David Schlesinger

**Ancestralidade da população de São Paulo e correlação
com alterações neuropatológicas no idoso.**

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com alterações neuropatológicas no idoso.**

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Orientadora: Profa. Dra. Mayana Zatz

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Profa. Dra. Mayana Zatz

*Para minha esposa Beatriz,
e nosso filho Elias,
com quem compartilho essas
e outras alegrias na vida.*

*“Discovery consists of seeing what everybody has seen
and thinking what nobody has thought.”*

Albert Szent-Gyorgyi

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Capítulo I: Introdução à Genética de Populações

Variação Populacional Humana

Existe significativa variação genética entre indivíduos da espécie *Homo sapiens sapiens*, apesar desta ser muito menor que em outras espécies, como *Drosophila melanogaster* (Li and Sadler 1991) e primatas (Kaessmann, Wiebe et al. 2001; Yu, Jensen-Seaman et al. 2003; Fischer, Wiebe et al. 2004). Diversos processos moldaram a distribuição da variação genética humana – isolamento geográfico, cultural e linguístico (Cavalli-Sforza 2000), endogamia e seleção natural (Jorde, Bamshad et al. 1998; Bamshad and Wooding 2003). Apesar de mudanças tecnológicas como novos meios de transporte e antibióticos/vacinas alterarem significativamente os agentes de variação, os processos são contínuos, dinâmicos e atuais. (Byars, Ewbank et al. 2010)

Populações Humanas na Pré-História

Humanos anatomicamente modernos (esqueleto leve, crânio arredondado e face retraída) surgiram na África sub-sahariana aproximadamente 200.000 anos atrás, na atual Etiópia. (Klein 1999; White, Asfaw et al. 2003; Lahr 2005; McDougall, Brown et al. 2005) Nesta época existiam diversas populações de humanídeos em outras partes do planeta, como *Homo erectus* na Ásia (Ilha de Java) e *Homo neanderthalis* na Europa. (Swisher, Curtis et al. 1994; Alves-Silva, da Silva Santos et al. 2000; Carvalho-Silva, Santos et al. 2001; Carvalho-Silva, Tarazona-Santos et al. 2006) Entre 100.000 e 50.000 anos atrás, grupos de *Homo sapiens* migraram para o Oriente Médio, provavelmente através do deserto do Sinai para a atual Israel e/ou através do Estreito Bab-el-Mandeb para a atual Arábia Saudita.

(Lahr and Foley 1998; Forster 2004; Mellars 2006) Esta espécie altamente capacitada expandiu-se rapidamente para outros continentes, sendo encontrados esqueletos nas distantes Austrália (datado de ~42.000 anos atrás) (Alves-Silva, da Silva Santos et al. 2000; Carvalho-Silva, Santos et al. 2001; Bowler, Johnston et al. 2003; Carvalho-Silva, Tarazona-Santos et al. 2006), Rússia (datado de ~42.000 anos atrás) (Anikovich, Sinitsyn et al. 2007), Romênia (datado de ~36.000 anos atrás) (Trinkaus, Moldovan et al. 2003) e Ártico (datado de ~24.000 anos atrás) (Richards, Pettitt et al. 2001; Pitulko, Nikolsky et al. 2004).

O modelo “*Out of Africa*” é corroborado por diversas evidências independentes. Nas populações africanas, encontra-se maior diversidade genética (Yu, Chen et al. 2002; Tishkoff and Verrelli 2003), assim como maior desequilíbrio de ligação e menores blocos haplotípicos quando comparados com europeus ou asiáticos (de Bakker, Burt et al. 2006; Frazer, Ballinger et al. 2007). Estudos populacionais usando marcadores polimórficos no cromossomo Y (Underhill, Shen et al. 2000; Chiaroni, Underhill et al. 2009), DNA mitocondrial (Ingman, Kaessmann et al. 2000; Behar, Villems et al. 2008), cromossomo X (Kaessmann, Heissig et al. 1999) e autossomos (Harpending and Rogers 2000; Tishkoff, Reed et al. 2009) todos enfatizam a nossa origem africana. (Templeton 2002)

Os grupos que migraram da África para outras partes do mundo substituíram gradativamente os humanídeos lá existentes, porém houve significativa quantidade de cruzamentos com estes povos e sua genética e características persistem nas populações locais atuais. (Nordborg 1998; Hawks, Hunley et al. 2000; Templeton 2002; Zietkiewicz, Yotova et al. 2003; Mellars 2006) Recentemente, o sequenciamento do genoma neandertal levou à conclusão que 1-4% do genoma de europeus e asiáticos é de origem neandertal. (Green, Krause et al. 2010) A ausência do genoma neandertal em africanos apoia de maneira sólida o modelo “*Out of Africa*”.

Cavalli-Sforza, em seu livro clássico “História e Geografia de Genes Humanos”, propôs que a população da Ásia formou-se a partir de duas migrações distintas: a “Sul”, que deu origem aos Negritos

e povos da Oceania, e a “Norte”, que deu origem às populações da Índia, China, Japão e subsequentemente, Ameríndios. (Cavalli-Sforza, Menozzi et al. 1994) Alguns estudos sugeriram que esta teoria estava errada, apontando para uma migração rápida e única pela costa, ~65.000 anos atrás. (Macaulay, Hill et al. 2005; Thangaraj, Chaubey et al. 2005) Em 2009, um estudo envolvendo 1928 pessoas de 73 populações asiáticas diferentes demonstrou conclusivamente que a migração da África para Ásia se deu em uma única onda, do sul para o norte. (Abdulla, Ahmed et al. 2009) As migrações (duas paleolíticas e uma neolítica) para a Europa, ~45.000 anos atrás, ocorreram a partir de populações do sudeste asiático e/ou Oriente Médio. (Cavalli-Sforza, Menozzi et al. 1994; Semino, Passarino et al. 2000)

O povoamento da América se deu por asiáticos através do estreito de Bering ~16.000 anos atrás, com rápida expansão até a Terra do Fogo em menos de mil anos. (Fagan 1987; Haynes 2002) Há significativa discordância a respeito do número de migrações – geneticistas sugerem uma única (Bianchi, Catanesi et al. 1998), enquanto alguns arqueologistas ainda defendem múltiplas migrações em períodos diversos (Neves and Hubbe 2005). De uma maneira ou de outra, a distinção genética dos Ameríndios é nítida e a maioria de sua população atual foi derivada de uma única população ancestral. (Bianchi, Catanesi et al. 1998; Wang, Lewis et al. 2007; Li, Absher et al. 2008)

Formação Histórica da População Brasileira

Na antiguidade havia quantidade significativa de cruzamentos entre populações - poucas eram verdadeiramente isoladas. (Cavalli-Sforza, Menozzi et al. 1994; Hoerder 2002) No entanto, mudanças em transportes e comunicações após o século XV e mais acentuadamente no século XX levaram a um grande aumento de misturas entre populações prévias. Neste contexto, o povoamento das Américas

após sua descoberta pelos europeus em 1492 é particularmente interessante, pela mistura de três populações providas de continentes distintos: ameríndios, africanos e europeus. Como consequência, cada país e cada população das Américas tem sua característica de mistura e seu peculiar reflexo político, social e biológico.

