutazioni *de novo* o di una diagnosi non corretta in piccole famiglie. Nei casi a presentazione sporadica, anche in assenza di consanguinità parentale, è necessario testare i geni per le forme AR ritenute fenotipicamente più probabili. Non raro il riscontro di una eterozigosi composta in geni ARHSP relativamente frequenti (i.e. *CYP7B1*/SPG5, *SPG7*, *KIAA1840*/SPG11).

1.7 I meccanismi patogenetici

A seguito della identificazione di alcuni geni e dei loro prodotti, è emerso che le principali funzioni cellulari alterate nella HSP possono essere raggruppate in poche principali categorie. Ciò è abbastanza interessante se si prende in considerazione l' eterogeneità clinica e genetica. Le alterazioni molecolari nella HSP coinvolgono il traffico intracellulare, l'omeostasi ed il metabolismo lipidico e l'attività mitocondriale.

1.7.1 Il traffico intracellulare

La maggior parte dei prodotti dei geni HSP, a funzione nota o supposta, è rappresentata da proteine coinvolte nel traffico intracellulare e nel conseguente trasporto assonale. Il prodotto del gene *KIF5A*/SPG10 è la kinesina5A, una "motor protein" che controlla il trasporto assonale anterogrado lungo i microtubuli. La kinesina5A mutata è caratterizzata da una ridotta affinità per i microtubuli e determina una ridotta velocità di flusso durante il trasporto assonale [55]. La spastina, codificata da

SPAST/SPG4, appartiene alla famiglia delle AAA-proteins *(ATPase associated with diverse cellular activities*). E' costituita da tre domini strutturali principali: all'estremità N-terminale vi è il dominio deputato all'interazione con i microtubuli ed al traffico endosomale (MIT, *microtubule interacting and endosomal trafficking domain*), un secondo dominio centrale di interazione con i microtubuli ed all'estremità C-terminale un dominio ad attività ATPasica [56]. Nelle cellule di mammifero la spastina è localizzata in aree citoplasmatiche importanti per la dinamica dei microtubuli [57] e sembra implicata nel disassemblaggio di questi anche in modelli animali di malattia [12,58]. La sua attività di " rottura" dei microtubuli sembra essere in relazione, nelle colture neuronali, con la crescita assonale [59]. Studi in vitro hanno confermato che la *spastina* può inoltre raggruppare i microtubuli polimerizzati [60].

Topi knock-out per *SPAST* hanno presentato un rigonfiamento assonale in prossimità del cono di crescita dei motoneuroni spinali, con un accumulo abnorme di organelli e componenti del citoscheletro, suggerendo un difetto nel trasporto assonale [13]. L'evidenza di un ruolo della *spastina* nel turn-over dei microtubuli, implica che la proteina mutata possa danneggiare il trasporto assonale ed essere causa di crescita neuronale anomala e conseguente degenerazione. La spastina interagisce con un'altra proteina HSP relata: l'atlastina-1 sintetizzata dal gene *ATL1*/SPG3A [61,62], una proteina dinamina con domini transmembrana, espressa sopratutto a livello del SNC. La sua localizzazione subcellulare nel reticolo endoplasmatico (ER), nelle vescicole del cono di crescita assonale e nei punti di diramazione dell'assone, suggerisce un ruolo funzionale sia nel traffico intracellulare di membrana che nella crescita dell'assone. Più recentemente, è stato proposto che atlastina-1sia sopratutto coinvolta nella morfogenesi e rimodellamento del ER e dell'apparato del Golgi [63].

La proteina *NIPA1*, sintetizzata da *SPG6,* è una proteina transmembrana altamente espressa nei neuroni. In alcune cellule neuronali ed epiteliali appare localizzata nel compartimento endosomale

precoce e sulla plasma membrana, dove si pensa sia un trasportatore del magnesio [64]. E' stato dimostrato che *NIPA1* è un partner di atlastina-1 legandosi direttamente ad essa, è un inibitore del fattore di crescita multifunzionale BMP (*bone morphogenic protein signaling*), importante per la funzione distale dell'assone, ed inoltre interagisce con il suo recettore BMP II [65]. Spastin/SPG4 e spartin/*SPG20* sono altre proteine inibitrici di BMP [65].

Il gene *SPG20* sintetizza la spartina che analogamente alla spastina è una AAA protein con un dominio MIT. Studi controversi hanno dimostrato la sua localizzazione subcellulare nei mitocondri [66], come anche nel nucleo e citoplasma [67]. E' di rilievo il fatto che la *spartina* interagisca con la E3 ubiquitina-ligasi e la cardiolipina, fosfolipide mitocondriale maggiore, e sia coinvolta nella determinazione della dimensione delle vescicole lipidiche. Di conseguenza, nella SPG20 potrebbero essere compromessi il traffico endosomale ed l' uptake del calcio mitocondriale e di membrana [68].

La spatacsina sintetizzata dal gene *KIAA1840/*SPG11, è particolarmente espressa nei neuroni corticali e del midollo spinale [69] con una distribuzione citopasmatica. La spatacsina è stata rilevata a livello delle vescicole di trasporto citoplasmatiche, ER, superficie dei mitocondri e microtubuli ed interagisce con i componenti del complesso AP5 [70] (deputato a facilitare il traffico intracellulare arruolando altre proteine in specifiche vescicole di trasporto), ha inoltre verosimilmente un ruolo nell'autofagia. Uno studio ha dimostrato nei pazienti SPG11 l'accumulo di materiale membranaceo pleomorfo in assoni non mielinizzati del nervo surale, suggerendo un disturbo nel trasporto assonale [71].

La spastizina sintetizzata dal gene *ZFYVE26/*SPG15 è una "*zinc finger protein*" con un dominio FYVE che è noto legare il fosfatidil-inositolo 3-fosfato di membrana[50]. Ciò fa ipotizzare che abbia un ruolo nel traffico endosomale. La spastizina è tra i partners preferenziali della spatacsina.

1.7.2 La funzione mitocondriale

Alcune proteine HSP sono direttamente coinvolte nella funzione mitocondriale. La proteina paraplegina, prodotta da *SPG7,* appartiene alla famiglia delle metalloproteasi AAA mitocondriali [72].

I membri di questa famiglia di proteine formano un complesso proteolitico sulla membrana mitocondriale interna che controlla l'assemblaggio ed il *folding* dei componenti della catena respiratoria, la degradazione delle subunità disassemblate e l'attivazione delle altre proteine [73].

La perdita del complesso metalloproteasi AAA nei fibroblasti dei pazienti con HSP associata a mutazioni *SPG7* sembra causare una riduzione dell'attività del complesso I della catena respiratoria mitocondriale ed aumenta la sensibilità allo stress ossidativo [42,74]. La perdita dell'energia mitocondriale può, di conseguenza, danneggiare il trasporto assonale. Almeno nel modello murino di malattia [75], la perdita della paraplegina sembra disorganizzare il substrato di clivaggio piuttosto che influenzare l'attività mitocodriale delle AAA proteasi, probabilmente a causa della ridondanza dei membri della famiglia delle proteine AAA [76]. SPG13 è associata ad una mutazione missense del gene *HSPD1* che sintetizza per la proteina mitocondriale *HSP60.* Si ritiene che questa abbia una funzione di cheperone molecolare prevenendo il misfolding proteico e l'aggregazione di un subset di proteine localizzate nei mitocondri [77]. Una piccola proporzione di *HSP60* è localizzata inoltre nel citoplasma, dove è stato dimostrato legarsi al BAX, promuovendo di conseguenza l'apoptosi se mutata [78]. Il gene *REEP1*/SPG31codifica per la *receptor expression-enhancing protein (*REEP1*)* una proteina mitocondriale che contiene i domini TB2/DP1/HVA22 caratteristici delle *heat-shock proteins*, capaci di esercitare un'attività chaperone simile [79]. Inoltre, *REEP1* sembra importante nella dinamica di divisione dei mitocondri. Ci sono evidenza che *REEP1* interagisca con le

membrane tubulari e microtubuli del reticolo endoplasmico, partecipando così alla sua formazione, sembra inoltre implicata nella formazione delle vescicole lipidiche.

1.7.3 La crescita assonale e la mielinizzazione

L1CAM (SPG1) codifica per una glicoproteina transmembrana espressa sopratutto nei neuroni e nelle cellule di Schwann, con funzione di adesione cellulare critica per la migrazione e differenziazione dei neuroni. La sua alterazione nei topi knockout è causa di alterazioni nello sviluppo del SNC. molto simili a quelle riscontrate nei pazienti, che sono verosimilmente il risultato di una aberrante crescita degli assoni [80]. Il gene *PLP1* sintetizza la proteina proteolipidica della mielina (PLP) che assieme alla sua più piccola isoforma, *DM20*, è la proteina più abbondante del SNC coinvolta nella formazione della mielina e nella maturazione degli oligodendrociti. Nei topi knockout il rivestimento mielinico è di normale spessore ma gli assoni sono rigonfi con seguente degenerazione disto-prossimale. All'esame autoptico del SNC dei pazienti con mutazione che annullino la funzione di *PLP1*, si è evidenziata una degenerazione assonale lunghezza dipendente [81]. L'effetto delle mutazioni di *PLP* e *DM20* potrebbe quindi essere un abnorme sviluppo del tratto cortico-spinale.

1.7.4 Il metabolismo lipidico

L a funzione fondamentale del reticolo endoplasmatico è la sintesi, il metabolismo e la distribuzione dei lipidi e degli steroli, attraverso dei meccanismi vescicolari e non. Un numero sempre maggiore di geni *SPG* appare coinvolto in diversi aspetti del metabolismo lipidico: *SPAST*/spastin nella formazione delle gocciole lipidiche ed *ATL1*/atlastin1 nelle loro dimensioni, *CYP7B1*/SPG5 nel metabolismo dell'oxisterolo (22-idrossicolesterolo), *FA2H*/SPG35 nel metabolismo degli acidi grassi, *BSCL2*/seipin nel deposito dei trigliceridi [82], *NTE*/SPG39 che codifica per l'enzima fosfolipasi B/lipofosfolipasi, coinvolto nell'idrolisi dei lipidi intrinseci di membrana con impatto

sulla composizione delle membrane neuronali [83]. Il coinvolgimento nel milieu degli acidi grassi è particolarmente rilevante per diversi geni *SPG* di recente scoperta. L' identificazione dei geni coinvolti nella SPG28, SPG49 e SPG54, *CYP2U1*, *DDHD1* e *DDHD2* rispettivamente, evidenzia come il metabolismo lipidico sia una via metabolica critica nella HSP. In particolare *CYP2U1* catalizza l'idrossilazione dell'acido arachidonico e dei relati acidi grassi a catena lunga, come l'acido eicosapentanoico e deicosapentanoico. Due noti metaboliti, gli acidi 19 e 20 idrossieicosatetranoici, sono dei mediatori locali della trasduzione del segnale [84]. Ci sono evidenze che altri enzimi coinvolti nel metabolismo degli acidi grassi e dei fosfolipidi siano implicati nella neudegenerazione [85]. Dato il ruolo fondamentale dei lipidi e degli steroli nella neuroprotezione e neurodegenerazione, è ipotizzabile che altri geni causativi della HSP appartengano a questa categoria.

1.8 Epidemiologia della HSP

Nonostante la HSP abbia una distribuzione mondiale, sono stati condotti pochi studi epidemiologici a riguardo e spesso in associazione con le Atassie Spino-Cerebellari (*Spino-Cerebellar Ataxia*, SCA) con le quali condividono la progressiva compromissione della deambulazione. I due gruppi di patologie presentano inoltre una certa sovrapposizione nella presentazione clinica, soprattutto nelle forme ad ereditarietà recessiva. Ciò unitamente alle storiche difficoltà nella diagnosi e classificazione di queste patologie hanno fatto si che i primi studi epidemiologici si avvalessero di criteri classificativi e di inclusione molto eterogenei [87,88]. Il punto di svolta è stato indicato da Anita Harding che nel 1983 [1] ha definito per la prima volta dei criteri di diagnosi e classificazione per la HSP e SCA basati sulle modalità di ereditarietà, consentendo studi epidemiologici molto più accurati e confrontabili [89-93]. Inoltre, lo sviluppo nelle ultime decadi di nuove tecniche di diagnosi genetica ha permesso, oltre che diagnosi più precise, un ulteriore sviluppo della

classificazione in base alle mutazioni patogenetiche e loci identificati. Gli studi epidemiologici degli ultimi anni, condotti in epoca "post-genetica" hanno potuto così indagare anche la prevalenza delle mutazioni più rappresentate sia nell'ambito delle AD-HSP che AR-HSP [94-97]. Tuttavia, nonostante i notevoli progressi c'è ancora molta incertezza riguardo la distribuzione globale e prevalenza della HSP: negli studi finora compiuti le stime di prevalenza variano da 1,27-12,1 su 100.000 [87,94]. Questa ampia variabilità è imputabile sia alle differenze metodologiche, di cui si è detto, che al background etnico delle popolazioni esaminate. Le stime di prevalenza per le AD-HSP variano da 0,5 a 5,5 su 100.000, con le stime più basse riportate in Tunisia e quelle più elevate nel Sud-Est della Norvegia [96,97]. In Portogallo la prevalenza delle AD-HSP è di 2,5 su 100.000, meno della metà che in Norvegia sebbene nei due studi i criteri diagnostici di inclusione e le fonti epidemiologiche siano simili. In tutti gli studi che si avvalgono dell'analisi genetica, a partire da quello di McMonagle et al. del 2002 che indaga la prevalenza delle AD-HSP in Irlanda [94], la forma più comune è la SPG4, con una frequenza relativa del 30-40% [97,98]. Anche SPG3A è una forma presente ma ad una frequenza inferiore, mentre SPG31 è ancora più rara [97,98]. Nei due studi in cui le famiglie vengono testate per mutazioni in questi tre geni, la percentuale dei soggetti senza una diagnosi genetica è del 45% [97] e 67% [98].

La prevalenza delle AR-HSP varia da 0,3 a 5,3 su 100.000, stime riportate da Brignolio et al. nella provincia di Torino nel 1986 e Boukhris et al. in Tunisia nel 2009, rispettivamente. L' elevata prevalenza riscontrata in Tunisia riflette verosimilmente l'alto grado di consanguineità tipico dei paesi del Nord Africa. Negli studi che si avvalgono dei test genetici, le forme più comuni sono in ordine di frequenza: SPG11, SPG15 e SPG5 [96-98]. Comunque, nella maggior parte dei soggetti con verosimile AR-HSP la mutazione patogenetica non viene identificata, variando tale percentuale dal 69% in Tunisia all'82% in Portogallo [96,98].

Le forme ad insorgenza sporadica hanno rappresentato un ulteriore motivo di variabilità delle stime di prevalenza, soprattutto negli studi eseguiti quando non erano ancora disponibili i test genetici. In alcuni di questi non viene contemplata tale evenienza e le forme a presentazione "isolata" vengono considerate come a trasmissione autosomica recessiva [87,91]. In altri studi variano la definizione e i criteri di inclusione delle forme sporadiche [92, 97], mentre nel recente studio di Coutinho et al. tali forme sono escluse [98].

2. Obiettivi dello studio

Le notevoli acquisizioni compiute negli ultimi anni nel campo della genetica della HSP, grazie alla introduzione delle tecnologie di *next-generation sequencing*, hanno consentito l'identificazione di un elevato numero di geni malattia e la definizione clinica dei corrispondenti fenotipi. Ciò ha apportato indiscutibili vantaggi nella diagnosi sia in fase sintomatica che pre-sintomatica. La conseguente e progressiva scoperta dei meccanismi patogenetici che sottendono le varie forme di malattia rappresenta il presupposto fondamentale per l'identificazione di appropriate comuni terapie risolutive. In questa prospettiva, assume rilievo l'approfondimento delle tematiche legate alla variabilità fenotipica intra-familiare ed ai fattori genetici e/o epigenetici che determinano l'espressività e la penetranza delle mutazioni.

Obiettivi del presente studio sono stati:

I. ottenere una stima di prevalenza di una malattia monogenica rara come la HSP nella popolazione della Sardegna. Questa, a motivo della sua insularità, presenta una popolazione con un profilo demografico e genetico peculiare che dà conto, ad esempio, della elevata incidenza di malattie poligeniche complesse come la sclerosi multipla ed il diabete mellito giovanile [99-101].

II. studiare la distribuzione delle forme AD, AR ed a presentazione sporadica

III. studiare la correlazione tra il genotipo ed il fenotipo

IV. avere altri spunti di ricerca nella prospettiva della "*proximity to cure*"

3. Materiali e Metodi

3.1 Disegno di studio (3.1- 3.7, Pubblicazione I)

Abbiamo condotto uno studio di prevalenza "*population based*". L'accertamento dei casi ha coperto un periodo di 11 anni (dal 1° Gennaio 2000 al 31 Dicembre 2010). Per agevolare la partecipazione allo studio, per la maggior parte i pazienti sono stati valutati in loco con la collaborazione dei Medici di Base di riferimento.

3.2 Area e popolazione in studio

L'area in studio è stata la provincia di Sassari, Sardegna nord-occidentale, che ha una superficie di 4.282 Km^2 (17,8% dell'intero territorio dell'isola) ed ha una popolazione di 333.576 persone (163.104 uomini e 170.472 donne; 20% di tutti gli abitanti della Sardegna) [102]. L'area in studio comprende 66 comuni, 63 con meno di 20.000 abitanti e tre con un numero maggiore. La provincia di Sassari fa riferimento all'Azienda Sanitaria Locale n.1, il cui territorio è suddiviso in tre distretti: Sassari (27 comuni), Alghero (23) ed Ozieri (16). L'area include inoltre la Clinica Neurologica di Sassari, afferente all' Università.

3.3 Definizione del caso prevalente

I pazienti sono stati valutati utilizzando i criteri clinici per la HSP universalmente accettati [97, 103-104]. I pazienti con una storia familiare positiva sono stati classificati come "definitivamente affetti" in presenza di una progressiva compromissione della deambulazione, segni clinici di coinvolgimento del tratto cortico-spinale con una marcata iperreflessia, risposte plantari in estensione, ed avendo escluso cause alternative di paraparesi spastica. Sono stati invece classificati come "probabilmente affetti" i soggetti asintomatici, senza una progressiva compromissione della deambulazione, ma con chiari segni di paraparesi spastica all'esame obiettivo neurologico. Questi

due gruppi di pazienti sono stati inclusi nello studio. Invece, i soggetti con storia familiare positiva, asintomatici e con segni clinici non chiari di coinvolgimento del tratto cortico-spinale, sono stati classificati come "possibilmente affetti" ed esclusi dal computo della prevalenza [105]. I soggetti con storia familiare non chiara, ma nei quali erano inequivocabilmente presenti segni clinici di malattia e per i quali era stata esclusa ogni diagnosi alternativa, sono stati classificati come casi a presentazione sporadica di malattia o con possibile ereditarietà AR e sono stati inclusi nello studio. Tutti i soggetti che hanno soddisfatto i precedenti criteri diagnostici e residenti nell'area in studio al 31 Dicembre del 2010, sono stati considerati casi prevalenti. In questi, previo consenso informato, è stato effettuato un prelievo di sangue venoso per l'analisi genetica.