A população ameríndia do Brasil no ano de 1500 era de ~3 milhões de pessoas. Após o início da colonização portuguesa, houve rápida queda da população devido a cruzamentos com europeus, guerras e epidemias diversas. Na época da independência do Brasil em 1822, existiam ~600.000 índios, caindo para ~300.000 na declaração da República em 1889. (Cunha and Salzano 1992) Em 1957, estima-se que a população indígena esteve no seu menor número: ~120.000. Desde então, estes grupos cresceram progressivamente, chegando atualmente a ~400.000 pessoas, divididos em mais de 200 tribos. (Instituto Brasileiro de Geografia e Estatística. Coordenação de População e Indicadores Sociais. 2005)

Desde o início da colonização, quando a maioria dos imigrantes europeus eram homens, houve maciço cruzamento entre estes e mulheres indígenas. Em 1570 o Rei Sebastião I decretou proibiu a escravidão indígena, mas a lei não “pegou”. Em 1755, com a abolição formal da escravatura indígena a miscigenação foi estimulada oficialmente como forma de aumento populacional. (Mörner 1967) Este padrão assimétrico de cruzamento é nítido quando se compara a ancestralidade do brasileiro contemporâneo no que diz respeito ao cromossomo Y versus o DNA mitocondrial. Em estudo de 247 brasileiros, 33% da herança mitocondrial originou-se de ameríndios, enquanto que nenhum dos cromossomos Y originaram-se dos mesmos. (Alves-Silva, da Silva Santos et al. 2000; Carvalho-Silva, Santos et al. 2001; Carvalho-Silva, Tarazona-Santos et al. 2006)

O influxo de africanos iniciou-se na metade do século XVI, trazidos como escravos de entrepostos comerciais portugueses em Moçambique (fundado em 1500) e Angola (fundado em 1575). Entre 1500 e 1888, aproximadamente 4 milhões de africanos chegaram ao Brasil, o que equivale a 40% de todos os escravos na Américas. Apesar da condição de escravos (ou talvez devido a ela), havia

grande quantidade de cruzamentos com europeus e índios, mas também de forma assimétrica – homens europeus com mulheres africanas. (Meltzer 1993; Hall 2005) No mesmo estudo em que se comparou as origens mitocondrial e do cromossomo Y de 247 brasileiros, encontrou-se 29% da herança mitocondrial proveniente de africanos, enquanto que somente 3% dos cromossomos Y originando-se deles. (Alves-Silva, da Silva Santos et al. 2000; Carvalho-Silva, Santos et al. 2001; Carvalho-Silva, Tarazona-Santos et al. 2006) É relevante ressaltar que os escravos africanos eram provenientes de diversos grupos étnicos trazidos de toda a África. (Meltzer 1993; Hall 2005)

A imigração europeia para o Brasil foi também diversificada como a africana, ocorrendo diversas ondas desde o descobrimento até o século XXI. Entre 1500 e 1808, houve nítida predominância portuguesa (~500.000 imigrantes), com contribuições locais de espanhóis na região Sul e holandeses na região nordeste. Após a abertura dos portos em 1808 e continuando durante o período imperial e republicano brasileiro, ocorreu progressivo estímulo à imigração, com maior diversificação dos países de origem. No período entre 1830 e 1933, chegaram ~5 milhões de imigrantes, dos quais 35% eram portugueses, 35% italianos, 16% espanhóis, 6% alemães, 4% japoneses, 2% árabes (principalmente libaneses e sírios) e 2% de outras nacionalidades (principalmente do leste europeu). (Fundação Instituto Brasileiro de Geografia e Estatística. 1996; Basto 1998; Fausto 1999; Centro de Documentação e Disseminação de Informações (Brazil) 2000)

A distribuição dos imigrantes europeus e asiáticos dentro do Brasil não foi uniforme – aproximadamente 2,5 milhões do total de 5 milhões de imigrantes entre 1830 e 1930 vieram para o estado de São Paulo. (Domingues 2003) Alemães, italianos, japoneses, árabes e judeus se concentraram principalmente nos estados do Sul e Sudeste. (Hajjar 1985; Luisi and Catanzaro 1997; Basto 1998; Grinberg 2005; Sakurai, Coelho et al. 2008) Em 1950, 95% dos japoneses encontravam-se em São Paulo e Paraná, por exemplo. (Sakurai, Coelho et al. 2008) Este processo de migração europeia e asiática predominante para São Paulo, fez com que a porcentagem de moradores da cidade de São

Paulo que se autodeclaravam brancos subisse de 60% em 1872 (primeiro censo brasileiro) para 90% em 1934. Em contrapartida, o número de pardos e negros caiu de 37% para 9%. (Domingues 2003)

Demografia da Cidade de São Paulo

Atualmente, a cidade de São Paulo é a sexta maior cidade do mundo (11 milhões de pessoas – 5,6% da população brasileira em 2009) e sua região metropolitana engloba 19 milhões de pessoas (10% da população brasileira). São Paulo é o centro econômico mais importante do Brasil, com 22% do PIB brasileiro em 2005. Apesar disto, há grandes contrastes locais no que se refere ao desenvolvimento social e econômico entre seus bairros e cidades próximas, com aproximadamente 10% de sua população vivendo abaixo da linha da pobreza. (Instituto Brasileiro de Geografia e Estatística 2010)

São Paulo concentra entre seus moradores as maiores populações de japoneses, libaneses e italianos fora de seus países de origem, além de significativas comunidades menores como armênios, lituanos, coreanos e judeus. Ocorreu (e ocorre) também grande fluxo de nordestinos para São Paulo. De acordo com o IBGE, em 2005, 68% de sua população se autodeclarou branca, 25% parda, 5% negra, 2% amarela e 0,2% índia. (Instituto Brasileiro de Geografia e Estatística. Departamento de População e Indicadores Sociais. 2002; Instituto Brasileiro de Geografia e Estatística 2010)

Raça e Etnia

Na antiguidade, ligações familiares, tribais, culturais e legais eram mais importantes que atributos físicos na aceitação de indivíduos às sociedades. (Snowden 1983; Lewis 1990; Magnoli 2009)

A categorização de diferentes grupos populacionais emergiu na era da exploração europeia, culminando

no livro “The Natural Varieties of Mankind” escrito por Blumenbach em 1775. (Blumenbach, Marx et al. 1969; Magnoli 2009) Blumenbach, assim como outros proponentes da ideologia das raças durante os 200 anos seguintes, incorporaram inúmeras crenças populares sobre supostas diferenças primordiais e imutáveis das raças. (Banton 1977; Smedley 1999)

A classificação racial variava (e varia) dependendo de diversos fatores: classe social, aparência física, país e época. (Mörner 1967; Davis 2001; Shriver, Parra et al. 2003) A autoidentificação de pessoas com raças também pode diferir da classificação feita por outras. (Kressin, Chang et al. 2003) A cor de pele é o principal fator utilizado na classificação das raças na maioria dos casos. Apesar de haver correlação de cor de pele (e outras características físicas) com ancestralidade genética, há grande variação dentro de cada grupo e grande sobreposição entre os diferentes grupos, conforme demonstrou o trabalho do Prof. Sérgio Pena e colegas. (Parra, Amado et al. 2003)

No século XX houve uma tentativa de troca do termo “raça” por “etnia”, para melhor classificar as populações humanas. Etnias são grupamentos populacionais que refletem aspectos culturais, socio-econômicos e religiosos, em oposição às características físicas e suposta ancestralidade. No entanto, os problemas foram mascarados, mas não corrigidos: há fluidez na definição de etnias dependendo de circunstâncias e etnias são igualmente falhas na classificação de similaridade genética. (Waters 1990; Smelser, Wilson et al. 2001) Além disto, há substancial sobreposição no uso dos termos “raça” e “etnia”. (Oppenheimer 2001)

Os problemas das definições de “raça” e “etnia” não alteram o fato de haver diferenças importantes entre estes diferentes grupos assim classificados. Nos Estados Unidos, os negros tem mortalidade maior em oito das dez principais causas de morte, enquanto que brancos morrem mais de câncer e doença cardíaca que outros grupos. (Anderson, Bulatao et al. 2004) Está claro que estas diferenças são uma combinação de fatores genéticos e ambientais, particularmente o nível sócio-econômico. (Cooper, Kaufman et al. 2003; Cooper and Koroukian 2004) Doenças mendelianas raras

também variam em frequência dependendo da raça ou etnia: exemplos são a doença Tay-Sachs em judeus Ashkenazi e anemia falciforme em negros.

Genética das Populações Atuais e Ancestralidade

Assim como os proponentes das diferenças raciais (leia-se cor de pele) (Kevles 1995) estão errados nas suas teses políticas por se basearem em interpretações científicas errôneas (quando às usam), também estão errados seus opositores mais ferrenhos. É recorrente entre o público leigo (Magnoli 2009) e especializado (Wolfsberg, McEntyre et al. 2001) a citação do dado inicialmente publicado por Lewontin em 1972 de que 85% da variação humana é comum a todas as populações e que portanto não é possível classificá-las em grupos. (Lewontin 1972) Apesar do dado genético estar correto (Hinds, Stuve et al. 2005), a conclusão estava errada. RA Fisher já havia demonstrado em 1925 que a combinação da frequência das características permite a separação em grupos, sejam elas genéticas ou físicas (Edwards 2003)

A medida de diferenciação populacional é o índice de fixação (F_{ST}), descrito por Weir e Cockerham em 1984 a partir dos trabalhos de Sewall Wright. (Weir and Hill 2002) De maneira simplificada, se 18% da variação entre africanos e europeus é inter-populacional e 82% individual, temos um F_{ST} de 0,18. A fração minoritária da variação não é desprezível – nela estão contidas milhões de variantes comuns em diferentes frequências e variantes raras privadas (específicas de cada população). (Risch, Burchard et al. 2002; Kittles and Weiss 2003)

Cavalli-Sforza e colaboradores demonstraram desde a década de 1950 o valor da utilização sistemática de marcadores polimórficos no estudo da populações humanas, incorporando a análise de componentes principais genéticos. (Cavalli-Sforza, Menozzi et al. 1994; Cavalli-Sforza 2000) Durante

e após o Projeto Genoma Humano (Lander, Linton et al. 2001; Venter, Adams et al. 2001), houve uma explosão de estudos das variantes genéticas nas diversas populações. (Tapper 2005; Khaja, Zhang et al. 2006; Redon, Ishikawa et al. 2006) O HapMap Project (“Mapa de Haplótipos) pode ser considerado uma segunda fase do Projeto Genoma (Frazer, Ballinger et al. 2007) e o 1000 Genomes Project, um terceiro (Kaiser 2008; Gamazon, Zhang et al. 2010) – ambos avaliando a variabilidade individual e populacional.

Em 2002, Rosenberg e colegas publicaram estudo de 1056 indivíduos de populações de todo o mundo genotipados para 377 microssatélites e mostraram o agrupamento por continente de origem baseando-se somente em informações genéticas. (Rosenberg, Pritchard et al. 2002) Estes resultados de ancestralidade foram replicados em outros estudos utilizando marcadores de diferente natureza, como inserções Alu (Bamshad, Wooding et al. 2003), inserções/deleções (Tuzun, Sharp et al. 2005) e SNPs (Li, Absher et al. 2008), reforçando a conclusão que raça/etnia são maneiras inadequadas de classificar as pessoas. (King and Motulsky 2002; Calafell 2003; Tishkoff and Kidd 2004)

Grupos populacionais distintos existem, mas este é um dado quantitativo, contínuo e mutável, não qualitativo e estático. Da mesma maneira que o mundo pode ser separado em continentes, continentes podem ser divididos em países, e subsequentemente em estados, cidades, bairros, etc. Estes subgrupos respeitam, de maneira simplificada, as divisões políticas (nações, estados), culturais (linguagem, religião) e geográficas (vales, montanhas), mas nem sempre. (Wang, Lewis et al. 2007; Novembre, Johnson et al. 2008; Abdulla, Ahmed et al. 2009; Tishkoff, Reed et al. 2009) Em estudos que examinaram grupos menores também foram encontrados gradientes genéticos – pode-se separar subgrupos da Estônia por geografia local (Nelis, Esko et al. 2009), subgrupos de judeus por origem histórica pré- e pós-Diáspora (Behar, Yunusbayev et al. 2010) e subgrupos de indianos por casta (Majumder 2010), sem no entanto denominá-los raças/etnias distintas. Até vilas separadas por alguns quilômetros podem ser classificadas e identificadas se usarmos suficientes marcadores genéticos, como

foi recentemente mostrado na Itália e Croácia. (O'Dushlaine, McQuillan et al. 2010)