3.4 Raccolta dei casi

Con la finalità di identificare il maggior numero di affetti nell'area in studio, abbiamo adottato un modello semplice a fonti multiple. L'acquisizione dei casi si è avvalsa delle seguenti fonti:

a) Le cartelle cliniche dei pazienti con diagnosi di HSP, paraparesi spastica non definita, tetraplegia spastica non definita, sindrome cerebello-piramidale, atassia, raccolte dai centri neurologici attivi nell'area in studio, come la Clinica Neurologica dell'Università di Sassari e la Divisione di Neurologia di Ozieri. Tutte le cartelle cliniche sono state esaminate e i casi sospetti sono stati sottoposti ad ulteriore valutazione clinica e test genetico specifico.

b) Sono stati posti al corrente della ricerca in corso ed invitati a partecipare con convegni e lettere informative, i neurologi, i neuropsichiatri infantili, gli urologi ed i medici di base dell'area in studio.

c) E' stata coinvolta la sezione sarda dell' Associazione italiana dei pazienti con HSP (*Vivere la Paraparesi Spastica Onlus,* VIPS, www.vipsonlus.it), costituitasi nel 2009, rivolgendo ai propri associati l'invito a partecipare.

3.5 Validazione dei casi

Ogni soggetto che ha partecipato allo studio è stato valutato obiettivamente ed i dati clinici sono stati raccolti in accordo al protocollo SPATAX, sviluppato dal network europeo e mediterraneo per lo studio delle malattie spino-cerebellari [106]. Per ciascun caso indice è stato ricostruito il pedigree da tre fino a dieci generazioni. E' stata supposta una ereditarietà di tipo AD in caso di trasmissione genitori-figli, sebbene quella padre-figli non fosse sempre presente. E' stata invece ipotizzata una ereditarietà di tipo AR, quando erano affetti due o più fratelli con genitori sani o quando era nota una consanguineità parentale. In alcuni casi a presentazione sporadica, il test genetico ha svelato una ereditarietà AR. Quando possibile i partecipanti allo studio hanno eseguito una RM dell'encefalo e del midollo spinale, come anche lo studio elettrofisiologico ed urodinamico. La severità di malattia è stata quantificata utilizzando la scala SPRS [107] e sulla base del punteggio totale, i pazienti sono stati suddivisi in cinque classi di disabilità: classe 0= score 0, classe 1= score 1-13, classe 2 = score 14-26, classe 3 = score 27-40, class 4= score 41-52. In accordo con gli studi precedenti, è stata esclusivamente valutata la disabilità motoria assegnando i pazienti a cinque classi funzionali nel seguente modo: 0= pazienti asintomatici, 1= con lievi sintomi e segni all'esame neurologico ma capaci di camminare senza sostegno e di correre, 2=capaci di camminare senza sostegno ma non di correre, 3= capaci di camminare con sostegno, 4= costretti alla sedia a rotelle [97].

3.6 Genetica

Nei partecipanti che hanno fornito il loro consenso, è stato eseguito un prelievo di sangue venoso ed stato purificato il DNA genomico in accordo alle metodiche standardizzate. Gli esoni di tutti i geni più frequentemente implicati nella HSP (ossia, *PLP*/SPG2, *ATL1*/SPG3A, *SPAST*/SPG4,

CYP7B1/SPG5, *NIPA1*/SPG6, *SPG7*, *KIAA0196*/SPG8, *KIF5A*/SPG10, *SPG11*, *RTN2*/SPG12, *HSPD1*/SPG13, *ZFYVE26*/SPG15 e *BSCL2*/SPG17) sono stati esaminati con sequenziamento diretto per la ricerca di mutazioni puntiformi e con l'analisi MLPA per la ricerca di delezioni/duplicazioni multiesoniche, in un unico centro di riferimento (IRCSS Fondazione Stella Maris, Università di Pisa). Tutti i pazienti a presentazione sporadica avevano già eseguito altrove nel corso del loro iter diagnostico, la ricerca per l'espansione della tripletta GAA nel gene *FXN* nel sospetto di una Atassia di Freidreich e per l'espansione della tripletta CAG nei geni *ATX1*/SCA1 and *ATX3*/SCA3 per le rispettive Atassie Spino-Cerebellari. Quando possibile, l'identificazione delle mutazioni è stata validata dall'uso di una seconda metodica, come la segregazione in ambito intra-familiare o la predizione della patogenicità *in silico*.

3.7 Statistica

L'analisi descrittiva ha incluso il calcolo delle medie, la deviazione standard (SD),), *t-test* per i campioni indipendenti e l'analisi dalle varianza per le variabili continue (come l'età all'esordio, la durata della malattia, l'età al giorno di prevalenza). Proporzioni e percentuali sono state utilizzate per descrivere le variabili categoriche (trasmissione ereditaria, mutazioni genetiche e disabilità motoria). E' stata calcolata per tutte le forme di HSP, ed in particolare per quelle AD, la prevalenza cruda sesso ed età specifica al giorno di prevalenza (31 Dicembre 2010), utilizzando come denominatore il censo della popolazione italiana al 2010 [108]. Il livello di significatività è stato fissato a $p<0,05$, test a due code. Il software SPSS (*Statistical Package for the Social Sciences*), versione 19.0 (SPSS Inc., Chicago, IL, USA) sarà utilizzato per l'analisi statistica.

4. Riassunto dei risultati

4.1 Stima di prevalenza e distribuzione delle forme (Pubblicazione I)

Dall'esame delle cartelle cliniche sono state raccolte settantacinque storie di pazienti con paraparesi spastica, tetraplegia spastica, sindrome cerebello-piramidale o atassia spastica. Dallo studio dei dati clinici, è emerso che 35 (46,7%) erano sclerosi multiple primariamente progressive, atassie ereditarie, degenerazione cerebellare di natura tossica o paraplegia/tetraplegia secondaria a lesioni dell'encefalo o del midollo spinale. Nei rimanenti 40 casi la diagnosi di HSP è stata confermata in 15, che sono stati inclusi nello studio. Nove ulteriori casi indice sono stati segnalati dai colleghi o dai membri della VIPS. A partire dai 24 casi indice, sono stati clinicamente valutati 195 familiari con l'evidenza di altri 70 individui che rientravano nei criteri per la HSP. Dei 94 pazienti individuati, cinque sono deceduti prima del giorno di prevalenza, 21 risiedevano al di fuori dell'area in studio e uno si è rifiutato di partecipare. Di conseguenza, 67 pazienti (40 uomini e 27 donne), tutti originari della Sardegna, erano eleggibili per lo studio di prevalenza. Cinquantanove fra i casi prevalenti (88,1%) appartenevano ad undici famiglie con trasmissione AD; la ricerca genealogica, l'anamnesi dei componenti più anziani ed i test genetici specifici hanno evidenziato che otto di queste famiglie erano fra di loro imparentate. Tre casi prevalenti (4,5%) appartenevano a due famiglie non imparentate con trasmissione AR, e cinque casi (7,5%) erano a presentazione sporadica. Consanguineità parentale era presente in un'unica famiglia *SPG7*. La prevalenza cruda totale è stata di 19,9/100.000 (95% CI: 18,4-21,4), 24,4 (95% CI: 23,3-25,4) negli uomini, e 15,7 (95% CI: 14,5-16,9) nelle donne, con un rapporto M:F di 1,55. La prevalenza standardizzata per il censo della popolazione italiana del 2010 è stata di 19,4/100.000 per entrambi i sessi, 23,9 per gli uomini e 15,1 per le donne. La prevalenza della HSP ha mostrato una distribuzione bimodale per età, con i più alti valori registrati tra i 30-39 anni (32,4/100.000) ed i 60-69 anni (32,9/100,000).

Negli uomini, la prevalenza più elevata è stata riscontrata tra i 70-79 anni (38,9/100.000), nelle donne tra i 30-39 anni (34,7/100.000). La prevalenza cruda per AD-HSP (N=59) è stata 17,5/100.000 (95%CI: 16,1-18,9) e 13,9 (95%CI: 12,8-15,0) per gli uomini e le donne rispettivamente. I casi prevalenti hanno presentato un'età media (SD) di 48,4 anni (15,8) (range 8-75), senza significative differenze tra i sessi. L'età media per le forme AD è stata di 47,6 anni (15,9) (range 8-75),è stata di 45,0 (9,8) per le forme AR (N=3) e di 59,0 anni (14,0) nelle forme a presentazione sporadica (N=5). Tutti i casi presentavano un'età media all'esordio clinico di 36,6 anni (13,6) senza una differrenza tra i sessi. Nelle forme AD, l'età media all'esordio era di 37,3 (13,3) anni, versus 26,0 anni (12,7) per le forme AR e 34,8 (17,2) in quelle sporadiche. La durata media di malattia è stata di 15,1 anni (12,4) in tutti i pazienti, senza differenze tra i sessi. La durata è stata di 14,4 anni (12,3) nelle forme AD, 16,0 anni (2,3) in quelle AR, e 21,2 anni (8,8) in quelle sporadiche. La durata media di malattia si è rivelata più lunga nei pazienti con esordio prima dei 35 anni rispetto a quelli con esordio successivo: 19,8 anni (15,7) versus 12,2 anni (7,6) (p=0,0048), come atteso in una malattia cronica che non compromette significativamente l'aspettativa di vita. Riguardo la distribuzione dei casi HSP prevalenti in base alle classe SPRS, il 20,9% dei casi apparteneva alla classe 0, il 38,8% dei casi alla classe 1, il 19,4% alla classe 2, il 14,9% alla classe 3 e lo 4,5% dei casi alla classe 4. Non è stato possibile assegnare un paziente ad alcuna classe. In base unicamente alla disabilità motoria (*functional score*), il 23,9% dei casi prevalenti era asintomatico, il 28,4% mostrava solo lievi sintomi e segni all'esame neurologico, 11,9% poteva camminare indipendentemente ma non era capace di correre, il 22,4% poteva camminare unicamente con sostegno ed il 13,4% era costretto alla sedia a rotelle. La severità espressa con le classi SPRS (da 0 a 4) aumentava con la durata di malattia (coefficiente chi-square di Pearson = 43.690, p<0.0001), così come la disabilità espressa con il *functional score* (da 0 a 4). Il *functional score* medio, corretto per l'età d'esordio, è stato di 1,05 (95% CI: 0,67-1,43) a 10 anni dall'esordio

di malattia, 2,26 (95% CI: 1,90-2,63) a 20 anni, e 3,14 (95% CI: 2,71-3,57) a 30 anni (ANOVA, $p<0.0001$). La diagnosi molecolare confermatoria è stata ottenuta in 55 dei 67 casi prevalenti (82,1%): 52 (77,6%) AD-HSP hanno presentato una forma pura di malattia, mentre tre (4,5%) una forma complessa in relazione ad una trasmissione AR. La maggior parte dei pazienti con AD-HSP hanno presentato mutazioni in *SPAST,* con una delezione multiesonica nel 76,1% dei casi ed una più piccola delezione nell'1,5%. Un solo paziente si è rivelato eterozigote composto in *SPG11,* mentre due pazienti hanno mostrato una mutazione omozigote in *SPG7.* Non è stato possibile ottenere una diagnosi molecolare in 12 casi prevalenti (17,9%), di cui 7 (58,3%) appartenevano a due famiglie con forma pura AD e 5 (41,7%) erano *bona fide* sporadici. Di questi ultimi, 4 presentavo una forma complessa.

4.2 Spot di malattia da effetto fondatore (Pubblicazione II)

In alcune aree della nostra isola l'isolamento geografico e la relativa stabilità delle popolazioni residenti dedite alla pastorizia ed agricoltura, ha favorito lo sviluppo di un vasto pedigree SPG4 i cui "*founder*" sono originari dell'Anglona.

L'albero genealogico, si compone di nove generazioni e 107 appartenenti sono stati studiati clinicamente e geneticamente. Di questi, 67 (M/F=1,3:1) sono risultati portatori eterozigoti dell'ampia delezione di *SPAST* comprendente gli esoni 2-17, che, come visto, dà conto della maggior parte dei casi prevalenti. Cinquanta pazienti (30M e 20F, età media di 53,2±15,4 anni) hanno mostrato all'esame clinico una forma pura di paraparesi caratterizzata dal lento incremento del tono muscolare agli arti inferiori con una minore compromissione della forza. L'età meta all'esordio era di 39± 13 anni, range 2-60.

Diciassette pazienti (8M e 9F con età media 37,5±14,8 anni, range 18-73) erano asintomatici, sebbene 11(16%) presentassero minimi segni all'esame obiettivo come isolato segno di Babinski, riflessi propriocettivi vivaci e clono del piede

I 50 pazienti sintomatici lamentavano soprattutto crampi ed un eccessivo senso di fatica durante la deambulazione. In 31 di questi le prime manifestazioni di malattia erano state rigidità agli inferiori con impaccio nella deambulazione, tre hanno invece riferito crampi e due urgenza urinaria. I rimanenti 14 pazienti non sono stati in grado di descrivere i sintomi d'esordio. L'impairment motorio era complessivamente moderato: 95% dei pazienti era in grado di deambulare autonomamente, 19% dei quali con supporto, e solo tre pazienti erano costretti alla sedia a rotelle all'età media di 61,3 ±9,3 anni (range 55-72) dopo 29,7± 15 anni (range13-42) di durata di malattia. Cinquantaquattro dei 67 pazienti hanno presentato riflessi propriocettivi patologicamente incrementati agli arti inferiori, sei vivacità dei riflessi anche agli arti superiori. Tre pazienti hanno presentato alterazioni della sensibilità profonda (ipo/apallestesia distale).Il Test del *Mini Mental State Examination* ha evidenziato un lieve decadimento cognitivo in 4 pazienti (età media 65,2±3,3 anni) con limitazione sopratutto della funzione mnesica. Le alterazioni sfinteriche non sono state spontaneamente riferite dai pazienti, ma dopo specifica anamnesi 28 hanno descritto sintomi urinari, come urgenza, incontinenza, difficoltà nella minzione. Due pazienti all'esame della motilità oculare hanno presentato nistagmo evocato dallo sguardo ed esotropia alternante.

Venti pazienti hanno eseguito la RMencefalo e 24 anche quella del midollo, senza evidenza di alterazioni morfologiche peculiari. In due dei sette pazienti che hanno eseguito lo studio delle velocità di conduzione motoria, è emersa una lieve polineuropatia sensori-motoria. La durata di malattia ha mostrato una correlazione diretta con lo score della SPRS ($r=0,83$, $p<0,001$), il *Motor Severity Score* (MSS: $r=0,81$, $p<0,001$) e con la *Ashworth Scale of Muscle Spasticity* (ASM: $r=0,70$, $p<0,001$). E' apparsa invece inversamente correlata con la *Medical Research Council*

Scale for Muscle Strength (MRC: r= -0,57, p<0,001) e con il *Barthel Index* (BI: r= -0,57, p<0,001).

Non sono emerse differenze statisticamente significative tra pazienti di sesso maschile e femminile, sebbene negli uomini sia stata riscontrata più frequentemente la spasticità agli arti inferiori. L'analisi statistica di Kaplan-Meier non ha mostrato differenze di outcome in relazione al sesso. L'analisi molecolare non ha evidenziato la presenza di varianti intrageniche o geni modificatori del fenotipo della HSP (p.G563A in *HSPD1*/SPG13) [109]. E' invece emerso il coinvolgimento del gene *SLC30A6*, che segue *SPG4/SPAST* lungo il cromosoma 2, e che risulta parzialmente deleto. Tale gene codifica per la proteina Znt6, appartenente alla famiglia delle proteine trasportatrici dello zinco [110].

4.3 Il ruolo dei geni fiancheggianti *SPG4* (Pubblicazione III)

Lo studio clinico-genetico di un'altra famiglia SPG4 della parte meridionale della Sardegna, ha evidenziato una più piccola delezione a carico degli esoni 1-4 di SPG4/*SPAST* che determina una riduzione nell'espressione del gene *DPY30* che lo precede, essendo localizzato a monte della 5'-UTR. Lo spettro fenotipico dei 5 affetti è sempre quello di una forma lieve-moderata di malattia a lenta progressione. Questo riscontro, unitamente al precedente, incoraggia ulteriori indagini circa il ruolo dei geni fiancheggianti SPG4/*SPAST* nella modificazione del fenotipo.

4.4 Infantile Ascending Hereditary Spastic Paraplegia (IAHSP), una nuova mutazione (Pubblicazione IV).

4.4.1 *ASL2*

Le mutazioni in *ASL2*, localizzato sul braccio lungo del cromosoma 2, codificante per l'alsina sono responsabili di uno spettro di rare patologie autosomiche recessive che vanno dalla paraparesi spastica ascendente infantile (IAHSP, MIM 607225) alla sclerosi laterale primaria giovanile (JPLS,

MIM 606353) con una degenerazione retrograda del motoneurone superiore, ed alla sclerosi laterale amiotrofica giovanile (MIM 205100), in cui si ha un coinvolgimento sia del motoneurone superiore che di quello inferiore [111-113]. Sebbene queste sindromi siano state inizialmente descritte in poche famiglie dell'area mediterranea, sono state identificate 14 famiglie con distribuzione mondiale. Abbiamo descritto la quindicesima famiglia, la prima in Sardegna, portatrice di una nuova mutazione.

4.4.2 Il caso clinico

Abbiamo studiato un giovane di 17 anni nato a termine dopo una gravidanza nella norma, da genitori sani ed apparentemente non consanguinei. Il paziente ha acquisito la posizione seduta all'età di sei mesi, ed ha pronunciato le prime parole a nove mesi. A 12 mesi, ha manifestato rigidità agli arti inferiori ed equinismo dei piedi. All' età di due anni, la deambulazione era possibile solo sulle punte con sostegno sottoascellare. A questa età l'obiettività neurologica evidenziava ipertono spastico delle estremità inferiori, incremento dei riflessi propriocettivi, clono delle caviglie e riflesso plantar-cutaneo in estensione bilaterale. Il controllo del capo e del tronco era buono e mostrava inoltre una buona coordinazione e manipolazione degli oggetti. Le sue capacità linguistiche erano adeguate per età ed era sintonico con l'ambiente circostante. Con la progressione della malattia, all'età di otto anni ormai costretto alla sedia a rotelle, il paziente ha presentato debolezza e spasticità degli arti superiori, lieve disfagia e disartria. Nell'anno seguente ha perduto la capacità di stare seduto senza supporto ed ha iniziato a manifestare una scoliosi. Due anni dopo ha presentato disartria e difficoltà nella masticazione. A questa età, anche la scrittura era diventata impossibile ma poichè le sue facoltà intellettive erano adeguate ha continuato a frequentare le scuole medie e superiori con l'aiuto di insegnanti di sostegno e di un comunicatore. All'età di 17 anni, l'obiettività neurologica ha mostrato un giovane collaborante e ben orientato. I movimenti

oculari erano pienamente conservati e non vi era evidenza di nistagmo, mentre la ridotta mobilità dei muscoli del volto conferiva una facies tipica con "sorriso forzato". Il paziente mostrava una ridotta mobilità della lingua in tutte le direzioni, ma non erano presenti fascicolazioni o amiotrofia. Un esame videofluoroscopico ha confermato la disfagia per i liquidi. Agli arti superiori era evidente un ipertono spastico con residua conservazione della motilità globale e segmentaria, mentre gli arti inferiori erano plegici. Non erano evidenti amiotrofia, fascicolazioni, deficit di natura sensitiva e non sono state riferite alterazioni degli sfinteri. Il paziente presentava retrazioni tendinee a livello delle articolazioni delle ginocchia e caviglie. Lo studio elettromiografico ha mostrato una marcata riduzione del reclutamento volontario, in particolare agli arti inferiori, ma senza segni di denervazione. Le velocità di conduzione motorie e sensitive erano nella norma. I potenziali evocati somatosensoriali hanno mostrato, solo a livello degli arti inferiori, un incremento della latenza della componente corticale P37, bilateralmente. La RM dell' encefalo ha presentato delle iperintensità nelle aree periventricolari posteriori nelle immagini T2- pesate e FLAIR, già presenti all'età di dieci anni ma di cui non è stato possibile valutare la progressione per mancanza delle immagini precedenti. Il paziente presentava inoltre una severa scoliosi. L'RX della colonna e la RM del midollo ha mostrato una severe rotoscoliosi dorsale e lombare, destro convessa nel tratto dorsale, ma senza compressione o assottigliamento del midollo. Il sequenziamento diretto degli esoni di *ALS2* su DNA genomico ha rivelato una mutazione omozigote c.3836+1G>T a livello del sito donatore di *splicing* dell'introne 24. La mutazione era nuova, presente in eterozigosi nei genitori e non riscontrata in 500 cromosomi di controllo. La nuova mutazione c.3836+1G>T era capace, con analisi di predizione *in silico*, di causare uno *skipping* degli esoni 22 e 23. La proteina alsina è costituita da tre domini " *guanine exchange factor* (GEF)", che regolano l'attività dei membri delle proteine RAS, appartenenti alla superfamiglia delle GTPasi: un dominio N-terminale regolatore della condensazione della cromatina (RCC1), un dominio omologo della plecstrina al centro della

proteina, otto consecutivi motivi MORN (Membrane Occupation and Recognition Nexus) ed il dominio VPS9 (Vacuolar Protein Sorting 9) all'estremità C-terminale [114]. La maggior parte delle mutazioni è predittiva per una proteina prematuramente troncata e per l'assenza del dominio VPS9, che agisce specificamente come GEF per le RAB5 GTPasi, proteine regolatrici della endocitosi durante la fusione e i processi di scambio degli endosomi [114]. Nel nostro paziente entrambi i trascritti rilevati a livello dei fibroblasti cutanei sono predittivi di una proteina prematuramente tronca a livello dell'estremità C-terminale. Come risultato, la nuova mutazione potrebbe perturbare il "*trafficking*" intracellulare con un meccanismo simile a quello di diverse altre proteine coinvolte nelle malattie del motoneurone [115], sebbene non si possa escludere l'alterazione di altre probabili funzioni dell'alsina, come la crescita del neurite e la neuroprotezione [116].

4.4.3 La revisione della letteratura

Risale al 1995 la prima descrizione di una famiglia con IAHSP. Si trattava di tre fratelli appartenenti ad una famiglia del Kuwait che hanno sviluppato una progressiva paresi con seguente progressione della compromissione motoria agli arti superiori ed alla muscolatura ad innervazione bulbare [117]. Subito dopo è seguita la descrizione di tre fratelli della Giordania con un quadro clinico dalla medesima evoluzione ed in associazione una paresi di sguardo [118]. In questo articolo venne usato per la prima volta nella letteratura neurologica il termine di "sclerosi laterale primaria infantile". Con la identificazione di ulteriori mutazioni patogenetiche in *ASL2*, sono state identificate ulteriori 13 famiglie con questo fenotipo, ed i termini di IAHSP e JPLS sono entrati nell'uso comune [111-113, 117-128]. Attualmente, mutazioni in *ASL2* sono state identificate in 27 pazienti, 20 da 12 famiglie con diagnosi di IAHSP e sette pazienti con diagnosi di JPLS basata sulla precoce comparsa dei sintomi bulbari [128]. Da un punto di vista clinico, la malattia, caratterizzata da un esordio infantile di una paraparesi spastica pura seguita da una paresi rapidamente ascendente

agli arti superiori ed alla muscolatura oro-faringea, appare abbastanza omogenea. Si manifesta nell'arco della prima decade di vita (tempo medio di progressione 5,96 ± 3,23 anni) e determina una severa una severa tetraparesi con sindrome bulbare nella seconda decade di vita (tempo medio di progressione 12,8 ± 12,2 anni), con conservazione delle capacità intellettive [114-127]. Nelle famiglie in cui è stata riportata una mutazione in *ASL2*, inclusa la nostra, l'età media d'esordio della disabilità motoria è stata 19,7 ± 5,7 mesi (range 12-24) nei casi con diagnosi di JPLS e di 13,3 ± 4,35 mesi (3-24) nei casi di IAHSP. L 'età media d'esordio di compromissione della muscolatura bulbare è stata di 5.14 ± 2.9 anni (2-10) nelle famiglie con JPLS, e di 6.53 ± 4.8 anni (3-16) in quelle con IAHSP. I pazienti erano confinati sulla sedia a rotelle all'età media di 17,4 ±19,3 anni (range 2-50) nella JPLS ed 8,4 ±3,2 anni (4-12) nella IAHSP. Tutti i pazienti con diagnosi di JPLS e solo tre con IAHSP (11% del totale), hanno presentato anomalie dell'oculomozione estrinseca, variabili da un rallentamento dei movimenti volontari alla paresi di sguardo. Si ritiene che le alterazioni dell'oculomozione estrinseca nella JPLS siano determinate da un coinvolgimento dei motoneuroni della corteccia frontale (cellule di Betz dell'area 8) che controllano la motilità oculare [118]. Il nostro paziente ha presentato alla RM encefalo delle iperintensità a livello delle aree periventricolari posteriori, che sono un comune riscontro nella IAHSP [119,126]. Ulteriori caratteristiche neuroradiologiche erano la presenza di atrofia corticale sopratutto a livello delle aree motorie, lieve atrofia cerebellare, iperintensità bilaterali a livello del braccio posteriore della capsula interna e del tronco [117,119,123]. Gli studi neurofisiologici condotti nei pazienti con IAHSP hanno rivelato un marcato coinvolgimento delle vie corticospinali e corticobulbari; i Potenziali Evocati Somatosensoriali erano normali o di latenza leggermente aumentata; l'elettromiografia ha mostrato una marcata riduzione del reclutamento volontario, come nel nostro paziente [118-120, 122, 125, 127]. La scoliosi non è un riscontro frequente nelle mutazioni di *ASL2*. E' stata descritta nel 15% dei pazienti con IAHSP ma in nessuno dei pazienti con JPLS. Alterazioni sfinteriche sono state

riportate in soli tre pazienti con IAHSP (11,1%). Di conseguenza le differenze principali fra la JPLS e la IAHSP, sono in relazione ad un precoce coinvolgimento motorio con rapido decorso e la possibile associazione di scoliosi nella IAHSP, e la presenza di una alterata oculomozione estrinseca nella JPLS. Tuttavia, poiché sia la IAHSP e la JPLS sono patologie dello stesso spettro clinico e poiché non vi sono, attualmente, correlazioni con diverse mutazioni in *ASL2,* i tentativi di stabilire delle differenze nosografiche non appaiono rilevanti. In conclusione, l' identificazione di una nuova mutazione di *ASL2* espande ulteriormente la eterogeneità genetica. Il nostro paziente ha presentato un decorso clinico tipico. Con la progressione della malattia ha sviluppato una severa scoliosi, ponendolo a rischio di insufficienza respiratoria e crolli vertebrali potenzialmente mortali. Data la presentazione clinica abbastanza omogenea della malattia, è auspicabile una pronta definizione diagnostica clinico-molecolare per la messa in atto di adeguati provvedimenti fisioterapici ed ortopedici.

5. Discussione

Tra Gennaio 2000 e Dicembre 2010, nella Sardegna nord-occidentale, sono stati riscontrati 67 soggetti affetti da HSP, determinando una prevalenza cruda di 19,9/100.000, che è la più elevata finora riscontrata nei paesi Occidentali. Gli studi epidemiologici condotti in diversi paesi e per la maggior parte in era " pre-genetica" hanno ottenuto stime eterogenee di prevalenza di malattia. Tali differenze, come detto, sono imputabili sia a differenze di ordine metodologico, come la numerosità della popolazione in studio, le fonti utilizzate, la classificazione della malattia ed i criteri di inclusione, che alle caratteristiche socio-demografiche delle popolazioni studiate. Nello studio di Skre condotto nella Norvegia occidentale si riporta una prevalenza di 12,1/100.000 [87]. L'autore fa riferimento ad un'unica fonte, utilizza criteri clinici che considerano la modalità di trasmissione e quelle peculiarità del fenotipo in base alle quali Harding formulerà successivamente la sua classificazione in forme pure e complesse [1]. Per il calcolo della prevalenza usa un metodo di

estrapolazione, correggendo per la probabilità di accertamento e duplicando la prevalenza cruda ottenuta. Anche nello studio di Polo *et al.* condotto in Cantabria, regione del nord della Spagna, si riscontra una prevalenza elevata di 9,6/100.000 [90]. La popolazione studiata ha dimensioni inferiori rispetto allo studio di Skre ma superiori alla nostra, i criteri clinici di inclusione si avvalgono della classificazione di Harding e della modalità di trasmissione escludendo però le forme a presentazione sporadica. La fonte è singola essendo rappresentata dall' unica struttura di valutazione neurologica (l' Ospedale Nazionale), ma gli autori a partire da 9 probandi esaminano un elevato numero di familiari con evidenza di 46 "casi secondari". L'accertamento intrafamiliare, aspetto particolarmente curato nel nostro studio, rappresenta un elemento fondamentale nella epidemiologia di una malattia geneticamente determinata e spesso a bassa espressività come la HSP.

Nel nostro studio la strategia di ricerca a fonti multiple ed i criteri di inclusione su base clinica e genetica sono comparabili agli ultimi [94,95,97], in particolare a quello di Erichsen condotto nel sudest della Norvegia, la dimensione della popolazione è tuttavia inferiore. A parte gli aspetti metodologici, tuttavia, l'alta prevalenza della HSP riscontrata in Sardegna può essere attribuita alla insularità e quindi all'isolamento geografico della sua popolazione, che ha favorito l'espansione nel tempo, di uno spot di malattia riconducibile a pochi soggetti fondatori originari dell'Anglona, sub-regione del nord dell'isola. Infatti, le forme AD contribuiscono per la maggior parte alla stima di prevalenza riscontrata. In comparazione con gli studi precedenti, il nostro è caratterizzato da una elevata proporzione di casi (82,1%) confermati geneticamente [94,95,97]. Sia la proporzione delle famiglie AD (88,1%) che la frequenza delle mutazioni *SPAST*/SPG4 (77,6%) sono più elevate nella popolazione sarda rispetto a quelle riscontrate negli studi precedenti [6, 129]. Al contrario, sono state riscontrate poche famiglie AR (4,5%, complessivamente), con mutazioni in *SPG7* ed *SPG11*, nonostante l'elevato tasso di consanguineità della popolazione sarda [130,131]. La prevalenza della

HSP nella nostra popolazione stratificata per età, ha una distribuzione bimodale registrando i valori più alti tra i 30-39 anni, range nel quale si ha per la maggior parte l'esordio della malattia, ed i 60-69 anni (negli uomini 70-79 anni). L'aumento della prevalenza con l'età è contemplato in una malattia che non compromette l'aspettativa di vita e che presenta un esordio variabile [97, 103]. D'altra parte, non riscontriamo alcun caso dopo gli 80 anni in linea con studi precedenti che riportano nelle età più estreme una riduzione della prevalenza [89,91, 93-95]. Questa condizione potrebbe anche conseguire all' occorrenza di complicanze in relazione alla malattia tali da ridurre l'aspettativa di vita. Al contrario, abbiamo osservato una bassa prevalenza tra i 0 ed i nove anni (7,1/100,000 negli uomini e 0/100,000 nelle donne), che verosimilmente riflette pochi casi congeniti o ad insorgenza infantile. Nella nostra popolazione, l'età d'esordio è più alta per le forme AD (37 anni) che per quelle recessive (26 anni) e per i casi ad insorgenza sporadica (34 anni). L'età media all'esordio per tutte le forme è 36,6 anni, più alta rispetto a quanto riportato in studi precedenti [97,98], forse anche in relazione alla peculiarità delle mutazioni patogenetiche. Nella nostra popolazione abbiamo infatti prevalentemente riscontrato delezioni di *SPG4* con il coinvolgimento dei geni localizzati a monte ed a valle della 5'-UTR e della 3'-UTR, rispettivamente. Questo riscontro può parzialmente spiegare l'elevata prevalenza di HSP nella popolazione sarda, poichè la malattia non interferisce in maniera significativa con la fitness riproduttiva. Con il 37.3% dei pazienti con uno score SPRS tra 1 e 13 e quindi a bassa severità globale, con il 28.4% dei pazienti con una disabilità motoria limitata a sintomi lievi e solo il 13.4% costretti alla sedia a rotelle, il decorso clinico della malattia appare relativamente benigno. Nella nostra popolazione il 23.9% dei pazienti sono asintomatici, tutti AD-HSP da mutazione *SPAST* tranne uno AR-HSP da mutazione *SPG7,* di questi il 20.9% con score 0 alla SPRS. Poiché questa situazione rispecchia l'atteso [94,132], riteniamo di aver data una stima abbastanza accurata anche di coloro che ancora non hanno manifestato sintomi, che può essere un

elemento prospettivamente importante pur tenendo conto della variabilità nel decorso della malattia [133, 134].

6. Conclusioni

La prevalenza della HSP riportata in Sardegna è verosimilmente la più elevata tra le popolazioni dell'Europa Occidentale. Nonostante l'elevato tasso di consanguineità della popolazione sarda, solo una minoranza delle forme riscontrate sono recessive, la quasi totalità è a trasmissione autosomica dominante e quindi indipendenti nella loro etiologia da questo fattore. Il fenotipo prevalentemente riscontrato è in relazione con delezioni del gene *SPAST* ed è caratterizzato da età media all'esordio superiore rispetto a quanto precedentemente descritto, lenta progressione, disabilità moderata. Questa evidenza epidemiologica ha delle implicazioni prognostiche per la popolazione generale, soprattutto in relazione alla presenza di una elevata proporzione di casi asintomatici. Una maggiore consapevolezza delle malattie cronico-degenerative come la HSP, dovrebbe portare ad una sorveglianza epidemiologica maggiore ed incoraggiare l'adozione di specifiche misure sanitarie, come il counseling genetico dedicato ed un regolare follow-up. Dal punto di vista molecolare, il coinvolgimento dei geni fiancheggianti *SPG4* in diversi nuclei familiari offre lo spunto ad ulteriori ricerche per valutarne l'eventuale ruolo nella modificazione del fenotipo ed allo studio dei fattori modulatori l'espressività e la penetranza di una mutazione genetica.

7. Bibliografia

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ORIGINAL COMMUNICATION

The high prevalence of hereditary spastic paraplegia in Sardinia, insular Italy

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Abstract The few epidemiological studies conducted to date on the heterogeneous group of hereditary spastic paraplegias (HSPs) indicate a prevalence of 1.27–12.1 per 100,000. This study aims to explore the epidemiological, clinical, and genetic variability of HSPs among Sardinians, a population of peculiar ethnicity.A population-based prevalence study was performed in north-western Sardinia between January 2000 and December 2010. Multiple sources were used for case ascertainment. Familial and sporadic cases were diagnosed according to generally accepted criteria, and clinical diagnoses were validated by expert neurological examination. Clinical data and pedigree information were recorded and blood samples drawn for genetic testing.Sixty-seven HSP patients were included in the study: 59 belonged to 11 families with autosomal dominant transmission (AD-HSP), three cases were from two unrelated autosomal recessive families, and the remaining five cases were apparently sporadic. On 31 December 2010, the total crude prevalence was 19.9 per 100,000 (95 % CI 18.4–21.4), while the crude prevalence of AD-HSP was 17.5 (24.4 M, 15.7 F; M:F ratio 1.55). The mean age at examination was 48.4 years, and the mean age at onset of HSP was 36.6 years. A molecular diagnosis was obtained in 82.1 % of the cases (52 cases with mutations in *SPAST/SPG4*, two in *SPG7*, and one in *SPG11*).The prevalence of HSP among Sardinians is high compared with other Western European populations. The multiple search strategy used in this study and the specific socio-demographic characteristics of Sardinians may account for this finding.

Keywords Hereditary spastic paraplegia · Prevalence · Sardinia · Epidemiology

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Introduction

The hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous neurodegenerative disorders of the motor system characterized by progressive spasticity and weakness in the lower limbs [1, 2]. HSPs are traditionally classified as "pure" or "complicated" forms, depending on whether or not the patient presents additional neurological and extra-neurological manifestations [3]. Autosomal dominant (AD), autosomal recessive (AR), X-linked, and cytoplasmic patterns of inheritance are described. Mutations in *SPAST*/SPG4, *ATL1*/SPG3A, and *REEP1*/SPG31 in the AD-HSP forms and in *CYP7B1*/SPG5, *SPG7*, and *SPG11* in the AR-HSP forms are the most common causes both within and outside Europe, although a significant number of cases (especially sporadic) still lack a molecular diagnosis [4, 6–8]. The few epidemiological studies of HSPs so far reported indicate a prevalence of 1.27–12.1 per 100,000 (Table 1), the wide range likely being attributable to methodological differences and ethnic characteristics of the populations

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Table 1 Prevalence of HSPs (per 100,000) in previous studies (1968–2010)

Country	Population	Source of case ascertainment	Inclusion criteria	No. of HSP cases	Prevalence day	Prevalence (95 % CI)	Reference
Western Norway	725,000	University hospital in Bergen	AD-HSP, AR-HSP	34	1 January 1968	12.1 (n.a.)	[9]
Benghazi (Libya)	519,000	Polyclinics, disability center, four university hospitals	HA, AD-HSP, AR-HSP, sporadic HSP	11	31 August 1984	2.1 (n.a.)	[10]
Turin (Italy)	2,327,996	Regional hospital, neurology divisions, nursing homes, patients' association	HA,AD-HSP, AR-HSP sporadic HSP	31	31 December 1982	1.33 (1.73–1.93)	[11]
Cantabria (Spain)	510,000	Neurology department of regional hospital	HA, AD-HSP, AR-HSP	49	31 December 1986	9.6 (n.a.)	[5]
Molise (Italy)	335,211	Mail and phone survey of 847 hospital physicians and GPs, hospital records	HA, AD-HSP, AR-HSP	9	1 January 1989	2.7 (1.2–5.1)	[12]
Valle d'Aosta (Italy)	115,275	Departments of regional hospital, diagnostic services, disability centers.	HA, AD-HSP, sporadic HSP	5	31 December 1991	4.3 (n.a.)	[13]
Viano de Castelano (Portugal)	250,061	District and regional hospital, disability centers, GPs	HA, AD-HSP, AR-HSP	5	1 January 1994	2 (n.a.)	[14]
Ireland	5,436,000	Neurologists, geneticists, hospital records	AD-HSP	69	1 June 2000	1.27 (0.99–1.61)	[15]
Estonia	1,300,000	Regional neurological center records, neurologists, GPs	AD-HSP,AR-HSP, sporadic HSP	59	1 May 2005	4.42 (3.36–5.70)	[16]
South-eastern Norway	2,633,893	Neurologists, computer searches, patients' association, families	HA, AD-HSP, AR-HSP sporadic HSP	194	1 February 2008	7.4 (6.4–8.5)	[17]
Portugal	10,322,000	GPs, records, neurologists and geneticists, families	HA, AD-HSP, AR- HSP	418	1994–2004	4.1 (3.8–4.8)	[18]
Present study	336,632	Neurologists, patients' association, families	AD-HSP, AR-HSP apparently sporadic HSP	67	31 December 2010	19.9 (18.4–21.4)	[This study]

AD-HSP autosomal dominant HSP, *AR-HSP* autosomal recessive HSP, *HA* hereditary ataxia, *n.a.* not available

investigated [5, 9–18]. Most of them were performed in the pre-genetic era, when HSP cases were often included in the broad category of hereditary ataxias.