A partir da genotipagem de milhares de polimorfismos em populações diversas, pode-se escolher uma pequena amostra de marcadores autossômicos que apresentem discordância extrema entre as populações. Estes marcadores são denominados “ancestry informative markers”, ou marcadores informativos de ancestralidade (AIMs). Diversos grupos geraram e validaram os seus AIMs para diferenciar as populações mundiais. (Halder, Shriver et al. 2008; Kosoy, Nassir et al. 2009; Nassir, Kosoy et al. 2009; Pena, Bastos-Rodrigues et al. 2009; Santos, Ribeiro-Rodrigues et al. 2010) Quanto mais distantes geneticamente, menor o número de marcadores que são necessários para diferenciar duas populações. Para as populações continentais, bastam 24 SNPs para avaliações com grandes intervalos de confiança, porém a acurácia melhora significativamente com 64 SNPs ou mais. (Kosoy, Nassir et al. 2009) Para diferenciar populações de países europeus, por exemplo, são necessários números maiores de SNPs (300-1000). (Tian, Kosoy et al. 2009)

Existem inúmeros algoritmos e programas computacionais para a separação genética dos grupos e estudo de genética populacional. (Excoffier and Heckel 2006) Atualmente, os mais utilizados são Eigenstrat (Price, Patterson et al. 2006) (baseado em análise de componentes principais) e Structure (Pritchard, Stephens et al. 2000) (que utiliza métodos bayesianos). De maneira geral, análise de componentes principais tem um desempenho melhor no estudo de muitos (>1000) marcadores, porém resultados insatisfatórios com pequenas quantidades (<50). É também interessante por não necessitar da determinação do número de grupos pré-teste. Em contrapartida, programas como Structure apresentam resultados robustos com poucos marcadores (<50), mas tornam-se inviáveis do ponto de vista computacional quando avaliamos muitos indivíduos e marcadores (>1000). Outros métodos existem que possibilitam a análise de ancestralidade para cada segmento cromossômico individualmente, mas estão fora do escopo desta tese. (Patterson, Hattangadi et al. 2004; Sankararaman, Sridhar et al. 2008; Price, Tandon et al. 2009)

Ancestralidade da População Brasileira

Conforme discutido previamente, há grande diferença entre ancestralidade quando utilizados os marcadores do cromossomo Y e do DNA mitocondrial. Apesar do valor antropológico destes marcadores devido à transmissão contínua e sem recombinação, quando comparados com autossomos são de menor importância para doenças complexas. Mesmo tratando-se da história populacional, os autossomos refletem mais pormenorizadamente os diferentes influxos gênicos.

Diversos estudos já publicados compararam a contribuição das populações ancestrais (africana, indígena e europeia) ao “pool” genético brasileiro, utilizando AIMs. (Callegari-Jacques, Grattapaglia et al. 2003; Lins, Vieira et al. 2009; Pena, Bastos-Rodrigues et al. 2009) Pena e colegas identificaram em São Paulo frequências de 12% africana, 11% indígena e 78% europeia em brancos e 50% africana, 13% indígena e 37% europeia em negros. No entanto, o estudo era caso-controle, não permitindo o cálculo específico da ancestralidade total da população. Além disso, não foram utilizados AIMs que diferenciasssem indígenas de asiáticos. (Pena, Bastos-Rodrigues et al. 2009) Em outro estudo, Lins e colegas encontraram frequências de 14% africana, 6% indígena e 80% europeia em Minas Gerais. (Lins, Vieira et al. 2009)

Doenças Complexas e Uso de Marcadores de Ancestralidade

As doenças comuns, como hipertensão, diabetes tipo I e tipo II, dislipidemia e as principais doenças neurológicas, são doenças complexas. Doenças complexas são causadas por múltiplos fatores ambientais em combinação com diversas variantes genéticas e são herdadas de forma não-mendeliana.

As variantes genéticas associadas aos fenótipos complexos (não só doenças), podem ser de qualquer natureza – polimorfismos de base única (SNPs), variantes do número de cópias (CNPs), inserções, deleções e translocações. A frequência destes polimorfismos é muito variável, desde uma frequência do alelo minoritário de ~50%, até alelos raríssimos. (Lander and Schork 1994; Burton, Tobin et al. 2005; Hardy and Singleton 2009)

Existe um intenso debate a respeito da importância relativa de alelos raros versus comuns nas doenças complexas. De um lado, pesquisadores como Eric Lander e David Altshuler defendem a teoria “Doença Comum-Variante Comum”, enquanto que outros como Joseph Terwilliger e David Goldstein defendem que polimorfismos raros são mais importantes. (Weiss and Terwilliger 2000; Pritchard and Cox 2002; Cohen, Kiss et al. 2004) A realidade provavelmente fica no meio termo. (Pritchard and Cox 2002; Chen, Jorgenson et al. 2007; Bodmer and Bonilla 2008; Nejentsev, Walker et al. 2009) Entre populações distintas os alelos comuns e raros podem variar de maneira sistemática, conforme descrevi anteriormente. Os alelos raros tem maior chance de variar desta maneira (pelo maior peso de deriva genética e mutação), mas os comuns também o fazem.

A maioria dos estudos genéticos bem sucedidos de fenótipos complexos até o momento foram estudos de associação em genoma inteiro (“genomewide association studies”, ou GWAS), utilizando chips de até um milhão de SNPs (comuns) das companhias Affymetrix e Illumina. (Hardy and Singleton 2009) Estes estudos identificaram inúmeras associações genéticas, causando uma explosão de conhecimento a respeito das doenças. Porém, há ainda uma “herdabilidade perdida” - estas variantes comuns não respondem por toda a herdabilidade detectada em estudos familiares populacionais e não podemos esquecer também que ambiente tem uma relevância grande em diversas doenças. (Manolio, Collins et al. 2009; Eichler, Flint et al. 2010) Um exemplo recente foi o sequenciamento completo do genoma de gêmeos monozigóticos discordantes para esclerose múltipla, não revelando diferenças genéticas entre eles. (Baranzini, Mudge et al. 2010)

Os resultados de GWAS são suscetíveis a diversos problemas metodológicos, entre eles a estratificação populacional. Quando comparando frequências alélicas entre casos e controles, se houver distribuição de subpopulações de maneira desigual entre os grupos, pode-se obter resultados falso-positivos e falso-negativos. (Cardon and Palmer 2003; Choudhry, Coyle et al. 2006; Price, Patterson et al. 2006; Tian, Gregersen et al. 2008) Num extremo, se os casos forem constituídos de africanos e controles de europeus, por exemplo, os alelos encontrados serão aqueles com alto F_{ST} – os AIMs. Da mesma maneira, os GWAS para fenótipos quantitativos são suscetíveis ao mesmo fenômeno estatístico se duas subpopulações (como negros e brancos) tiverem distribuições distintas de um fenótipo, mesmo que este seja causado inteiramente por fatores ambientais.