Sardinia, because of its insularity, has a population with a peculiar and very interesting demographic and genetic profile, likely driving the exceptionally high incidence of complex polygenic diseases such as juvenile diabetes mellitus and multiple sclerosis [19–21]. We performed a prevalence study of HSPs among Sardinians, seeking to identify possible peculiar epidemiological patterns and to establish clinical and genetic correlates.

Methods

Study design

This is a population-based prevalence study. Case ascertainment was performed in reference to an 11-year period (1 January 2000 to 31 December 2010). To facilitate study participation, on-site examinations were performed in collaboration with local general practitioners (GPs).

Study area and population

The study area was the province of Sassari, north-western Sardinia, Italy, which covers 4,282 km^2 (17.8 % of the whole area of the island) and has a population of 333,576 (163,104 men and 170,472 women; 20 % of all Sardinian inhabitants) [22]. The study area comprises 66 municipalities, 63 with fewer than 20,000 population and three larger ones. The province of Sassari comes under the Sassari local health authority (Azienda Sanitaria Locale n. 1), whose territory is divided into three districts: Sassari (27 municipalities), Alghero (23) and Ozieri (16). The area also includes a neurological teaching center (University of Sassari).

Case definition

Patients were evaluated using generally accepted clinical diagnostic criteria for HSP previously described [17, 23, 24]. Patients with a disease-positive family history were classified as "definitely affected" if they manifested progressive gait disturbance, involvement of the corticospinal tract with marked hyperreflexia, extensor plantar responses, and if secondary causes of spastic paraplegia had been excluded; instead, they were classified as "probably affected" if they were asymptomatic without any progressive gait disturbance but with clear signs of spastic paraparesis on neurological examination such as Babinski sign or increased tendon reflexes and lower limb hypertone. These two groups of subjects were included in the study, even if a confirmatory molecular diagnosis had not been reached. Conversely, subjects with a positive family history for HSP who were found to be asymptomatic and to have questionable pyramidal tract involvement were classified as "possibly affected" or "not affected" and were excluded from the prevalence study, unless a confirmatory molecular genetic diagnosis could be obtained [25].

Subjects with an unclear family history, but undoubtedly presenting neurological signs on expert clinical examination—and in whom no other possible diagnosis was entertained—were defined as apparently sporadic or with possible AR inheritance and were included, regardless of positive DNA testing. All subjects fulfilling the aforementioned inclusion criteria for HSP, and residing in the study area on 31 December 2010 were considered prevalent cases. Peripheral blood samples were drawn from all the subjects participating in the study, who provided informed consent to perform genetic analyses.

Data collection

To identify all possible subjects with HSP in the study area, we adopted a multiple search strategy gathering data from the following epidemiological sources.

1. Case histories. We collected case histories of patients diagnosed with HSP, undefined spastic paraplegia, undefined spastic tetraplegia, cerebellar-pyramidal syndromes, or ataxia, consulting medical records obtained from the neurology centers operating within the area covered by the Sassari local health authority, i.e., the Unit of Clinical Neurology, Sassari University Hospital, and the Division of Neurology, Ozieri Hospital. All case histories were reviewed by two expert investigators (L. R. and C. C.) and suspected cases underwent further clinical evaluation and possibly specific gene testing.

2. Neurologists, pediatricians, urologists, and GPs across the area were informed about the study through meetings and letters. Colleagues were asked to refer patients with known or suspected HSP to a local investigator (L. R.) for specialized clinical examination.

3. The Sardinian HSP Patients' Association (*Vivere la Paraparesi Spastica Onlus,* VIPS, www.vipsonlus.it), established in 2009, was also involved in the study and VIPS members with confirmed or suspected HSP were invited to participate [26].

Case validation

Every subject who agreed to participate in the study was evaluated individually by one of the investigators (L. R. or R. DF.) either at a local outpatient clinic or at his/her own home. We adopted the standardized clinical chart developed by SPATAX, the European and Mediterranean

network for the study of spinocerebellar degenerative disorders, to record clinical data and pedigree information and patients were classified according to the established clinical classification [3, 27].

Three- to ten-generation pedigrees were drawn for each index case. Dominant inheritance was presumed in cases of parent-to-child transmission, although father-to-son transmission was not always present. Recessive inheritance was presumed when two or more siblings were affected and the parents were clinically normal, or when parental consanguinity was evident. In some sporadic cases, genetic testing showed recessive inheritance.

Whenever possible, the patients participating in the study underwent brain and spine MRI as well as neurophysiological and neuro-urological studies. Disease severity was scored using the Spastic Paraplegia Rating Scale (SPRS), and HSP patients were assigned to five disability classes on the basis of their SPRS total score: class 0 = score 0, class 1 = score 1–13, class 2 = score 14–26, class 3 = score 27–40, class 4 = score 41–52 [28]. Furthermore, in accordance with previous investigations, HSP patients were also evaluated for motor disability only and assigned to five functional classes, scored as follows: 0 = asymptomatic, 1 = mild symptoms and/or signs on neurological examination, able to walk without help and able to run; 2 = able to walk without help but unable to run; 3 = able to walk with aids; 4 = wheelchair-bound [17].

Genetics

In consenting individuals, total genomic DNA was purified according to standard methods and the coding exons of all the most frequent *SPG* genes (namely, *PLP*/SPG2, *ATL1*/SPG3A, *SPAST*/SPG4, *REEP1*/SPG31, *CYP7B1*/SPG5, *NIPA1*/SPG6, *SPG7*, *KIAA0196*/SPG8, *KIF5A*/SPG10, *SPG11*, *RTN2*/SPG12, *HSPD1*/SPG13, *ZFYVE26*/SPG15, and *BSCL2*/SPG17) were screened for punctuate mutations and multi-exon deletion/duplications by direct sequencing and MLPA analyses, respectively, in a single molecular neurogenetic center (F. M. S.). All sporadic patients had already been tested elsewhere for pathological expansion of the GAA tract in *FXN* and the CAG tract in *ATX1*/SCA1 and *ATX3*/SCA3 at some point during their disease course. Identification of mutations was corroborated by a second validated method whenever possible, namely segregation in the families and in silico predictions of pathogenicity.

Statistics

Descriptive analyses included the computation of means, standard deviations (SD), *t*-test for independent samples, and analysis of variance for continuous variables (e.g., age at clinical onset, disease duration, age on the prevalence day). Counts and percentages were used to describe categorical variables (hereditary transmission, genetic mutations and motor disability). Gender- and age-specific crude prevalence with 95 % confidence intervals (95 % CI) were computed for all the HSP forms and specifically for AD-HSP on the prevalence day (31 December 2010), using the 2010 Italian census population as denominator [22]. Standardized prevalence was obtained based on 10-year age grouping and the 2010 census population. Significance was set at $p < 0.05$, two-tailed test. The Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM SPSS Inc., USA) was used for statistical analyses.

Ethical aspects

The overall study was approved by the Sassari local health authority institutional review board (Prot. N. 893/CE, 21 July 2010, Azienda Sanitaria Locale n. 1, Sassari, Italy) and performed in compliance with the Italian ethical rules on data collection for statistical and scientific purposes.

Results

A total of 75 case histories of patients with spastic paraplegia, spastic tetraplegia, cerebellar-pyramidal syndromes or spastic ataxia were collected from hospital records. Review of the clinical data showed that of these, 35 (46.7 %) were primary progressive multiple sclerosis, hereditary ataxia, toxic cerebellar degeneration or paraplegia/tetraplegia due to brain or spinal cord lesion. Of the remaining 40 cases, the diagnosis of HSP was confirmed in 15, who were thus included in the study. A further nine index cases were referred by colleagues or VIPS members. A total of 195 relatives of the 24 index cases were clinically evaluated. Examinations disclosed 70 additional individuals fulfilling a diagnosis of HSP. Of 94 HSP patients ascertained, five had died prior to prevalence day for intercurrent diseases (cancer, cardiac failure, cerebrovascular diseases), 21 were not prevalent cases, and one individual refused to participate. Thus, 67 patients (40 men and 27 women), all of Sardinian origin and still alive at the time of this writing, were eligible for the prevalence study (Fig. 1).

Of the cases, 42 (62.7 %) fulfilled the criterion for 'definitely affected', 12 (17.9 %) for 'probably affected', 8 (11.9 %) were asymptomatic carriers of the pathogenetic mutation with no signs at neurological examination and five (7.5 %) were apparently sporadic cases.

Of the prevalent cases, 59 (88.1 %) belonged to 11 families with AD transmission; genealogical surveys, interviews with senior members of the families, or review

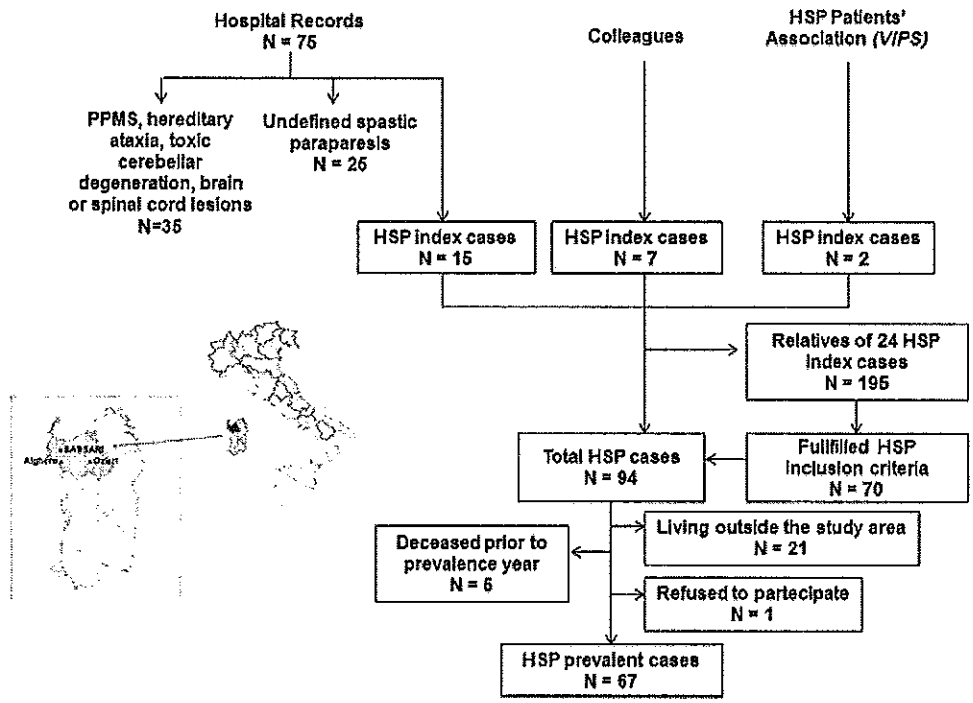

Fig. 1 Flowchart of case ascertainment in north-western Sardinia

Table 2 Age- and gender-specific prevalence of HSP (all forms) (per 100,000) on 31 December 2010

Age group (years)	Men			Women			Total		
	Population	Number of HSP cases	Prevalence	Population	Number of HSP cases	Prevalence	Population	Number of HSP cases	Prevalence
0–9	14,131	1	7.1	13,520	0	0.0	27,651	1	3.6
10–19	16,071	0	0.0	15,122	0	0.0	31,193	0	0.0
20–29	19,817	4	20.2	18,854	1	5.3	38,671	5	12.9
30–39	26,508	8	30.2	25,955	9	34.7	52,463	17	32.4
40–49	27,072	7	25.9	27,690	5	18.1	54,762	12	21.9
50–59	22,803	8	35.1	23,341	4	17.1	46,144	12	26.0
60–69	18,898	7	37.0	20,582	6	29.2	39,480	13	32.9
70–79	12,860	5	38.9	16,417	2	12.2	29,277	7	23.9
80+	6,049	0	0.0	10,942	0	0.0	16,991	0	0.0
Total	164,209	40	24.4	172,423	27	15.7	336,632	67	19.9
95 % CI			23.3–25.4			14.5–16.9			18.4–21.4
Standardized prevalence to 2010 census pop. [20]		23.9				15.1			19.4

of specific genetic tests showed that eight of these families were related. Three subjects (4.5 %) belonged to two unrelated families with AR transmission. Parental consanguinity was present in a single *SPG7* family.

The total crude prevalence of HSPs was 19.9/100,000 (95 % CI 18.4–21.4), 24.4 (95 % CI 23.3–25.4) in men, and 15.7 (95 % CI 14.5–16.9) in women, giving an M:F ratio of 1.55. The prevalence standardized to the Italian 2010 census population was 19.4 per 100,000 for both sexes, 23.9 for men and 15.1 for women. The prevalence of HSPs showed a bimodal age distribution with the highest values recorded in the age groups 30–39 years (32.4/100,000) and 60–69 years (32.9/100,000). In men, the highest prevalence was found for the age group 70–79 years (38.9/100,000) and in women for the 30–39 year group (34.7/100,000) (Table 2). The total crude prevalence of AD-HSP ($N = 59$) was 17.5/100,000 (95 % CI 16.1–18.9), 21.3 (95 % CI 20.3–22.3) and 13.9 (95 % CI 12.8–15.0) for men and women, respectively.

The prevalent cases had a mean age (SD) of 48.4 (15.8) (range 8–75) years, with no significant differences by gender. The mean age for AD-HSP cases was 47.6 (15.9) (range 8–75) years, 45.0 (9.8) for AR-HSP cases ($N = 3$), and 59.0 (14.0) years in apparently sporadic individuals ($N = 5$). All cases had a mean age at clinical onset of 36.6 (13.6) years with no gender difference. In AD-HSP, the mean age at onset was 37.3 (13.3) years, versus 26.0 (12.7) years in the AR-HSP group and 34.8 (17.2) years in the apparently sporadic cases.

The mean disease duration in all patients was 15.1 (12.4) years, with no gender difference. The duration was 14.4 (12.3) in the AD-HSP, 16.0 (2.3) in the AR-HSP, and 21.2 (8.8) years in the apparently sporadic forms. It was longer in patients with onset before than in those with onset after 35 years of age: 19.8 (15.7) vs 12.2 (7.6) years ($p = 0.048$), as is to be expected in a chronic disease that does not significantly affect life expectancy.

With regard to the distribution of the HSP prevalent cases by SPRS classes, 20.9 % of the cases belonged to class 0, 38.8 % to class 1, 19.4 % to class 2, 14.9 % to class 3, and 4.5 % to class 4. One patient could not be assigned to any SPRS class.

On the basis of motor disability only (functional score), 11.9 % were asymptomatic, 38.8 % displayed only mild symptoms and/or signs at examination, 14.9 % could walk independently but were unable to run, 22.4 % could walk with aids and 11.9 % were wheelchair-bound. Figure 2 shows the distribution of the functional scores by type of transmission in prevalent cases.

Disease severity expressed by SPRS classes (0–4) increased with disease duration (Pearson chi-square coefficient = 43.690, $p < 0.0001$), as did disability expressed by functional scores (0–4). The marginal mean functional score, adjusted for age at onset, was 1.05 (95 % CI 0.67–1.43) at 10 years after onset, 2.26 (95 % CI 1.90–2.63) at 20 years, and 3.14 (95 % CI 2.71–3.57) at 30 years (ANOVA, $p < 0.0001$).

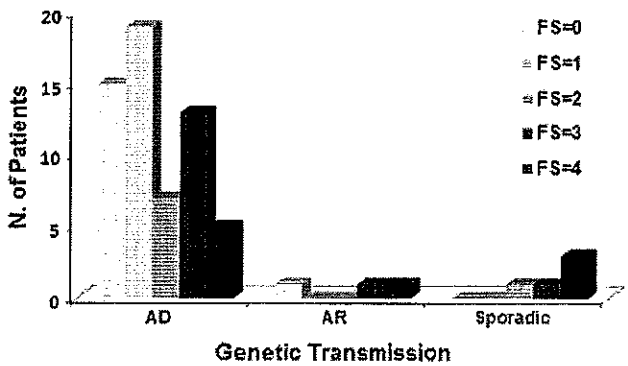

Fig. 2 Distribution of functional scores (FS) by type of transmission in Sardinian HSP prevalent cases: 0 = asymptomatic, 1 = mild symptoms and/or signs on neurological examination, able to walk without help and able to run; 2 = able to walk without help but unable to run; 3 = able to walk with aids; 4 = wheelchair-bound

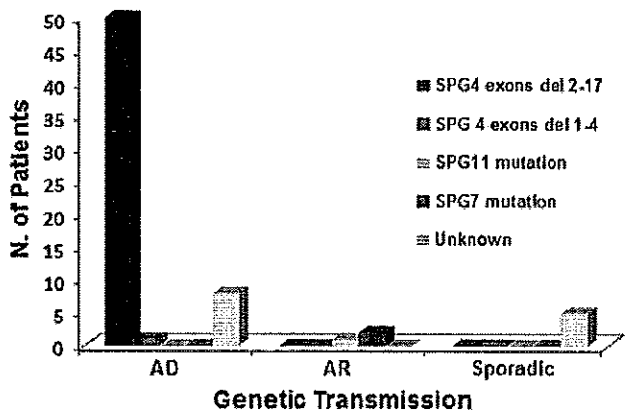

Fig. 3 Distribution of genetic mutations in Sardinian HSP prevalent cases according to their pattern of transmission

A confirmatory molecular diagnosis was obtained in 55 of the 67 (82.1 %) prevalent cases: 52 (77.6 %) AD-HSP presented the pure form of disease, whereas three (4.5 %) had a complicated form related to an AR-inherited gene. Figure 3 shows the distribution of genetic mutations by transmission. All AD-HSP patients presented mutations in *SPAST*/SPG4, with a multiexon spastin deletion occurring in 76.1 % of the cases and a smaller deletion (exons 1–4) in 1.5 %. A single patient was compound heterozygous for mutations in *SPG11*, whereas two patients harbored a homozygous mutation in *SPG7*. A confirmatory molecular genetic diagnosis could not be obtained in 12 prevalent cases (17.9 %), of which seven (58.3 %) belonged to two pure, apparently unrelated AD-HSP families and five (41.7 %) were *bona fide* sporadic. Of the latter, four had a complicated form of HSP.

Discussion

In 2010, in north-western Sardinia, insular Italy, HSP prevalence was found to be 19.9 per 100,000, the highest so far reported in a Western country. Epidemiological studies on HSP carried out in different regions, and mostly in the pre-genetic era, have produced heterogeneous estimates of the disease prevalence (Table 1), in relation to differences in study methodology (e.g., population sizes, sources of data, disease classification and inclusion criteria), and the ethnic and socio-demographic characteristics of the populations studied. Using a single data source, Skre and coworkers reported a prevalence of 12.1 per 100,000 in western Norway by means of extrapolation, based on the type of transmission and clinical peculiarities of the 'pure' and 'complicated' forms [3, 9]. High prevalence estimates have also been found in Cantabria, northern Spain (9.6 per

100,000), based on the Harding classification and not considering sporadic forms [5]. In that study, case ascertainment was carried out from a single data source (a tertiary hospital), though the authors examined a large number of relatives and discovered 46 additional cases. The use of extended intra-familial ascertainment is needed in epidemiological studies of inherited diseases with possible reduced penetrance, such as HSP. Our study, though in a smaller population, adopted a similar strong methodological approach and followed the design strategy recently adopted in HSP [17]. The higher prevalence of HSP found in Sardinians as compared to previous ascertainment may also be ascribed to insularity which favored the temporal expansion of familial clusters attributable to few founders. Compared with previous reports, our study features a high proportion (82.1 %) of molecularly confirmed HSP cases [15–17]. Both the proportion of AD families (88.1 %) and the frequency of *SPAST*/SPG4 mutations (77.6 %) are greater than reported by others [1, 29]. Conversely, fewer AR families (4.5 %) were detected, despite the high rate of consanguinity in the Sardinian population [30, 31].

The prevalence of HSP in our population by age showed a bimodal distribution with peaks between 30 and 39 years, and 60 and 69 years (70–79 years in men). A high prevalence in the elderly is typically seen in genetic diseases without reduced life expectancy and with variable onset [17, 32]. On the other hand, and in line with previous studies, no cases aged 80 years or older were recorded, when possible long-term complications of HSP may have occurred [12, 13, 15–17]. A low prevalence in the age group 0–9 years (7.1/100,000 in males and 0/100,000 in females) was found, reflecting few congenital or infantile onset cases. This finding seems to conflict with what shown for mainland Italy (C. C., personal observation).

In the Sardinian cohort, the age at onset was higher in AD-HSP (37 years) than in AR-HSP and in sporadic conditions. A higher age at onset differing from previous reports [17, 18] might be attributed to different sets of mutations and explain, at least in part, the observed higher prevalence, since neurological disability does not seem to significantly interfere with patients' reproductive fitness.

In the present study, the symptomatic patients were found to show a relatively benign clinical course of HSP. More than a third showed low global disease severity, mostly determined by mild motor signs, and only 12 % of patients were wheelchair-bound.

In this study, the proportion of asymptomatic patients, 29.8 % (about 18 % with and 12 % without pyramidal signs), is in line with other reports [15, 33]. Due to the lack of a cure for HSP, proposing diagnostic ascertainment to asymptomatic individuals implies ethical problems but offers a better awareness and, more importantly, facilitate family planning, especially in the youngest. To this end, we offered at-risk subjects the choice on to whether undergo neurological examination or genetic testing, or both.

In summary, the prevalence of HSP in Sardinians herein reported is likely the highest ever recorded in Western countries. The proportion of asymptomatic and benign cases, as well as the older age at onset and the relatively high number of large pedigrees characterizing the population of north-western Sardinia may contribute to the high disease burden, with prospective and prognostic implications for the general population [34, 35]. Increased medical awareness of chronic-degenerative diseases like HSP should foster greater epidemiological surveillance and encourage the adoption of specific healthcare policies, including ad hoc counseling and follow-up.

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Conflicts of interest The authors declare that they have not conflict of interest.

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Pubblicazione II

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SHORT COMMUNICATION

Large deletion mutation of *SPAST* in a multi-generation family from Sardinia

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Background and purpose: The hereditary spastic paraplegias (HSP) are characterized by progressive spasticity of the lower limbs, mostly inherited as an autosomal dominant trait. Analyses of large HSP pedigrees could help to better characterize the phenotype due to a single causative mutation. Patients in a seven-generation kindred carrying a large deletion in *SPAST*/SPG4 are described.

Methods: Individuals originating from Sardinia were clinically and genetically studied.

Results: Sixty-seven subjects carried a heterozygous deletion encompassing exons 2–17 of *SPAST*. Fifty patients (53.2 ± 15.4 years) presented a pure form of spastic paraparesis characterized by mild impairment and slow progression. Most patients showed spasticity, increased tendon reflexes in the lower limbs and Babinski sign, whilst weakness was rarely detected and urinary disturbances occasionally reported. Amongst the 17 asymptomatic carriers of the mutation, minimal neurological signs were detected in 11 cases.

Conclusions: A focus on spasticity, increased tendon reflexes and Babinski sign, more than on weakness, could help clinicians to promote early diagnosis in asymptomatic carriers of *SPAST* deletions.

Introduction

Autosomal dominant hereditary spastic paraplegias (AD-HSP) are clinically characterized by progressive spasticity and weakness of the lower limbs [1], mostly due to mutations in the *SPAST*/SPG4 gene encoding spastin [1]. Large pedigrees might provide a unique resource for addressing clinically relevant issues in these disorders.

Sixty-seven individuals from a single multi-generation Italian kindred found to harbor the same large deletion in the spastin gene were studied.

Methods

The index case (VII:04) and her cousin (VII:36) displayed progressive spastic paraparesis. A total of 107 members in the patients' extended family, living in the same area in northern Sardinia and ascertained on the basis of local parish registers and direct interviews, were included in the survey (Fig. 1). The Spastic Paraplegia Rating Scale (SPRS), the Medical Research Council Scale for Muscle Strength (MRC), the Motor Severity Score (MSS), the Ashworth Scale of Muscle Spasticity (ASM) [2] and the modified Barthel Index (BI) [3] were used. The annual rate of progression of the disease was also considered [2].

Standard methods for DNA purification, sequencing of frequent AD-HSP genes and multiplex ligation-dependent probe amplification analyses were used. Further molecular and statistical methods are illustrated in Data S1. This study received approval from the ethics committees of our institutions.

Results

Of the 107 individuals tested, 67 (M/F ratio 1.3:1) harbored a heterozygous deletion in *SPAST*/SPG4 encompassing exons 2–17 and the flanking *SLC30A6* (*SPAST*c.415+806_*SLC30A6*:4-5302del) (Table 1). Of these, 17 (eight men, nine women, 37.5 ± 14.8 years, range 18–73) were asymptomatic, although 11 (16%)

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presented minimal signs on neurological examination (isolated Babinski sign, brisk tendon reflexes and ankle clonus). The 50 clinically affected individuals (30 men, 20 women, 53.2 ± 15.4 years, range 9–76) complained mostly of cramps and excessive sense of fatigue whilst walking. In 31 patients the first manifestations were walking abnormalities and leg stiffness, whereas three patients initially reported cramps and two patients urinary urgency. The remaining 14 patients were unable to define the manifestations of the disease at onset. Ninety-five percent of the patients walked independently (19% using a support). Three patients were confined to a wheelchair at 61.3 ± 9.3 years (55–72), after 29.7 ± 15 years (13–42) of disease duration. Fifty-four of the 67 patients showed brisk tendon reflexes in the lower limbs, and six also increased reflexes in the upper limbs. Vibratory sensation was impaired in three subjects. Mini Mental State Examination ascertained slight cognitive impairment in four patients (median age 65.2 ± 3.3 years), with limitations mainly in recall. Urinary disturbances were not spontaneously reported, although 28 patients, when specifically questioned, complained of various combinations of micturition discomfort, urgency and incontinence. Two patients showed gaze-evoked nystagmus and intermittent exotropia. There were no statistically significant differences between men and women, although men more commonly displayed lower limb spasticity (Table 1). The Kaplan–Meier analysis did not show gender-related differences in outcome.

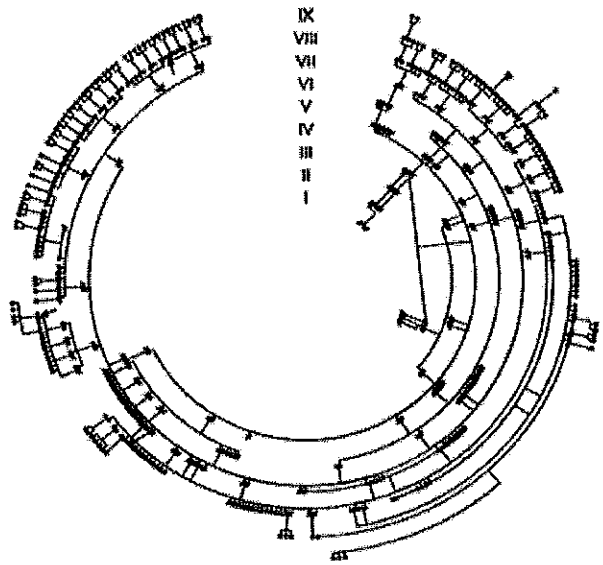

Figure 1 Pedigree of the extended Nulvi kindred studied in this work.

Twenty patients underwent a brain MRI and 24 also a spine MRI, which in all cases were normal. Two of the seven patients who underwent nerve conduction studies displayed a slight sensorimotor polyneuropathy.

Disease duration correlated with ASM ($r = 0.70$, $P < 0.001$), MSS ($r = 0.81$, $P < 0.001$) and SPRS ($r = 0.83$, $P < 0.001$), whereas it was inversely correlated with MRC ($r = -0.57$, $P < 0.001$) and BI ($r = -0.57$, $P < 0.001$).

Table 1 Clinical features in 67 patients harboring a multi-exon deletion in the *SPAST* gene

	Total	Men	Women	*P*
Patients, *n* (symptomatic)	67 (50)	38 (30)	29 (20)	
Clinically affected, %	85	92	76	ns[a]
Age at evaluation, years	50.7 ± 16.6	52.1 ± 18.2	48.9 ± 18.2	ns[b]
Age at onset, years	39 ± 13	38.9 ± 15	39.2 ± 9.9	ns[b]
Disease duration, years	11.7 ± 11.6	13.2 ± 13.1	9.9 ± 9.2	ns[b]
SPRS score[c]	12.2 ± 11.7	14.2 ± 12.5	9.8 ± 10.4	ns[b]
SPRS item 13, %	47	44	51	ns[a]
Motor severity score[d]	2.5 ± 1.5	2.8 ± 1.6	2.1 ± 1.3	ns[b]
Spasticity score[e]	1.3 ± 1.1	1.7 ± 1.1	0.8 ± 0.9	**0.04**
Muscle strength score[f]	4.4 ± 1.1	4.3 ± 1.1	4.6 ± 1.2	ns[b]
Barthel Index score[g]	94.2 ± 13.4	92.3 ± 15.5	96.7 ± 9.8	ns[b]
Rate of progression[h],%/year	5.2 ± 5.7	5.4 ± 5.5	4.8 ± 6.1	ns[b]
Pes cavus, %	42	37	48	ns[a]
Brisk reflexes, %	81	84	76	ns[a]
Babinski, %	70	74	62	ns[a]

[a]Chi-squared; [b]unpaired two-tailed Student *t* test (bold values are significant); [c]SPRS (Spastic Paraplegia Rating Scale) score [16] ranges from 0 (normal) to 52 (severely affected); [d]the motor severity score [18] ranges from 0 (normal) to 7 (confined to bed); [e]the Modified Ashworth Scale of Muscle Spasticity in the lower limbs score [17] ranges from 0 (no increase in tone) to 4 (affected parts rigid in flexion or extension); [f]the Medical Research Council Scale for Muscle Strength in the lower limbs [19] ranges from 0 (no contraction) to 5 (normal strength); [g]the Barthel Index [5] ranges from 0 (no autonomy) to 100 (normal); [h]the rate of progression was calculated as follows: (motor severity score/7) × 100/disease duration.

None of the 67 patients harbored additional variants in *SPAST*/SPG4 [1], or *SLC30A6*, or the p.G563A in *HSPD1*/SPG13 invoked as modifier in spastin disease [4].

Discussion

The clinical features observed in the 'Nulvi kindred' (NK) – the largest pedigree thus far described with a single genomic deletion encompassing *SPAST* and the flanking *SLC30A6* – did not seem to differ greatly from those observed in other kindred harboring large *SPAST* deletions, although the mean onset age is higher than was previously reported [5–7]. At our latest examination 25% of patients (median age 37.5 ± 14.8 years) were asymptomatic, and 16% had minimal neurological signs. In the few individuals examined well beyond the mean age at onset, this finding may be taken as an indication of true reduced penetrance of the disorder. Moreover, the low SPRS scores recorded, indicating a slight functional impairment, as well as the high BI, consistent with partial reduction of autonomy in daily living activities, support our opinion that the mutation caused only mild disability in this family. Only three patients in NK were wheelchair-bound after a long disease course.

In the NK, the presence of asymptomatic patients, the low overall disability, the slow progression of the neurological impairment together with the geographical isolation and a likely founder effect may have favored the high prevalence of the disease in that area of Sardinia. Notably, <30% of patients were aware of their clinical status even when gait disturbances were already manifest (SPRS >10), and limited perception of the disorder delayed appropriate medical counseling.

In accordance with literature data on spastin mutations [8], the NK contained more men than women (M/F ratio 1.3), and men were also more likely to show signs of the disease (92% vs. 76%). Along with a bias in selection, protection due to estrogens [9] is one possible explanation for this discrepancy.

The spastin mutation found in the NK family almost completely removes a copy of the gene, leads to severely reduced mRNA (Data S1) and predicts a hypofunctioning protein [1]. It is believed that patients with gene deletions/duplications tend to show less severe gait disability than cases with point mutations (especially those residing in the AAA domain) [7]. This difference might be related to a reduced likelihood of dominant-negative consequences on spastin function [10].

The variable disease progression in the NK family (that included up to 25% of mutation carriers who were asymptomatic), as in others carrying a *SPAST* deletion [7], suggests a possible role for modifiers. In this regard, partial damage of the neighboring *SLC30A6* was detected, encoding the Znt6 subtype of zinc transporters [11]. Nonetheless, additional intragenic variants in *SPAST*/SPG4 and *SLC30A6* were ruled out, as well as the possible contribution of the p.G563A in *HSPD1*/SPG13 [4] (Data S1). Thus, the issues of genetic or epigenetic modifiers require further investigation.

In summary, our focus was on the variability and severity of the disease in a large SPG4 kindred. Like other families with deletions in *SPAST*/SPG4, the NK cohort showed relatively mild disease, which had little impact on everyday activities. Asymptomatic relatives of patients should be carefully checked for neglected signs, in particular brisk reflexes and Babinski sign, to promote early diagnosis.

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Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The distribution of age at onset in the family members of the Nulvi kindred. Yrs, years.
Figure S2. Rapid diagnostic genotyping of the multiexon deletion detected in the NK family.
Figure S3. Bar chart showing the relative expression of mRNA/*SPAST* in blood.
Data S1. Supplementary methods.

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Pubblicazione III

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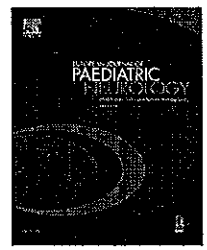

Case study

Infantile-onset ascending hereditary spastic paralysis: A case report and brief literature review

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ABSTRACT

Background: Infantile-onset ascending hereditary spastic paralysis (IAHSP) is a rare, early-onset autosomal recessive motor neuron disease associated with mutations in *ALS2*.
Aim: We studied a 17-year-old boy who had features of IAHSP. We also reviewed the current literature on *ALS2*-related syndromes.
Methods: Clinical and neuroimaging studies were performed. Blood DNA analyses were combined with mRNA studies in cultured skin fibroblasts.
Results: Like previously described cases, the patient presented with severe spastic paraparesis and showed rapid progression of paresis to the upper limbs. He also developed bulbar involvement and severe scoliosis during childhood. In blood DNA we identified a novel splice-site homozygous mutation in *ALS2* (c.3836+1G > T), producing exon skipping in fibroblast mRNA and predicting premature protein truncation.
Conclusions: This case adds to the allelic heterogeneity of IAHSP. Review of the pertinent literature indicates a fairly homogeneous clinical picture in IAHSP that should facilitate molecular confirmation and prevention of long-term complications.

1. Introduction

The hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous neurodegenerative disorders of the motor system characterized by insidiously progressive weakness and spasticity in the lower limbs (pure forms), which may be combined with additional neurological or non-neurological manifestations (complicated phenotypes). The HSPs are associated with a plethora of loci (about 60) and related genes (more than 30)[1,2] and it is therefore hardly surprising that genotype–phenotype correlations are weak.

Mutations in *ALS2*, located on chromosome 2q33 and encoding alsin, are responsible for a spectrum of rare autosomal recessive disorders ranging from infantile ascending hereditary spastic paralysis (IAHSP, MIM 607225) to juvenile primary lateral sclerosis (JPLS, MIM 606353) with retrograde degeneration of the upper motor neurons, and juvenile amyotrophic lateral sclerosis (MIM 205100), in which there is both upper and lower motor neuron involvement.[3–5]

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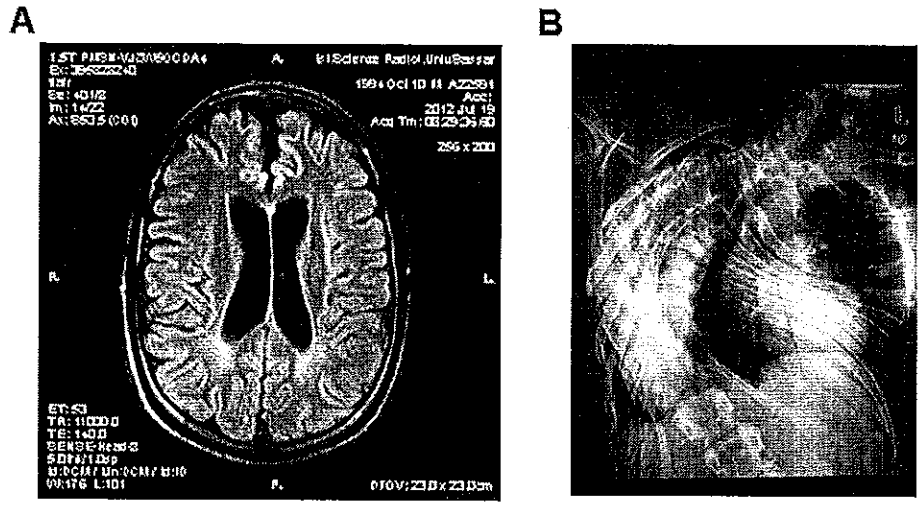

Fig. 1 – Imaging studies in a patient harboring a novel mutation in *ALS2*. A Brain MRI at age 17 years, FLAIR image: hyperintensities in posterior periventricular areas. B Spine X-ray of the dorsal tract: severe deformity due to right convex rotoscoliosis.