A solução para a estratificação passa por métodos estatísticos que quantifiquem a variação populacional dentro do estudo. Métodos como controle genômico (GC), componentes principais (PCA) e mínimos quadrados parciais (PLS) são os mais utilizados. (Lee 2004; Tsai, Choudhry et al. 2005; Price, Patterson et al. 2006; Divers, Vaughan et al. 2007; Tian, Gregersen et al. 2008) O problema central destes métodos é a necessidade de genotipar todas as amostras, encarecendo o estudo com genotipagens que serão descartadas. A alternativa é a seleção prévia das amostras a serem genotipadas utilizando AIMs, para obtermos grupos mais homogêneos. Isso pode gerar uma economia de até 30% no custo final do estudo. Nos raros casos em que estudos de associação caso-controle simples (poucos SNPs) são aceitáveis, AIMs também servem como controle de estratificação. (Pritchard, Stephens et al. 2000)

Além do controle de estratificação, outra modalidade de investigação tem surgido nos últimos anos: o uso de AIMs em estudos epidemiológicos. Como citei previamente, apesar de existirem diferenças importantes em fenótipos entre as raças e etnias, estes são ineficientes por diversas razões, especialmente por embutirem fatores genéticos e ambientais dentro do mesmo classificador. Ao utilizar AIMs, podemos detectar o efeito genético com maior poder e precisão.

Alguns estudos publicados mostraram resultados interessantes. Fejerman e colegas relataram que maior ancestralidade europeia em mulheres latinas nos Estados Unidos e México aumenta o risco de câncer de mama, mesmo quando fatores ambientais foram incluídos no modelo. (Fejerman, John et al. 2008; Fejerman, Romieu et al. 2010) Estudando negros americanos, Reiner e colegas mostraram um padrão de colesterol melhor em pessoas com maior ancestralidade africana. (Reiner, Carlson et al. 2007) Outros trabalhos compararam incidências de diabetes, fatores endócrinos, lupus e função renal. (Peralta, Ziv et al. 2006; Martinez-Marignac, Valladares et al. 2007; Shaffer, Kammerer et al. 2007; Keene, Mychaleckyj et al. 2008; Chung, Tian et al. 2009; Casazza, Thomas et al. 2010; Casazza, Willig et al. 2010; Hunter, Chandler-Laney et al. 2010) Trabalho recente no New England Journal of Medicine também demonstrou correlação de ancestralidade com função pulmonar. (Kumar, Seibold et al. 2010)

Apesar da grande diferença relatada entre raças na incidência de demência, não há estudos clínicos nem anátomo-patológicos comparando ancestralidade. No campo da neurologia há somente um estudo de malformações arteriovenosas em mexicanos. (Kim, Hysi et al. 2008) No Brasil, um estudo na Bahia comparou ancestralidade africana (30 marcadores, separação em 2 populações) com dengue. Tanto ancestralidade africana quanto baixo nível socio-econômico se mostraram protetores em relação à forma hemorrágica. (Blanton, Silva et al. 2008) Outros estudos no Brasil estão em andamento em doenças cardiovasculares e malformações cranio-faciais, porém estes não foram publicados ainda.

Capítulo II: Introdução às Demências

Epidemiologia das Demências no Mundo

Demência é uma síndrome clínica caracterizada por perda de habilidades cognitivas previamente adquiridas com intensidade suficiente para interferir no desempenho social ou profissional do indivíduo. Classicamente, o déficit de memória é *sine qua non* para o diagnóstico, acompanha a perda de ao menos uma outra função cognitiva (ie. linguagem, gnosis, praxias, funções executivas ou alterações de comportamento). No entanto, síndromes cognitivas como a Afasia Primária Progressiva se apresentam com memória intacta (inicialmente) e são manifestações clínicas dos mesmos processos patológicos de outras demências. (Mesulam, Wicklund et al. 2008; Bigio, Mishra et al. 2010)

Demência, particularmente no idoso, foi considerada tema de importância menor pela Medicina até 1950. Só então, com o grande aumento de expectativa de vida experimentado na Europa, as demências se tornaram objeto de crescente interesse. De acordo com a OMS, demência é responsável por 11% dos anos vividos com incapacidade funcional em pessoas acima de 60 anos de idade. Esse número é maior que o causado por neoplasias ou doenças cardio e cerebrovasculares. (2000) Com o aumento da população de idosos no mundo, este problema tende a piorar exponencialmente. (Christensen, Doblhammer et al. 2009; Vaupel 2010) A prevalência de demência dobra a cada 5 anos a partir dos 65 anos. A prevalência de demência passa de 0,7% no grupo etário de 60 a 64 anos, para 39% no de 90 a 95 anos. (Jorm, Korten et al. 1987; Corrada, Brookmeyer et al. 2010)

O maior desafio para a definição exata da prevalência das demências é a ausência de teste clínico diagnóstico que possa ser aceito como “padrão ouro”. (Erkinjuntti, Ostbye et al. 1997) Em países em desenvolvimento, dois outros obstáculos também estão presentes, os testes neuropsicológicos

validados em outros países podem ter acurácia muito diferente por causa de fatores socioculturais e heterogeneidade no nível educacional.

Em 2005, foi publicada uma meta-análise sobre a prevalência de demência no mundo. Apesar dos dados serem pouco precisos em vários países, estima-se uma prevalência atual de 24 milhões de casos de demência no mundo e uma incidência anual de aproximadamente 5 milhões de casos. O número de afetados irá dobrar a cada 20 anos, chegando a 81 milhões em 2040, sendo 71% deles em países em desenvolvimento. Para a América Latina é estimada uma prevalência de demência de 1,8 milhões em 2001 e 9,1 milhões em 2040, um aumento de cinco vezes. (Ferri, Prince et al. 2005)

Epidemiologia das Demências no Brasil

Os estudos de prevalência de demências realizados no Brasil podem ser divididos em estudos de registros de casos e populacionais. Nitrini e colegas avaliaram 100 pacientes ambulatoriais com demência atendidos em hospital público e em clínica privada em São Paulo, com diagnóstico de demência baseado no DSM-III-R e no Mini Exame do Estado Mental. (Folstein, Folstein et al. 1975; Spitzer, Williams et al. 1992) Constatou-se que doença de Alzheimer (DA) é causa principal de demência (54%), independentemente do nível sócio-econômico dos pacientes, seguida por demência vascular (20%). (Nitrini, Mathias et al. 1995) Outros estudos revelam prevalências de 13% em pacientes idosos ambulatoriais e de 52% a em pacientes idosos institucionalizados no Brasil. (Engelhardt, Laks et al. 1998)