Although these syndromes were initially confined to few kindred in the Mediterranean area, 14 *ALS2* families have now been identified worldwide.

We here present clinical and molecular findings in an additional patient from Sardinia, Italy, and review the literature dealing with alsin phenotypes.

2. Case study

This 17-year-old boy was born after an uneventful pregnancy to healthy, apparently unrelated parents. His six-year-old sister is healthy. The patient sat up unsupported at the age of six months, and uttered his first words at the age of nine months. At 12 months, he manifested leg stiffness and bilateral clubfoot. At the age of two years he needed bilateral assistance while walking on tiptoes, and he has never been able to walk independently. At two years, neurological examination revealed lower extremity spastic hypertonia with enhanced deep tendon reflexes, bilateral ankle clonus, and Babinski sign. He could control his head and maintain the sitting position and he also showed good coordination and hand manipulation. His language skills were adequate and he interacted well both with people and with the environment. The disease progressed and, at the age of eight years, the patient showed weakness and spasticity in the upper limbs, mild dysphagia and dysarthria, and was wheelchair-bound. Within the following year he lost the ability to sit unsupported. He also began to develop scoliosis. Towards the age of 11 years the patient showed anarthria and difficulty chewing. By this time, handwriting was impossible. However, he continued to be mentally unimpaired and was able to attend both primary and secondary school profitably using special aids for communication.

We first saw him at the age of 17 years when, on neurological examination, he was found to be collaborative and responsive to stimuli. He was able to achieve a full range of eye movements with no nystagmus, while the reduced mobility of his facial muscles resulted in a "forced smile".[8] He had decreased tongue mobility in all directions, but no fasciculations or amyotrophy. A videofluoroscopy confirmed inhalation of liquids. His upper limbs showed hypertonia with residual gross and fine motor functions, whereas his lower limbs were plegic. We did not observe lower limb fasciculations, muscle atrophy, or gross sensory deficits, and did not record sphincter dysfunction. The patient displayed retractions at the knee and ankle joints. EMG showed a marked reduction of voluntary recruitment, in particular in the lower limbs, without signs of denervation. Motor and sensory nerve conduction velocity studies were normal. Somatosensory evoked potentials (SEPs) showed, only in the legs, increased latency of a cortical component (P37) bilaterally. Brain MRI showed hyperintensities in posterior periventricular areas (T_2-weighted images and FLAIR) (Fig. 1A). These abnormalities had been reported since the patient was aged 10 years, but we did not have access to earlier scans and were therefore unable to assess any progression. The patient also presented severe scoliosis. His spine X-ray and MRI images showed severe dorsal and lumbar rotoscoliosis, right convex in the dorsal tract (Fig. 1B), but without spinal cord thinning or compression.

Direct sequencing of the coding exons of *ALS2* (NM_020919.3) in blood DNA revealed a homozygous c.3836+1G > T mutation at the consensus splice-donor site of intron 24 (Supplementary Fig. 1). The mutation was novel, found to be heterozygous in the healthy parents, and not detected in 500 healthy, ethnically-matched control chromosomes. The new c.3836+1G > T predicts *in silico* exon skipping (www.fruitfly.org/seq_tools/splice.html). To test this, we purified and reversely transcribed polyA + RNA from cultured skin fibroblasts, amplified alsin cDNA with primers located in exons 20 and 27, and directly sequenced PCR-amplified fragments with the corresponding primers. In cultured cells, we detected two equally abundant *ALS2* transcripts, one missing

Table 1 – Clinical features and ALS2 mutations in families with upper motor neuron phenotype.

Family ID and origin	Phenotype	Age at onset of motor disability (months)	Age when confined to a wheelchair (years)	Age at onset of bulbar involvement (years)	Consanguinity	Mutation	E/I	Predicted protein	References[c]
ID1/KWT	IAHSP	14/11/3	nr/6[a]/21[b]	7/5/nr	Y	c.1548delAG	E5	p.T475Tfs*70	3, 9
ID2/SA	JPLS	24/18/12	10[a]/6/10	10/7/3	Y	c.1867delCT	E9	p.L623Vfs*24	4, 10
ID3/DZ	IAHSP	12/12/12	nr	nr	Y	c.3742delA	E22	p.M1206*	5, 6
ID4/FR	IAHSP	16	8	4	N	c.1471_1480del10	E6	p.V491Gfs*3	5, 6
ID5/IT	IAHSP	16	5	10	Y	c.2660delAT	E13	p.N845fs858	5, 6
ID6/IT	IAHSP	18	4	9	N	c.1130delAT	E4	p.I331fs335	5, 6
ID7/PK	IAHSP	18	nr	nr	Y	c.4844delT	E32	p.V1573fs*43	11
ID8/IL	IAHSP	12/14	9[a]/9[a]	3/3	Y	c.2992C > T	E18	p.R998*	12
ID9/IT	JPLS	24	19	6	N	c.1619G	E6	p.G540E	7
ID10/TR	IAHSP	12/12	12/10	16/nr	Y	c.467G > A	E4	p.C156Y	13
ID11/NL	IAHSP	8/18	13/8[a]	5/4	Y	c.2143C > T	E10	p.Q715*	14
ID12/HU	IAHSP	10/12	11/6	5/5	N	[c.1825_1826ins5] + [c.2529G > T]	E9 + E13	p.E609fs*9 + p.G1177*	15
ID13/DE	IAHSP	18	7	7	N	c.2000-2A > T	I9	p.E724fs*32	16
ID14/CY	JPLS	24/12/24	50/2/16[a]	6/2/2	Y	c.2980-A > G	I17	p.T993fs*7	17
ID15/IT	IAHSP	12	8	8	N	c.3836 + 1G > T	I24	p.K1234fs*3	This case

KWT, Kuwait; SA, Saudi Arabia; DZ, Algeria; FR, France; IT, Italy; PK, Pakistan; IL, Israel; TR, Turkey; NL, The Netherlands; DE, Germany; CY, Cyprus; nr = not reported; E/I = exon/intron.

[a] Use of walker.

[b] Bilateral support.

[c] Numbers in brackets refer to reference list.

exon 24 and another also retaining 225 bp of intron 24 (Supplementary Fig. 2A). Both transcripts predict a prematurely truncated protein, missing key protein domains (Supplementary Fig. 2B).

3. Discussion

The first report of IAHSP, in 1995, described three Kuwaiti children who developed progressive paresis in the lower limbs with subsequent progression to the upper limbs and bulbar muscles.[9] This was followed, shortly afterwards, by a description of three Jordanian children who showed a similar clinical evolution and associated gaze paresis.[10] This latter paper marked the introduction of the term "childhood primary lateral sclerosis" into the neurological literature. With the identification of further pathogenic mutations in *ALS2*, 12 additional families with the typical phenotype have been described (Table 1) and the terms IAHSP and JPLS have become commonly used.[3–7,11–17] To date, *ALS2* variants have been reported in a total of 27 patients, 20 with a diagnosis of IAHSP and seven patients with a diagnosis of JPLS based on earlier bulbar manifestations.[18]

From a clinical point of view the disease, characterized by childhood-onset pure spastic paraparesis followed by rapid ascent of paresis to the upper and oro-pharyngeal muscles, appears rather homogeneous. The disease manifests itself within the first decade of life (mean progression time 5.96 ± 3.23 years) and leads to severe tetraparesis and bulbar syndrome in the second decade (mean progression 12.8 ± 12.2 years), while mental function continues to be preserved.[3–7,9–17] In the reported *ALS2* families, including our case, the mean age at onset of motor disability was 19.7 ± 5.7 months (range 12–24) in JPLS and 13.3 ± 4.35 months (3–24) in IAHSP, whereas the mean onset of bulbar involvement was 5.14 ± 2.9 years (2–10) in JPLS, and 6.53 ± 4.8 years (3–16) in IAHSP. Patients are confined to a wheelchair by the mean age of 17.4 ± 19.28 years (range 2–50) in JPLS and 8.4 ± 3.2 years (4–12) in IAHSP. All the JPLS patients thus far described, as opposed to only three IAHSP ones (11% of the total), displayed impaired ocular motility ranging from slow voluntary eye movements to gaze paresis. Abnormal oculomotor activity in JPLS is thought to be associated with involvement of the upper motor neurons in the frontal cortex (Betz cells of area 8) that control ocular motility.[10]

Our patient showed brain MRI hyperintensities in posterior periventricular areas, which seems to be a common finding in IAHSP.[6,16] Additional imaging features include brain cortical atrophy predominant in the motor areas, subtle cerebellar atrophy, and bilateral hyperintense signals in the posterior arms of the internal capsule and brainstem.[6,9,13] Neurophysiological studies conducted in IAHSP patients consistently revealed a selective involvement of corticospinal and corticobulbar pathways; SEPs were normal or mildly delayed, and EMG showed a marked reduction of voluntary recruitment, as seen in our patient.[6,7,10,12–15,17] Scoliosis is rare in *ALS2*-disorders; it is described in 15% of IAHSP patients, but has not been reported in JPLS. Sphincter disturbances have been reported in only three IAHSP patients (11.1%). Thus, the main clinical differences between JPLS and IAHSP seem to be an earlier motor impairment and a more rapid course with possible occurrence of scoliosis in IAHSP, and the presence of oculomotor signs in JPLS. However, given that both IAHSP and JPLS appear to be part of the same clinical spectrum and there are, as yet, no clear correlations with the *ALS2* mutations, efforts to establish distinct nosographic groups may be of little value.

Alsin contains three putative guanine exchange factor (GEF) domains, which regulate the activity of members of the RAS superfamily of GTPases: the N-terminal regulator of chromatin condensation (RCC1) domain, the pleckstrin homology domain in the middle portion of the protein, and the vacuolar protein sorting 9 (VPS9) domain at the C-terminal.[19] The great majority of mutations predict early protein truncation and absence of the VPS9 domain, which specifically acts as a GEF for RAB5 GTPase, a key regulator of endocytosis during endosome fusion and trafficking.[19] In our patient, both transcripts detected in skin fibroblasts predict an abnormal C-terminus lacking at least one membrane occupation and recognition nexus motif and, in one case, also incorporating a region extremely similar to the STYKc domain (see SM00221 at smart.embl-heidelberg.de) needed for protein phosphorylation (Supplementary Fig. 2B). As a result, the new mutation may affect intracellular trafficking with a mechanism highly reminiscent of that of several other proteins involved in motor neuron diseases,[2,20] even though perturbation of other putative functions of alsin, such as neurite outgrowth and neuroprotection, cannot be excluded.[21]

In conclusion, we identified a novel *ALS2* mutation that further expands the allelic heterogeneity at the locus. Our patient presented a typical clinical course. As the disease progressed he developed severe scoliosis, placing him at risk of severe respiratory difficulties and life-threatening events.[6,9] Given the rather homogeneous presentation of IAHSP, a definitive molecular diagnosis can be essential for the provision of appropriate counseling and also quick and appropriate physiotherapy and orthopedic measures. Such a diagnosis should therefore be promptly sought.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejpn.2013.09.009.

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CASE REPORT Open Access

Novel *SPAST* deletion and reduced *DPY30* expression in a Spastic Paraplegia type 4 kindred

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Abstract

Background: The hereditary spastic paraplegias (HSPs) are pleiomorphic disorders of motor pathway and a large number of affected genes have been discovered. Yet, mutations in SPG4/*SPAST* represent the most frequent molecular etiology in autosomal dominant (AD) patients and sporadic cases. We describe a large, AD-HSP Sardinian family where 5 out of several living members harbored a novel deletion affecting also the 5'UTR of *SPAST* and resulting in reduced expression of *DPY30*, the gene located upstream *SPAST* in a head-to-head manner.

Case presentation: A 54-year-old woman manifested leg stiffness at age 39 and required a cane to walk at age 50. Neurological examination disclosed mild spasticity and weakness in the legs, hyperreflexia in all limbs, and bilateral Babinski sign. She also complained of urinary urgency, but no additional neurological symptoms or signs were detected at examination. The clinical examination of 24 additional relatives disclosed three further affected individuals, two men and one woman. In the four symptomatic patients the initial manifestations were walking abnormalities and leg stiffness with a mean age at onset (SD) of 46.75 (5.44) years (range 39–51). The mean disease duration was 13.2 (13.4) years (range 6–35), and it correlated well with clinical severity (SPRS score) ($r = 0.975$, $p = 0.005$). One patient was confined to bed and displayed knee and ankle contractures, another case needed a cane to walk, and two individuals were able to walk without aids. Interestingly, a patient had also had a miscarriage during her first pregnancy.
Gene testing revealed an heterozygous deletion spanning from the 5'-UTR to intron 4 of *SPAST* in the affected individuals and in one clinically unaffected woman. In three affected patients, the deletion also determined low mRNA levels of *SPAST* and *DPY30*, a component of the Set1-like multiprotein histone methyltransferase complex located upstream, head-to-head with *SPAST*.

Conclusion: Together with data described in a Japanese family, our findings seem to suggest that genes close to spastin might be candidates in modulating the clinical phenotype. This report endorses future research on the role of neighboring genes as potential players in SPG4 disease variability.

Keywords: SPG4, *DPY30*, Genetic modifier, Deletion

Background

Heterogeneity is a key feature of the hereditary spastic paraplegias (HSPs). To date, autosomal, sex-linked, and cytoplasmic inheritance have been reported, an ample array of complicated phenotypes disclosed, more than 70 loci mapped, and roughly 50 disease genes cloned [1,2]. In common clinical practice, the gene-after-gene testing strategy allows definition of the molecular basis in about half of HSP cases, unless peculiar features emerge during examination or follow-up.

Mutations in *SPAST*/SPG4 encoding spastin represent the most common cause of autosomal dominant hereditary spastic paraplegias (AD-HSP) and also account for about 15% of sporadic cases [3,4]. In north-west Sardinia the relative frequency of HSP is higher than what is calculated in other Western European populations with an estimated crude prevalence of about 17.5/100,000 for AD-HSP [5]. As documented in several families and different populations [4,6], the SPG4 phenotype is usually pure and inter- and intra-familial variability of the clinical presentation are well established [7]. In some cases, single nucleotide polymorphisms in *SPAST* and variants

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in additional genes are invoked as modifiers of age at onset, disease course and severity [8,9].

We identified a novel *SPAST* mutation segregating in a Sardinian kindred (family IK).

Case presentation

The proband, a 54-year-old woman (IV-13) (Figure 1), manifested leg stiffness at age 39 and required a cane to walk at age 50. Neurological examination disclosed mild spasticity and weakness in the legs, enhanced deep tendon reflexes in all limbs, and bilateral Babinski sign. Case IV-13 also complained of urinary urgency, but no additional neurological symptoms or signs were detected at examination. The patient reported that her deceased father (III-03) and two uncles (III-02, III-04) had manifested similar gait abnormalities in adult age, although with different degree. Detailed clinical information and examinations were gathered from her living relatives.

Materials and methods

Total genomic DNA was purified by peripheral blood with standard methodologies. Analysis of common AD genes associated with HSP used traditional Sanger sequencing and the BigDye 3.1 Chemistry, as reported [5]. Search for copy number variation and gene deletion/duplication adopted reported array-comparative genomic hybridization (aCGH) and multiple ligation-dependent probe amplication (MLPA) methodologies [10].

Twenty-four additional relatives were examined and sampled with written informed consent in family IK. Using oligonucleotide primers (5'-3') SPASTPrimer F, SPASTPrimer R1 and SPASTPrimer R2 that were designed flanking the mutation deletion breakpoints (sequences available upon request), we PCR-amplified genomic DNA to generate a single 955-bp fragment in wild-type individuals. The presence of the heterozygous *SPAST* deletion produced an additional fragment of 303-bp (Figure 2).

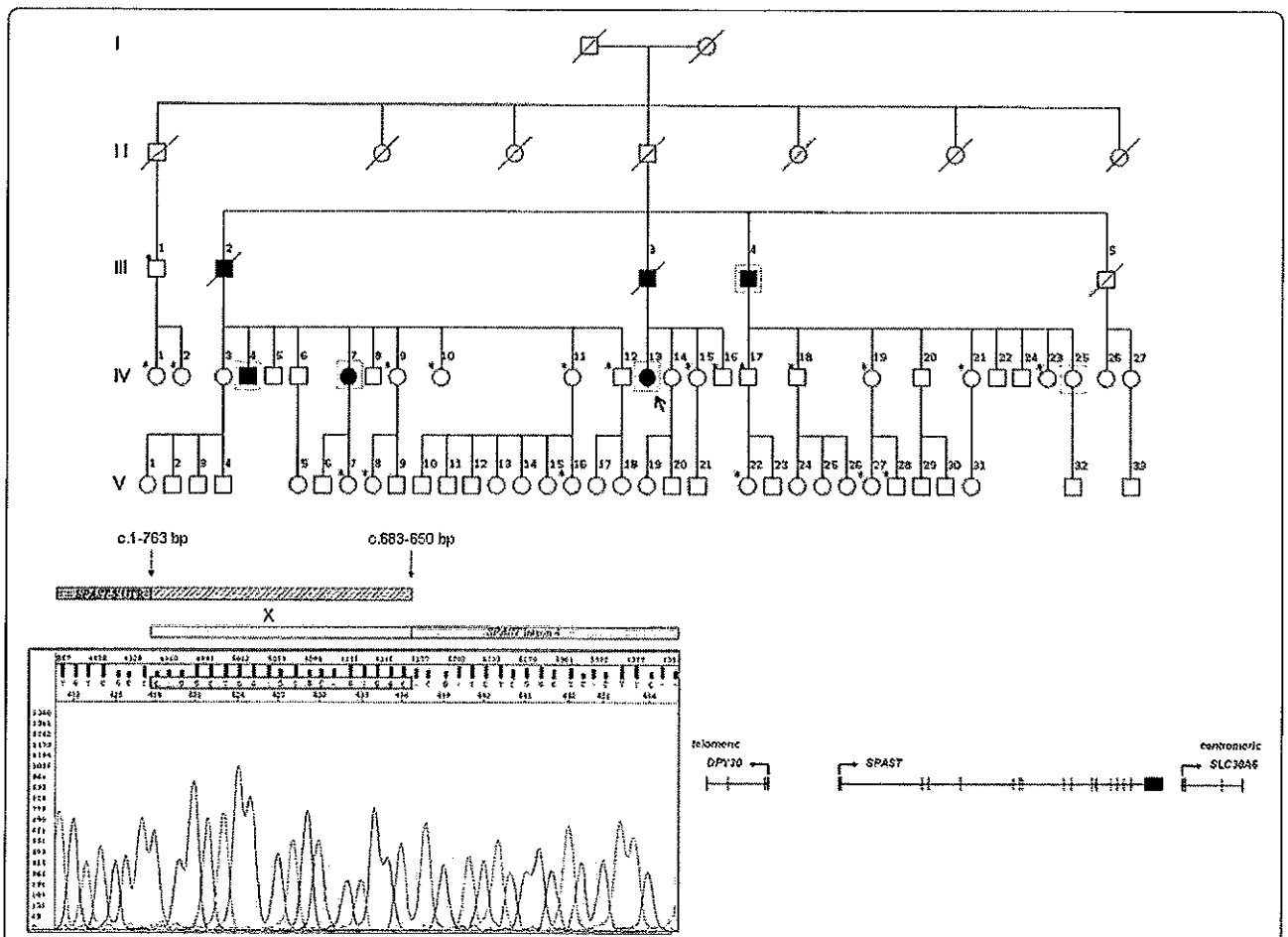

Figure 1 (*Top*) **Family pedigree.** The arrow indicates the index case. Squares, men; Circles, women. Black symbols indicate clinically affected individuals; empty symbols show clinically healthy relatives. Carriers of the novel mutation in SPG4/*SPAST* are indicated with a dotted frame. Star symbols indicate individuals who underwent DNA studies but then tested normal. (*Bottom*) Electropherogram flanking the novel mutation c.1-763_c.683-650del affecting the 5'UTR and exons 1–4 of *SPAST*, and the position of spastin neighboring genes.