Almeida Filho e colegas realizaram em 1984 o primeiro estudo populacional sobre prevalência de desordens mentais em idosos vivendo na comunidade. (Filho, Santana et al. 1984) A prevalência de “quadros orgânico-cerebrais” foi de 7% quando apenas indivíduos com mais de 65 anos foram

considerados. Desde então, outros estudos revelaram taxas entre 5 a 10%. (Kalache, Veras et al. 1987; Blay 1989; Veras 1991; Meguro, Meguro et al. 2001) Finalmente, estudo realizado em Catanduva no interior de São Paulo, encontrou DA como maior causa de demência, com 55% dos casos, seguida por DA associada a doença vascular cerebral (14%) e demência vascular isolada (9%). (Herrera, Caramelli et al. 2002)

Não obstante, a referência às demências nos atestados de óbito é infrequente. No estudo de Catanduva, apenas em 13% dos casos de demência havia referência à demência ou à DA em qualquer dos itens onde as condições mórbidas são listadas no atestado de óbito. (Nitrini, Caramelli et al. 2005)

Demências em Grupos Populacionais Diversos

As diferentes populações, grupos étnicos, sociais, religiosos e geográficos variam significativamente nas suas variantes genéticas e exposições ambientais (vide abaixo) e por consequência, variam significativamente na sua frequência e expressão de diversas doenças. Em estudos realizados nos Estados Unidos, detectou-se maior prevalência de demência em negros quando comparados a caucasianos. (Schoenberg, Anderson et al. 1985; Heyman, Fillenbaum et al. 1991; Froehlich, Bogardus et al. 2001; Demirovic, Prineas et al. 2003; Hou, Yaffe et al. 2006) No entanto, estas diferenças podem ser de natureza diagnóstica (Folstein, Folstein et al. 1975), genética (Tishkoff, Reed et al. 2009; Zakharia, Basu et al. 2009; Bryc, Auton et al. 2010) ou ambiental (Kaufman, Cooper et al. 1997; Bravata, Wells et al. 2005; Cook and Manning 2009; LaVeist, Thorpe et al. 2009).

Um estudo clínico de Hendrie e colegas revelou que a prevalência de demência em negros americanos é duas a quatro vezes maior que em nigerianos. (Hendrie, Osuntokun et al. 1995) A

interpretação destes estudos é difícil, considerando-se que as duas populações não somente provém de ambientes distintos, mas também tem características genéticas significativamente diferentes, mas não foram quantificadas. (Tishkoff, Reed et al. 2009; Zakharia, Basu et al. 2009; Bryc, Auton et al. 2010) Pesquisas de diferenças anátomo-patológicas, que eliminam as incertezas diagnósticas, pecaram por estudarem números insuficientes de pacientes e não quantificarem ancestralidade. (Pytel, Cochran et al. 2006; Wilkins, Grant et al. 2006) O maior estudo do gênero comparou 200 cérebros de brancos e negros americanos, não revelando diferenças significativas entre os grupos. (Riudavets, Rubio et al. 2006)

Principais Causas de Demência Senil

Os principais diagnósticos neuropatológicos em pacientes com demência senil são a doença de Alzheimer (DA), a demência vascular (DV) e a doença de Corpúsculos de Lewy (DCL). (Lobo, Launer et al. 2000; 2001; Fitzpatrick, Kuller et al. 2004; Ravaglia, Forti et al. 2005) Outras alterações, como taupatias (Demência Fronto-Temporal ou DFT, Degeneração Córtico-Basal, Paralisia Supranuclear Progressiva) (Cairns, Bigio et al. 2007), TDP43-patias (DFT) (Neumann, Sampathu et al. 2006), ubiquitinopatias (DFT) (Bigio, Mishra et al. 2010; Urwin, Josepht et al. 2010) também são encontradas mais raramente. Algumas alterações comuns tem significado desconhecido, como a doença de Grãos Argirofílicos (DGA). (Braak and Braak 1987; Ferrer, Santpere et al. 2008)

A frequência de demência clínica nas diversas populações é bastante consistente ($DA > DV > DCL > outras$). No entanto, há variações importantes. No Japão, por exemplo, um estudo relatou que DV era mais comum que DA. (Yoshitake, Kiyohara et al. 1995) Estudo populacional na Inglaterra, com diagnóstico anátomo-patológico de 456 casos, concluiu que 25% do risco populacional de demência é

dado por alterações de DA (placas, emaranhados e angiopatia amilóide), 21% por alterações vasculares, 4% por atrofia hipocampal e 2% por corpúsculos de Lewy. (Matthews, Brayne et al. 2009) É importante notar que o método usado para identificação de corpúsculos de Lewy neste estudo resultou em significativo subdiagnóstico. Em estudo subsequente utilizando anticorpos antisinucleína, a incidência de corpúsculos subiu de 10% para 37%. (Zaccai, Brayne et al. 2008) Nos Estados Unidos, estudo comparativo entre negros e brancos revelou aumento (não estatisticamente significativo) de DA e DV, mas também seguindo a relação $DA > DV$. (Fitzpatrick, Kuller et al. 2004)

Doença de Alzheimer (DA)

Descrita em 1906 por Alois Alzheimer, foi inicialmente relatada em uma paciente com menos de 60 anos, porém ocorre mais frequentemente em idosos. (Alzheimer 1907) A significância dos achados neuropatológicos em idosos só recebeu seu devido peso na década de 1950. O quadro neurológico clássico é a perda progressiva da memória recente, evoluindo para outras alterações mais tardiamente. Em estágios avançados da doença, a mortalidade chega a 54% em 18 meses. (Mitchell, Teno et al. 2009) O diagnóstico clínico, porém, tem acurácia limitada e a especificidade contra outras demências varia entre 23 e 88%. (Cullen, O'Neill et al. 2007; Jellinger 2009)

Os pacientes, antes de desenvolverem a síndrome clínica, passam por estágio intermediário comum a diversas patologias neurológicas, conhecida como distúrbio cognitivo leve (MCI). Os pacientes com MCI são um grupo heterogêneo, podendo ou não evoluir para demência franca. (Gauthier, Reisberg et al. 2006) Mesmo com uso de diversos marcadores biológicos e de imagem, o padrão-ouro continua sendo o diagnóstico anátomo-patológico. (McKeel, Price et al. 2004; van der Flier and Scheltens 2005; Perrin, Fagan et al. 2009; Savva, Wharton et al. 2009)

Apesar de resultados iniciais promissores, ainda não existe tratamento que previna ou modifique o processo patológico. (Vellas, Andrieu et al. 2007; Holmes, Boche et al. 2008) As medicações disponíveis (donepezila, rivastigmina, galantamina e memantina) melhoram transitoriamente os sintomas cognitivos. (Cummings 2004) Isto torna premente estudos que aumentem nossa compreensão da doença.