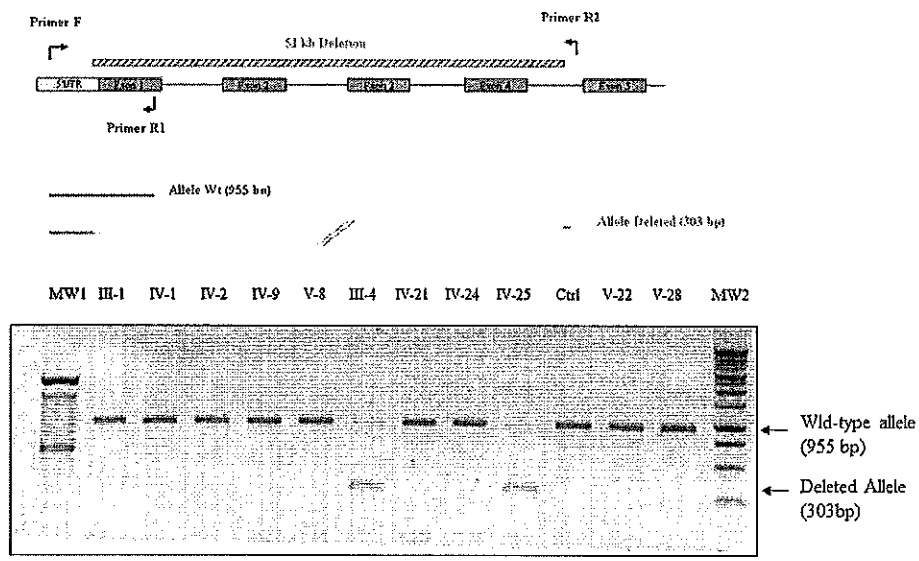

Figure 2 Rapid diagnostic genotyping of the multi-exon deletion detected in the IK family by a duplex-PCR method.

To test the effects on mRNA expression of the mutation deletion in *SPAST*, total blood RNA was extracted using a micro-scale total RNA separator kit (Ambion INC., Austin, TX). For standard gene expression experiments, the mRNA transcript levels were determined by qPCR runs in an ABI7500Fast system (Applied Biosystems, Foster City, CA) using the TaqMan Universal PCR Protocol, and human *SPAST* (Hs00368084_m1, Applied Biosystems), *SLC30A6* (Hs01071782_m1), and *DPY30* (Hs00261491_m1) as probes. *GAPDH* (Hs99999905_m1, Applied Biosystems) was used for endogenous normalization, and expressions were determined using the comparative Ct method [11]. Values were normalized in reference to the average control value obtained from three age-matched normal control subjects. Statistical analyses used unpaired two-tailed Student-test (significance was set at $p < 0.05$).

Results

No point mutations were found in *SPAST* and analyses of other frequent AD-HSP etiologies (namely, SPG3A/*ATL1*, SPG31/*REEP1*, SPG10/*KIF5A*, SPG8/*KIAA0196*) were all normal in the proband. Combination of customized aCGH, MLPA analysis, and direct sequencing identified a novel heterozygous mutation (c.1-763_c.683-650del) spanning 51 kb, from the 5'-UTR (and upstream regulatory elements) to intron 4 of *SPAST*. The deletion mutation was not found in the NCBI genomic structural variations database (http://www.ncbi.nlm.nih.gov/dbvar/?term=human+SPAST) nor in polymorphic databases.

Using a rapid PCR-based method to quickly genotype individuals in the family, and rule out the mutation in 500 ethnically-matched control chromosomes, we identified the new mutation in a total of five individuals, including the yet asymptomatic IV-25 (Figure 1). In all, global disease severity was assessed using the Spastic Paraplegia Rating Scale (SPRS) [12]. Table 1 summarizes clinical data in carriers of the novel *SPAST* mutation deletion. In the four symptomatic patients the initial manifestations were walking abnormalities and leg stiffness with a mean age at onset (SD) of 46.75 (5.44) years (range 39–51). The mean disease duration was 13.2 (13.4) years (range 6–35). Pearson's correlation coefficient indicated a positive correlation between disease duration and clinical severity (SPRS score) ($r = 0.975$, $p = 0.005$). One patient (III-04) was confined to bed and displayed knee and ankle contractures, another needed a cane to walk, and two individuals were able to walk without aids. Interestingly, subject IV-07 had also had a miscarriage during her first pregnancy. At this point, we cannot state if IV-25 will develop any clinical manifestation in the near future or if she is a true not penetrant carrier of the mutation, since her present age is slightly younger than the mean age of onset of motor disturbances in the family. Reduced or no penetrance has repeatedly been described in spastin-related HSP kindred [1,3], including cases from Sardinia [10].

In three affected patients, we observed that the deletion also determined low mRNA levels of *SPAST* and *DPY30*, a component of the Set1-like multiprotein histone methyltransferase complex located upstream, head-to-head with *SPAST* [15] (Figure 3).

Discussion

This report is the third description of a deletion characterizing the 5'-UTR of *SPAST*. Interestingly, the mutation also affected even more upstream sequences likely

Table 1 Clinical features in the five patients harboring the mutation in the IK family

	III-04	IV-04	IV-07	IV-13	IV-25
Age/Sex	86/M	53/M	60/F	54/F	42/F
Age at Onset, yrs	51	47	50	39	NA
Disease duration, yrs	35	6	10	15	0
SPRS score[a]	47	12	6	16	0
SPRS item 13[b]	4	1	1	1	0
Spasticity score LL[c]	4	2	1	2	0
Muscle strenght score LL[d]	0	5	4	4	5
Hyperreflexia UL/LL	Contracture	0/+ + +	0/+ +	+/+ + +	0/0
Babinski sign	+	+	Indifferent	+	Indifferent
Decreased vibration sense	na	No	No	No	No
EMG/NCS	na/na	Abnormal*/n	n/n	n/n	na/na
Miscarriage/Pregnancy			1/3	0/0	0/1

M man, *F* woman, *UL* Upper Limbs, *LL* Lower Limbs, *na* not available, *EMG* electromyography, *NCS* nerve conduction studies, *n* Normal; *slight impairment but no frank signs of denervation.
[a]The SPRS score ranges from 0 (normal) to 52 (severely affected) [12]; [b]1 = Urinary urgency (difficulties to reach toilet in time); 4 = Permanent catheterization; [c]The Modified Ashworth Scale of Muscle Spasticity score ranges from 0 (no increase in tone) to 4 (affected parts rigid in flexion or extension) [13]; [d]The Medical Research Council Scale for Muscle Strength score ranges from 0 (no contraction) to 5 (normal strength) [14].

regulating *SPAST* and the neighbor *DPY30* gene. Previously, a deletion was found in a Japanese family with clinical features and disease duration highly similar to our cases [16]. Also, six men and four women in a further Japanese kindred harbored a 70 kb deletion involving exons 1 to 4 of *SPAST* and also exons 1 to 3 of *DPY30* [17]. Those patients had on average a teenage onset and a slowly progressive course leading to wheelchair in four, and use of a walking stick in one case. Other clinical features in that family were mild cognitive impairment and slight peripheral neuropathy. We believe that the presence of the spastin deletion might well explain a tendency towards less severe walking disability in family IK and in the Japanese kindred as described before in other families [3,9]. It is, however, intriguing that all affected Japanese women experienced miscarriages of unknown etiology similar to subject IV-07 in our kindred. Whether the shared feature of birth interruption relates to a similar defect in *DPY30* is still questionable.

DPY30 is yet to be fully characterized but it seems to be essential for neural fate of embryonic cells, cell-cycling and cellular proliferation [15,18]. In nematodes, null mutations in dpy-30 cause XX-specific lethality and the gene is required for normal development of XO males [19]. As it orthologue, it can be speculated that human *DPY30* is also implicated in brain development and infertility [20] and when mutated might lead to miscarriages. The hypothetical function of the gene, however, cannot clearly explain how a low *DPY30*/mRNA expression (probably because of a position effect involving long-range gene regulatory elements) in the IK family with a milder phenotype agrees with the reduced mRNA levels (because of partial gene deletion) in the Japanese family with an earlier onset and apparently more severe neurological features [17]. We have no clear-cut explanation for this apparent "clinical riddle" other than raising the possibility of additional modifiers in the two families, maybe related to the different ethnic

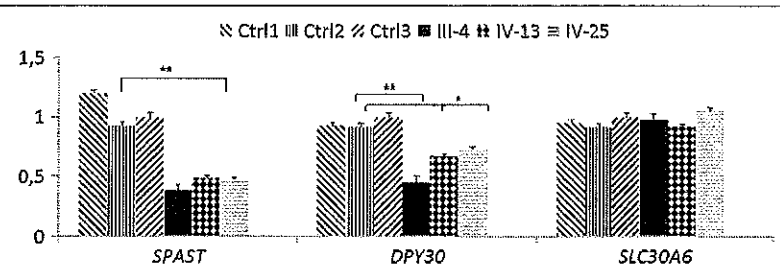

Figure 3 Bar chart showing the relative expression of mRNA/*SPAST*, mRNA/*DPY30*, and mRNA/*SLC30A6* in blood. Data are presented from three patients in the IK family (samples refer to pedigree in Figure 1) with error bars representing the mean ± SD of three different determinations. Values are reported as the ratio to the internal control (GAPDH), and are also expressed in reference to the average control value from three age-matched normal subjects (Ctrls). **, $p < 0.01$; *, $p < 0.05$.

origin. Yet, alike the recent identification of a small deletion of *SLC30A6* — the gene flanking *SPAST* 3'UTR — in another Italian SPG4 family [10], this report endorses future research on the role of neighboring genes as potential players in SPG4 disease variability.

Conclusion

We describe an AD-HSP Sardinian family where 5 out of several living members harbored a novel deletion affecting also the 5'UTR of *SPAST* and resulting in reduced expression of *DPY30*, the gene upstream *SPAST* in a head-to-head manner. If the presence of the spastin deletion might well explain a tendency towards less severe walking disability in our family, it is intriguing that a patient in our kindred experienced a miscarriage of unknown etiology similar to all affected women in a Japanese family harboring a *SPAST* and *DPY30* deletion. This report encourages future research on the role of neighboring genes as potential players in SPG4 disease variability.

Consent

Written informed consent was obtained from the patients in the family for publication of this Case report.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

LR was involved in the acquisition and analysis of clinical data. AT and ES carried out the molecular genetic studies and participated in drafting the manuscript. MP was involved in the collection of patients and in the acquisition and analysis of clinical data. VA participated in the coordination of the study and revised the draft critically. FMS and LR conceived the study, participated in its design and coordination and contributed to draft the manuscript. All authors read and approved the final manuscript.

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Pubblicazione V

Bridging Over the Troubled Heterogeneity of *SPG*-Related Pathologies: Mechanisms Unite What Genetics Divide

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Abstract: The hereditary spastic paraplegias (HSP) are characterized by spastic gait with weakness in the legs and additional neurological or extra-neurological signs in "complicated" forms.

The past two decades have witnessed major advances in our understanding of their molecular bases with the identification of a plethora of loci and the cloning of several *SPG* genes. Combined genetic and clinical information has permitted a modern, molecularly-driven classification and an improved diagnosis, with several new data on the possible disease mechanisms. Further heterogeneity will rapidly emerge with the diffusion of next-generation sequencing platforms and, under the shadow of common themes in the pathogenesis, new therapeutic options will likely emerge for a great number of patients.

Keywords: Hereditary spastic paraplegia, genetic, genotype/phenotype correlations, heterogeneity, mechanisms of disease, mutation, *SPG*.

INTRODUCTION

The hereditary spastic paraplegias (HSP) constitute a clinically and genetically heterogeneous group of neurodegenerative disorders characterized by insidiously progressive weakness and spasticity of the lower limbs (pure forms), which may be combined with additional neurological or non-neurological manifestations (complicated subtypes) [1]. HSP are rare conditions, with an estimated incidence of 1.27-9.6 cases/100,000 in the "pregenetic" era, and affect any age, from early childhood to adult life. Recent epidemiological studies in selected populations (i.e., Portugal, southeastern Norway) estimated a prevalence of 4.1-7.4/100,000 individuals, considering also cases in which a molecular diagnosis had been reached, regardless of the pattern of inheritance [2, 3].

On clinical grounds, the initial symptoms in HSP are stiffness in the legs usually progressing to a marked spastic gait requiring the use of a cane, a walker, or a wheelchair to move around. Additional features are mainly enhanced or brisk deep tendon reflexes, extensor-plantar responses, mildly diminished vibration or joint position sensation on the lower limbs, and pyramidal muscle weakness. Spasticity and weakness are maximal in the iliopsoas, hamstring, and tibialis anterior muscles [4]. When these signs are the only clinical features observed in the patients, the disease is described as "pure". "Complicated" forms are associated with a plethora of additional signs or symptoms, including among others mental retardation, peripheral neuropathy, cerebellar ataxia, epilepsy, optic atrophy, retinitis pigmentosa, deafness, and cataracts.

Unpredictability in progression and severity, both within and among families, and variable age at onset are well known in HSP, particularly in pure autosomal dominant (AD) forms. Reduced penetrance and expressivity also run in families [5], and are thought to be age- and sex-dependent [6]. Modifiers, even intragenic, of HSP genes [7, 8] are also invoked to modulate the phenotype.

There is frequent clinical overlap among most of the HSP forms limiting our possibility to predict the genotype on clinical grounds only. Clinical presentation and pattern of inheritance, however, may be a useful clue in prioritizing molecular diagnosis [9] even in children [10]. A larger knowledge of the molecular bases has also modernized classification criteria in HSP, even though combination of findings gathered at the neurological examination and neuroimaging remain the most important criteria for labeling a "definitely" or "probably" affected individual and to start a therapy. Importantly, it must be said that better understanding of the physiopathology and etiology can foster future therapies since current treatments continue to be largely symptomatic.

Physiopathological mechanisms responsible for HSP are also variable and largely unclear. Study of cellular and animal models [11-17] proposes impairment of intracellular trafficking, mitochondrial energy production, lipid metabolism, and defective axonal transport as the responsible mechanisms for

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degeneration in the longest fibers of the corticospinal tracts. This causes, in turn, a pyramidal tract dysfunction that accounts for spasticity and for the signs observed in patients at clinical evaluation [18]. In addition to the long fibers, other brain structures, including the cerebellum, cerebral cortex, or white matter structures may be affected in complicated forms of HSP, as evidenced by MRI [19]. This is best illustrated by a form of HSP associated with thinning of the corpus callosum (TCC) and mental deficiency, which has a worldwide distribution [20, 21] and seems to account for 30-35% of autosomal recessive (AR) forms of HSP [22].

This short review will focus on the latest genetic and physiopathological information in the study of HSP with particular attention to the most frequent genes.

GENETIC AND PHENOTYPIC HETEROGENEITY

The marked clinical variability observed in HSP is made even more complex by a large genetic heterogeneity. All modes of inheritance (AD, AR, or X-linked) have been reported and are associated with a multiplicity of genes or loci. Maternal inheritance is a more recent possibility described in five members of a family carrying the homoplasmic m.9176T > C (p.L217P) in the mtDNA-encoded ATPase6 and associated with a late-onset SPG-like disorder [23]. In addition, isolated cases represent the largest cohort of patients examined in the clinical practice and if they are the results of *de novo* mutations or even explained by a "missing" heritability or an overlooked AR transmission is a frequent question at bedside. To date, according to the latest classifications, 58 different loci and 39 disease-associated *SPG* (Spastic gait) genes have been identified. The ever growing list of the known forms of HSP is summarized in Table 1.

Overall, among the mendelianly-inherited forms of the disease, the AD forms (ADHSP) represent 70- 80% of the whole set in western countries [24], and are in general pure in clinical terms. In contrast, the AR forms (ARHSP) seem to be less frequent, even though common in inbred populations, more complex and usually associated with earlier age at onset [25].

X-Linked Forms

Five HSP loci have been localized on chromosome X. SPG1 is a rare form and results from mutations in the *L1CAM* gene which encodes the neural cell adhesion molecule involved in migration and differentiation of neurons. SPG2 is caused by mutations in *PLP1*, which encodes the myelin component proteolipid protein. SPG2 is allelic variant to the Pelizaeus- Merzbacher disease (PMD), a hypomyelineted leukodystrophy. PMD is caused mostly by gene duplication whereas point mutations (missense, nonsense) seem to be responsible for its allelic form SPG2 [26]. In SPG1, only affected boys present with a complex phenotype including spastic paraplegia and mental retardation, aphasia, shuffling gait, and adducted thumbs (MASA-syndrome). Rare, additional phenotypic manifestations include hydrocephalus due to aqueduct stenosis or agenesis of the corpus callosum. Pure, congenital spastic paraparesis is exceptional. SPG16, SPG22, and SPG34, whose genes are as yet unknown, relate to forms complicated by mental retardation.

Autosomal Dominant Forms (ADHSP)

Less than 20 AD loci and 11 genes are already known. The most common ADHSP is caused by mutations in the SPG4 gene (*SPAST*/SPG4), encoding spastin, a member of the AAA family of ATPases. The mutational spectrum of *SPAST* is ample, including missense, nonsense, and large scale rearrangements. The mutations detected so far account for about 40-50% of all the ADHSP cases [27, 28]. They are responsible for a pure spastic paraplegia of variable severity, with a bimodal age at onset (the two peaks are around age 9 and 30-35years), although it has been shown that sensory disturbances, sphincter problems, and mild peripheral nerve system symptoms occur in about half of the cases, and hand tremor is found in roughly 10% of the cases. More complex phenotypes (e.g., late cognitive decline, peripheral neuropathy, TCC) have seldom been recognized [8].

The second frequent ADHSP gene (*ATL1*/SPG3A), encoding atlastin 1, is mutated in approximately 10% of cases [29, 30]. The forms associated with mutations in this gene are mostly pure, and usually with an earlier onset, before age 10 years [31].