Neuropatologia da Doença de Alzheimer

Dois são os principais achados neuropatológicos na DA, placas neuríticas e amilóides e emaranhados neurofibrilares. As placas são constituídas de acúmulo extracelular de proteína beta-amilóide. O CERAD (Consortium to Establish a Registry for Alzheimer's Disease) é a escala patológica mais utilizada na quantificação do acometimento cerebral por placas, sendo compensado por idade. Pode ser 0 (sem alteração), A (possível), B (provável) ou C (definitivo), uma adaptação para a idade de placas ausentes, raras, moderadas e frequentes. (Mirra 1997; NIG/NIH 1997)

Os emaranhados neurofibrilares intracelulares são compostos de proteína tau fosforilada. A escala mais utilizada que quantifica os emaranhados foi criada por Braak e Braak. (Braak and Braak 1991; Braak and Braak 1995; Braak, Alafuzoff et al. 2006) A escala de Braak varia de 0 a VI, sendo VI o maior acometimento. Ambas as alterações podem ser encontradas em outras patologias e em pacientes normais, porém ambas tem correlação com perda cognitiva. As alterações também aumentam progressivamente com a idade. (Savva, Wharton et al. 2009) Existe acirrado debate relativo às escalas neuropatológicas, porém é crescente o uso da escala de Braak, por ser mais correlacionada com disfunção cerebral, apesar da proteína beta-amilóide ser relacionada à gênese da doença. (Geddes,

Genética da Doença de Alzheimer

A identificação da sequência da proteína precursora de amiloide (APP) (Glenner and Wong 1984), e a subsequente identificação de mutações no gene APP em famílias com formas precoces de DA, abriu caminho para a compreensão das bases genéticas da DA. (Goate, Chartier-Harlin et al. 1991) Recentemente, duplicações da APP também foram identificadas em famílias com DA precoce, tornando nítida a relação entre excesso de APP e DA. (Rovelet-Lecrux, Hannequin et al. 2006) Outros genes envolvidos diretamente na fisiopatologia da DA são os genes Presenilina 1 (PSEN1) e Presenilina 2 (PSEN2). Mutações nestes genes foram subsequentemente encontradas em pacientes com início precoce de doença e história familiar. (Levy-Lahad, Wijsman et al. 1995; Li, Ma et al. 1995; Rogaev, Sherrington et al. 1995; Sherrington, Rogaev et al. 1995)

As bases genéticas da forma tardia não seguem a genética Mendeliana, apesar de sua herdabilidade ter sido estimada em 79% em estudo de 11884 gêmeos com mais de 65 anos na Suécia. (Gatz, Reynolds et al. 2006) Alguns grupos procuraram identificar associação entre polimorfismos de APP e DA de início tardio, porém sem sucesso. (Li, Perry et al. 1998; Athan, Lee et al. 2002) O padrão de herança é multifatorial, com múltiplos genes envolvidos interagindo com fatores ambientais ainda desconhecidos. (Bodmer and Bonilla 2008; Hardy and Singleton 2009) O número de genes envolvidos é estimado entre 200 e 400. (Kraft and Hunter 2009) O banco de dados AlzGene (www.alzgene.org) foi criado para organizar os estudos genéticos na DA tardia. (Bertram, McQueen et al. 2007) No entanto, conforme discuti anteriormente, é o perfeito caso de GIGO (“garbage-in, garbage-out”). A maioria de

associações encontradas nos mais de 1300 estudos são falsos-positivos, o que elimina o valor prospectivo do banco. (Dahlman, Eaves et al. 2002; Wacholder, Chanock et al. 2004; Clayton, Walker et al. 2005; Ehm, Nelson et al. 2005). Os motivos para estas falhas graves incluem estratificação populacional de casos e controles, erros diagnósticos de casos avaliados clinicamente, número de amostras insuficientes, viés de publicação em favor de resultados positivos, entre outros. (Lander and Schork 1994; Cardon and Palmer 2003) Excetua-se desta lista o gene apolipoproteína E (APOE) e os genes identificados em GWAS.

O gene de maior importância na DA, comprovado em inúmeros estudos, é APOE. A associação de polimorfismos do gene APOE, situado no cromossomo 19, com a forma tardia da DA, representa uma das primeiras associações conclusivas entre variantes gênicas e doenças complexas. (Pericak-Vance, Bebout et al. 1991) Há 3 isoformas proteicas da APOE: epsilon-2 (e2), epsilon-3 (e3) e epsilon-4 (e4). A presença de alelo e4 da APOE aumenta o risco de doença e reduz a idade de surgimento dos sintomas. (Schmechel, Saunders et al. 1993; Strittmatter, Saunders et al. 1993) Em contraste, a presença de e2 tem efeito protetor para DA. (Corder, Saunders et al. 1993) O efeito atribuível populacional dos polimorfismos de APOE é de 20-25%, devido ao grande efeito de cada polimorfismo e sua alta frequência em todas as populações. A associação genética de APOE e DA é mais forte para endofenótipos radiológicos e alterações anátomo-patológicas, reforçando a imperfeição do diagnóstico clínico. (Bennett, De Jager et al. 2009; Langbaum, Chen et al. 2010; Vemuri, Wiste et al. 2010)

A associação da DA e de perda cognitiva com os polimorfismos comuns da APOE foi replicada inúmeras vezes em brancos de ascendência norte europeia e asiáticos. (Brousseau, Legrain et al. 1994; Caselli, Dueck et al. 2009) Em nigerianos e negros norte americanos (estes também predominantemente originários do oeste africano) (Tishkoff, Reed et al. 2009) a associação de APOE e4 com DA não se confirmou consistentemente. (Tang, Stern et al. 1998; Ogunniyi, Baiyewu et al.

2000; Hall, Murrell et al. 2006; Murrell, Price et al. 2006) Não está claro se a ausência de associação de risco da DA em portadores do alelo epsilon-4 em negros americanos e africanos se dá por questões metodológicas ou por diferenças na fisiopatologia da doença. Existem inúmeros outros polimorfismos na região genômica de APOE, sem aparente associação com DA. (Martin, Lai et al. 2000) No entanto, um trabalho recente em pacientes na Inglaterra sugere que outras variantes ainda desconhecidas de APOE podem alterar o risco para DA, de forma independente de e2-e3-e4 ou interagindo com estes. (Belbin, Dunn et al. 2007)

No Brasil, país notoriamente miscigenado (Lins, Vieira et al. 2009), alguns estudos de associação foram feitos entre DA e APOE, mas não há até o momento estudos genéticos de amostras analisadas do ponto de vista neuropatológico, somente do ponto de vista clínico. (Almeida and Shimokomaki 1997; Oliveira, Shimokomaki et al. 1999) Em nenhum momento estas amostras foram analisadas para marcadores de ancestralidade, o que é recomendado para populações miscigenadas como a brasileira. (Berg, Bonham et al. 2005)