KIAA0196/SPG8 encoding strumpellin, *SPG31*, encoding the mitochondrial protein REEP1, and *KIF5A*/SPG10, encoding kinesin 5A, are the other genes that are mutated in pure forms in several families, and have estimated frequencies of 8% [32], 5%, and 3%, respectively. The remaining ADHSP loci/genes are less common. In some cases, there are distinctive clinical features as in the case of *BSCL2*/SPG17 [33] that is associated with a complex form, also known as Silver syndrome, characterized by prominent distal amyotrophy in the hands. Sometimes memory impairment could be additionally observed in SPG6 patients. Interestingly, *RTN2*, the gene mutated in SPG12, [34] encodes reticulon 2 that interacts with spastin and is involved in endoplasmic reticulon shaping. Patients usually present with pure, rapidly progressive spastic paraparesis, requiring a wheel chair shortly after onset. SPG9, SPG29 and SPG38 are linked to complex forms of the disease. SPG9 was identified in two families where cataract, gastroesophageal reflux, amyotrophy and skeletal abnormalities emerged in their early 30's [35]. Table 1 illustrates the spectrum of genetic forms of ADHSP.

Autosomal Recessive Forms (ARHSP)

The overabundance of clinical features associated with ARHSP correlate well with the ever-growing list of

Table 1. Spectrum of genetic forms and *Spastic gait* genes (SPG) in hereditary spastic paraplegias. MIM, Mendelian inheritance in men.

Locus (MIM)	Gene (Protein)	Frequency	Age at Onset, *Years*
X-Linked Forms			
SPG1 (308840)	*L1CAM*	Over 100 families	Infancy
SPG2 (300401)	*PLP*	<100 families	Infancy
SPG16 (300266)	Unknown	1 family	Infancy
SPG22 (300523)	*SLC16A2*	Several	Infancy
SPG34 (300750)	Unknown	1 family	Infancy
Autosomal Dominant Forms			
SPG3A (182600)	*SPG3A*	10% (39% of young patients)	Early onset (most < 10 yrs)
SPG4 (182601)	*SPG4*	40% (12%–18% of sporadic cases)	Variable
SPG6 (600363)	*NIPA1*	< 1% (9 families)	8–40
SPG8 (603563)	*KIAA0196*	8% (6 families)	18–60
SPG9 (601162)	Unknown	1 family	First to third decade
SPG10 (604187)	*KIF5A*	3% (7 families)	2–51
SPG12 (604805)	*RTN2*	5 families	7–24
SPG13 (605280)	*HSPD1*	Rare (2 families)	Mostly early onset
SPG17 (270685)	*BSCL2*	Several	Variable
SPG19 (607152)	Unknown	1 family	36–55
SPG29 (609727)	Unknown	1 family	Childhood
SPG31 (610250)	*REEP1*	6.5%	Adulthood
SPG36 (613096)	Unknown	1 family	Adulthood
SPG37 (611945)	Unknown	1 family	8–60
SPG38 (612335)	Unknown	1 family	16–18
SPG41 (613364)	Unknown	1 family	>36
SPG42 (612539)	*SLC33A1*	1 family	4–42
SPG58 (609797)	*BICD2*	1 family	<40
Autosomal Recessive Forms			
SPG5 (270800)	*CYP7B1*	10%	1–40
SPG7 (607259)	*SPG7*	1%–4%	11–42
SPG11 (604360)	*KIAA1840*	21% (59% of ARHSP-TCC)	1–27
SPG14 (605229)	Unknown	1 family	Adulthood
SPG15 (270700)	*ZFYVE26*	2-4% (12% of ARHSP-TCC)	13–23
SPG18 (611225)	*ERLIN2*	1 family	Variable
SPG20 (275900)	*KIAA0610*	Amish founder	Early childhood
SPG21 (248900)	*ACP33*	Amish founder	20–40
SPG23 (270750)	Unknown	1 family	Early childhood
SPG24 (607584)	Unknown	1 family	Early childhood
SPG25 (608220)	Unknown	1 family	30–46
SPG26 (609195)	*B4GALNT1*	5 families	Infancy
SPG27 (609041)	Unknown	2 families	Variable

(Table 1) contd.....

Locus (MIM)	Gene (Protein)	Frequency	Age at Onset, *Years*
Autosomal Recessive Forms			
SPG28 (609340)	*DDHD1*	3 families	6–15
SPG30 (610357)	*KIF1A*	4 families	12–21
SPG32 (611252)	Unknown	1 family	Infancy
SPG35 (612319)	*FA2H*	4 families	6–35
SPG39 (612020)	*PNPLA6* (NTE)	2 families	Childhood
SPG43 (615043)	Unknown	1 family	Early childhood
SPG44 (613206)	*GJC2*	1 family	Early childhood
SPG45 (613162)	Unknown	1 family	Infancy
SPG46 (614409)	*GBA2*	4 families	10-20
SPG47 (614066)	*AP4B1*	1 family	Infancy
SPG48 (613647)	*AP5Z1*	1 family	Adulthood
SPG49 (615031)	*TECPR2*	3 families	Childhood
SPG50 (612936)	*AP4M1*	2 families	Congenital
SPG51 (613744)	*AP4E1*	4 families	Congenital
SPG52 (614067)	*AP4S1*	1 family	Congenital
SPG53 (614898)	*VPS37A*	2 families	Early onset
SPG54 (615033)	*DDHD2*	2 families	Early onset
SPG55 (615035)	*C12orf65*	2 families	Early onset
SPG56 (615030)	*CYP2U1*	4 families	Variable
SPG57 (604484)	*TFG*	3 families	Early onset

molecular etiologies among these subtypes: 33 genes have been cloned, and about 25 have been discovered, but more than 15 (60%) in the past two years (Supplementary Fig. 1).

SPG5A is due to mutations in the *CYP7B1* gene, which encodes the oxysterol 7-alpha-hydroxylase 1 [36], accounts for about 10% of the cases [37], and it is probably the second most frequent gene mutated in ARHSP. Most commonly, the phenotype in SPG5 is "pure" but in single patients the presentation is blurred with slight cerebellar signs and white matter changes on MRI [38], with a multiple sclerosis-like appearance. Extensive electrophysiological investigations in three families revealed normal nerve conduction, normal EMG, abnormal conduction along the central pathway on somatosensory- and motor-evoked potentials, and damage of the visual pathway Interestingly, optic atrophy is not rare in SPG5 [39]. Accumulation of 27-hydroxy-cholesterol in blood of SPG5 patients might have important impact on clinical practice as biomarker of disease status.

SPG7 is an AR condition due to mutations in the *SPG7* gene, which encodes paraplegin, a subunit of an ATP-dependent AAA-protease located within the inner mitochondrial membrane. *SPG7* accounts for about 4-7% of the ARHSP cases [40], but there are a large number of heterozygous missense variants with a still unclear role, that could represent susceptibility factors or causative mutations [40, 41]. SPG7, which was initially reported to be a pure form of the disease, has now been shown to be either pure or associated with cerebellar atrophy, optic atrophy and variable degrees of cerebellar dysfunction, neuropathy, and mental deficits [42]. Mutations in *SPG7* also cause about 10% of sporadic upper motor neuron disease without bulbar involvement [43] and spastic ataxia with optic atrophy [44].

KIAA1840/SPG11 encodes spatacsin and is the most frequent gene mutated in ARHSP (25% of the cases) [22, 45]. The phenotype is relatively homogeneous. It consists of early-onset (before age 25 years) spastic paraparesis and cognitive impairment, sometimes diagnosed as mental retardation, that progresses insidiously to severe functional disability over a period of 10 to 20 years. Some patients also develop involvement of the arms, pseudo-bulbar dysarthria, Parkinsonism, cerebellar signs and muscle atrophy. Neuroradiological examinations show TCC with variable cerebral cortical atrophy that worsens with time. Recent studies specified that the thinning of the corpus callosum does not proceed with the progression of the disease and brain MRI detects white matter hyperintensities in about 60% of the cases, with a specific and distinctive pattern [45] even in early disease stages [46]. A variable glucose hypometabolism in cortical and thalamic regions was

evidenced by PET, and electroneuromyography usually detects predominantly axonal motor or sensorimotor peripheral neuropathy [47].

A highly similar clinical and MRI phenotype is the one linked to *ZFYVE26*/SPG15 encoding spastizin, a protein interacting with spatacsin, and thought to account for 5% of ARHSP [48, 49]. The SPG15 phenotype was initially associated with Kjellin syndrome, a complex form of HSP with macular degeneration, cerebellar signs, mental impairment, and thinning of the corpus callosum, with onset before the third decade. The cerebellar signs and macular degeneration were not consistently found in subsequent studies, but the phenotype has been extended to Parkinsonism, and axonal neuropathy with distal wasting. Collectively, SPG11 and SPG15 are the two major forms responsible for ARHSP-TCC, of which they account for up to 60% and 12% of the cases, respectively [45, 49]. More rarely, mental impairment and TCC is also found in patients linked to *SPG21*, *SPG32*, *SPG7*, or *SPAST*/SPG4 [50]. Other ARHSP loci are less common, occurring in a few families in association with even more complex clinical features [51].

Sporadic Cases

Identification of the genetic defect in isolated cases of HSP is the most relevant question clinical neurologists and neurogeneticists ask in medical practice, and the most difficult to characterize at the molecular level. A large array of quite similar neurological diseases (e.g., leukodystrophies, multiple sclerosis, tumors, human T-lymphotrophic virus type 1 infection, Dopa-responsive dystonia, primary or amyotrophic lateral sclerosis (ALS), arginase deficiency, Friedreich ataxia, among others) should be excluded through MRI and biochemical analyses [52]. One should also consider that a sporadic presentation of spastic legs may occur at onset in ALS, Machado-Joseph disease (SCA3), early-onset Alzheimer disease due to *PS1* mutations, structural abnormalities of the brain and spinal cord, and mitochondrial disorders. The list of tests to be considered for a proper differential diagnosis is challenging particularly in children with progressive gait disturbance and stiff legs [10]. Mutations in known HSP genes can be tested at that point. In particular, about 15-20% of sporadic patients harbor mutations in *SPAST*, though they seem to have a less severe phenotype than typical familial forms [53]. "Apparently" sporadic cases of ADHSP can result from incomplete penetrance [53], *de novo* mutations or under diagnosis in small kindred. Sporadic patients may also carry two mutations in relatively frequent ARHSP genes (i.e. *CYP7B1*/SPG5, *SPG7*, *KIAA1840*/SPG11), even in the absence of parental consanguinity or close relationships.

PATHOGENETIC MECHANISMS

Upon the identification of several genes and their products, it has emerged that the main cellular functions altered in HSP can easily be grouped under few important categories. This is intriguing if one considers the large clinical and genetic heterogeneity.

Main abnormal functions in HSP involve intracellular trafficking, lipid homeostasis and metabolism, and mitochondrial activity.

Intracellular Trafficking

The largest group of HSP gene products with known or supposed function is represented by proteins involved in intracellular trafficking and the ensuing axonal transport. The gene product of *KIF5A*/SPG10, kinesin-5A, is a motor protein which controls microtubule-dependent anterograde axonal transport. A reduced gliding velocity during the transport and a lower affinity for microtubules characterize the mutant protein [54]. Spastin (*SPAST*/SPG4) is a member of the AAA family (ATPase with various cellular activities). It has three main structural domains: a microtubule interacting and endosomal trafficking domain (MIT) at the N-terminus, a second microtubule interacting domain, and the ATPase AAA domain at the C-terminus [55]. In mammalian cells, spastin localizes to cytoplasmic areas important for microtubule dynamics [56] and it seems to be implicated in the disassembling of microtubules even in animal models [13, 57]. *In vitro* studies have confirmed that spastin can also bundle polymerized microtubules [58]. Its microtubule-severing activity in cultured neurons seems related to axonal branching [59]. *Spast* knock-out mouse models showed axonal swellings close to the growth cone of spinal cord axons with abnormal accumulations of organelles and cytoskeletal components, suggesting a defect in axonal transport [14]. The evidence for a role of spastin in microtubule turnover implicates that the mutated protein could affect axonal transport and lead to abnormal neuronal growth and degeneration.

Spastin interacts with another HSP-related protein, atlastin-1 (*ATL1*/SPG3A) [60, 61], a dynamin/guanylate-binding protein with transmembrane domains, predominantly expressed in the CNS. Its subcellular localization (endoplasmic reticulum (ER), vesicular structures in axonal growth cones, and axonal branch points) suggested a functional role in intracellular membrane trafficking but also neurite outgrowth in axonal development. More recently, it has been proposed that atlastin is rather involved in ER and Golgi morphogenesis and shape [62].

The NIPA1 protein (SPG6) is a neuron-specific transmembrane protein. In a variety of neuronal and epithelial cells it appeared localized in the early endosomal compartment and on the plasma membrane, where it is thought to be a magnesium transporter [63]. Interestingly, the NIPA1 protein is a direct binding partner of atlastin-1 and it is also an inhibitor of the bone morphogenic protein (BMP) signaling and interacts with the BMPII receptor [64]. BMP signaling is important for distal axonal function. Other inhibitors of the BMP signaling are spastin/SPG4 and spartin/SPG20.

SPG20 encodes spartin, an AAA protein with an MIT domain, like spastin. Controversial studies have demonstrated its subcellular localization in mitochondria [65] as well as in the nucleous and cytoplasm (synapse-like structures, neurites and trans-Golgi network in differentiated neurons) [66]. Importantly, spartin interacts with the E3 ubiquitine ligase and cardiolipin, a major mitochondrial phospholipid, and it is involved in determination of the lipid droplet size. Thus, endosomal trafficking and mitochondrial calcium uptake and membrane potential may be impaired in *SPG20* [67].

Spatacsin (*KIAA1840*/SPG11) is particularly expressed in cortical and spinal motor neurons [68] with a cytoplasmic distribution. Spatacsin co-localizes with protein-trafficking vesicles, ER, mitochondrial surface, and microtubules and interacts with members of the AP5 complex [69], and likely has a role in autophagy. One study has shown the accumulation of intra-axonal pleomorphic membranous material in unmyelinated axons of sural nerves of SPG11 patients, suggesting a disturbed axonal transport [70].

Spastizin (*ZFYVE26*/SPG15) is a zinc finger protein with a FYVE domain, known to bind membrane phosphatidyl-inositol 3-phosphate during endosomal trafficking. This lends hands to the hypothesis of a role in membrane trafficking. Spastizin is among the preferred partners of spatacsin.

Mitochondrial Function

Few HSP proteins are directly involved in mitochondrial function. Paraplegin (the gene product of *SPG7*) belongs to the mitochondrial AAA metalloprotease family [71]. Members of this protein family form an ATP-dependent proteolitic complex on the inner mitochondrial membrane that controls assembly and folding of the components of the respiratory chain, degradation of unassembled subunits, and activation of other proteins [72]. Loss of the metalloprotease AAA complex in fibroblasts from patients with *SPG7*-associated HSP seemed to cause reduced activity of complex I in the mitochondrial respiratory chain and increased sensitivity to oxidative stress [40, 73]. A loss of mitochondrial energy could, in turn, affect axonal transport. Loss of paraplegin could, at least in murine brain [74], disorganize substrate cleavage rather than influence mitochondrial AAA protease activity, probably because of redundancy of AAA family members [75].

SPG13 is associated with missense mutations in *HSPD1*, encoding the mitochondrial HSP60, believed to act as molecular chaperone to prevent protein misfolding and aggregation of a subset of proteins located in mitochondria [76]. A small proportion of HSP60 is also located in the cytoplasm and was shown to bind Bax, hence promoting apoptosis if mutated [77].

Receptor expression-enhancing protein, encoded by *REEP1*/SPG31, is a mitochondrial protein that contains the conserved TB2/DP1/HVA22 domain, characteristic of heat-shock proteins, able to exhibit a chaperone-like property [78]. Moreover, REEP1 seems to be important in mitochondrial dynamic and fragmentation. There are indications that REEP1 interacts with the tubular ER membrane in corticospinal neurons to coordinate ER shaping, ER-microtubule interaction and microtubule dynamics and it is also implicated in formation of lipid droplets.

Axon Guidance and Myelination

L1CAM (SPG1) encodes a transmembrane glycoprotein mainly expressed in neurons and Schwann cells, with cell adhesion functions critical for neuronal migration and differentiation. Its alteration in patients and in knockout mice produces highly similar developmental phenotypes, likely the result of failed axonal guidance [79].

PLP1-gene product and its smaller isoform DM20 are the most abundant proteins in the CNS involved in myelin formation and oligodendrocyte maturation. *Plp1*$^{-/-}$ mice have myelin sheaths of normal thickness and axonal swellings followed by late degeneration of long axons. A length-dependent axonal loss in the CNS was also seen in postmortem tissue from a patient with null mutation of *PLP1* [80]. The resulting effect of mutations in both these proteins might be an abnormal development of the corticospinal tract.

Lipid Metabolism

A key function of the ER is the synthesis, metabolism, and distribution of lipids and sterols, through both vesicular and nonvesicular mechanisms. A growing list of *SPG* genes appear to be involved in several aspects of lipid metabolism, including lipid droplet formation (*SPAST*/spastin) and size (*ATL1*/atlastin1), oxysterol metabolism (*CYP7B1*/SPG5), fatty acid metabolism (*FA2H*/SPG35) triglyceride storage (*BSCL2*/sepin) [81], hydrolysis of intrinsic membrane lipids with impact on neuronal membrane composition (*NTE*/SPG39 coding for phospholipase B/lysophospholipase enzyme) [82]. Involvement of fatty acid milieu is particularly crucial for several novel *SPG* genes. The identification of the causative mutations in SPG28-, SPG49-, and SPG54-affected kindred highlights lipid metabolism as a critical pathway in HSP given that *CYP2U1*, *DDHD1*, and *DDHD2*, respectively encode fatty acid- and phospholipid-metabolizing enzymes. In particular, *CYP2U1* catalyzes the hydroxylation of arachidonic acid and related long-chain fatty acids such as eicosapentaenoic and docosahexaenoic acids. Two known metabolites, 19- and 20-hydroxyeicosatetraenoic acids, are local mediators of signal transduction [83]. Other enzymes involving the metabolism of fatty acids and phospholipids have also been implicated in neurodegeneration [84]. On the whole, given the fundamental roles played by lipids and sterols in neuroprotection and neurodegeneration, it is tempting to hypothesize that additional genes will be detected under this category.

CONCLUSIVE REMARKS

This overview on the clinical, genetic, and mechanicistic state-of-the art in the different forms of HSP highlights how this group of disorders is "paradigm" of how a human disease can foster insights into cellular processes and metabolisms. The discovery that common themes are detectable in an extremely heterogeneous class of disorders, models also for more common pathologies affecting the motor neurons or the peripheral nerves, is crucial for future research.

Rapid advances have been made in the past few years, both in the genetics field and the associated clinical phenotypes, and this is largely due to the increasing throughput and falling cost of next-generation sequencing technologies, including whole-exome sequencing (WES). Indeed, WES allowed discovery of more *SPG* genes in the last year and this will improve correlations with clinical presentations, facilitate pre-symptomatic testing and counseling, make possible an even more appropriate use of brain and spinal cord imaging, and will likely reduce the need for extensive biochemical screening needed to distinguish the clinical conditions said to mimic HSP. Besides, uncovering the fine mechanisms that could lead to disruption of the membrane trafficking or axonal transport of macromolecules and other cargoes, or organelle metabolisms will further facilitate the identification of common drugs targeting common themes. In perspective, with the advantage of the new "OMIC" methodologies [85], and their combination with insights into network structures, interest should also be directed towards the issues that explain intrafamilial phenotypic variability and the factors that impact on penetrance and expressivity of the mutations.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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