Até o momento, foram publicados 11 GWAS da doença de Alzheimer ou alterações radiológicas compatíveis. (Coon, Myers et al. 2007; Grupe, Abraham et al. 2007; Reiman, Webster et al. 2007; Bertram, Lange et al. 2008; Li, Wetten et al. 2008; Beecham, Martin et al. 2009; Carrasquillo, Zou et al. 2009; Harold, Abraham et al. 2009; Lambert, Heath et al. 2009; Potkin, Guffanti et al. 2009; Seshadri, Fitzpatrick et al. 2010) Os genes que atingiram significância “genômica”, com valor de p menor que 5×10^{-8} , foram poucos: GAB2 (Reiman, Webster et al. 2007), PCDH11X (Carrasquillo, Zou et al. 2009), CLU (Harold, Abraham et al. 2009; Lambert, Heath et al. 2009), PICALM (Harold, Abraham et al. 2009), CR1 (Lambert, Heath et al. 2009), TOMM40 (Potkin, Guffanti et al. 2009), BIN1 (Seshadri, Fitzpatrick et al. 2010) e EXOC3L2 (Seshadri, Fitzpatrick et al. 2010). É importante ressaltar que todos os estudos, sem exceção, identificaram APOE de maneira estatisticamente

significativa e que o gene TOMM40 está em desequilíbrio de ligação com o gene APOE, dificultando sua análise isoladamente. O papel destes genes na fisiopatologia da DA ainda não é conhecido. Estudos funcionais prévios sugeriram que CR1, assim como APOE, está envolvido na retirada de acúmulo de beta-amilóide. (Rogers, Li et al. 2006)

Demência Vascular

Demência vascular é um agregado de alterações cognitivas diversas de difícil diagnóstico, causado por múltiplos mecanismos fisiopatológicos: doença de pequenos vasos, doença de grandes vasos, lesões hipóxicas-reperfusionais e infartos estratégicos. (Katz, Alexander et al. 1987; Mendez, Adams et al. 1989; Tatemichi, Desmond et al. 1992; Andin, Gustafson et al. 2005) Domínios cognitivos acometidos incluem atenção, memória, linguagem, orientação visuo-espacial, e funções executivas. {Roman, 1999 #3023} Síndromes disexecutivas são as alterações clássicas associadas às lesões subcorticais. {Price, 2005 #3030; Roman, 1999 #3022}

A associação de fatores de risco para doença cardiovascular é robusta, com múltiplos estudos grandes mostrando forte correlação. No estudo de Framingham, demonstrou-se ao longo de dez anos um risco duas vezes maior de demência nos pacientes que tiveram AVCs quando comparados com controles sem AVC. (Ivan, Seshadri et al. 2004) No estudo Atherosclerosis Risk in Communities (ARIC), pacientes com grande quantidade de hiperintensidades de substância branca na ressonância magnética apresentaram significativa diminuição de capacidade cognitiva. (Mosley, Knopman et al. 2005)

No estudo de Rotterdam em 1995, também correlacionou-se fatores como hipertensão,

espessura de íntima média da carótida e lesões vasculares na ressonância ao desenvolvimento de demência. (Breteler 2000; Prins, van Dijk et al. 2005) Mesmo na ausência de AVCs, ocorre maior perda cognitiva nos pacientes com fatores de risco cardiovascular que em pacientes controle. (Manolio, Kronmal et al. 1996; Elkins, O'Meara et al. 2004)

Os fatores clínicos mais associados (independentes e combinados) com demência no North Manhattan Aging Study (NOMAS) foram diabetes, tabagismo, hipertensão e doença cardíaca. (Luchsinger, Reitz et al. 2005) No Austrian Stroke Prevention Study, o nível de hemoglobina glicada (um marcador de diabetes) correspondeu a 13% da variância de atrofia cerebral. (Schmidt, Enzinger et al. 2003)

O nível sérico de homocisteína, em si um preditor de risco cardiovascular, também correlaciona-se com demência. (Longstreth, Katz et al. 2004) Disfunção renal (Buchman, Tanne et al. 2009), sedentarismo (Buchman, Boyle et al. 2009), tabagismo e etilismo (Harwood, Kalechstein et al. 2010), em si fatores de risco para doença cardiovascular, estão associados ao diagnóstico de demência.

Neuropatologia da Demência Vascular

Em muitos estudos clínicos, o diagnóstico de demência (global), DA e DV tem uma sobreposição importante. Por isso, estudos anátomo-patológicos são mais precisos, especialmente quando se considera a coincidência de DA e DV nos mesmos pacientes. (White, Petrovitch et al. 2002) O NUN Study, estudo longitudinal prospectivo de freiras com alto nível educacional e ambientes similares, infartos cerebrais foram associados à apraxia de construção. Mais importante, nas pacientes com lesões neuropatológicas compatíveis com DA porém sem infartos, o diagnóstico clínico de demência era de 57%, enquanto que naquelas com infartos lacunares, este valor aumentava para 93%.

(Snowdon, Greiner et al. 1997) No Honolulu Ásia Aging Study (HAAS), presença de infartos também aumentou significativamente o diagnóstico de demência. (White, Petrovitch et al. 2002) Em outros estudos, tanto patologia compatível com DA quanto infartos se associaram de maneira independente ao diagnóstico de demência. (Schneider, Wilson et al. 2003; Schneider, Wilson et al. 2004; Troncoso, Zonderman et al. 2008)

Genética da Demência Vascular

Além dos fatores ambientais como tabagismo e etilismo (Harwood, Kalechstein et al. 2010), existe nítida predisposição genética aos AVCs e DV. (Jousilahti, Rastenyte et al. 1997; Liao, Myers et al. 1997; Bak, Gaist et al. 2002) Estes fatores genéticos podem agir indiretamente, aumentando a incidência de diabetes (Manolio, Rodriguez et al. 2007; Zeggini, Scott et al. 2008), hipertensão (Cho, Go et al. 2009; Ehret 2010) e dislipidemia (Kathiresan, Willer et al. 2009), ou podem agir diretamente (Ikram, Seshadri et al. 2009).

Até o momento sete GWAS de AVCs (nenhum de demência vascular) foram realizados, com a identificação de poucos genes: PRKCH (Kubo, Hata et al. 2007), NINJ2 (Ikram, Seshadri et al. 2009), ZFHX3 (Gudbjartsson, Holm et al. 2009), CELSR1 (Yamada, Fuku et al. 2009), MACROD2 (Debette, Bis et al. 2009) e o SNP rs2200733 (gene não descrito) (Gretarsdottir, Thorleifsson et al. 2008). Alguns estudos não obtiveram resultados estatisticamente significativos. (Matarín, Brown et al. 2007) As associações descritas também se deram diretamente com outros fenótipos envolvidos na fisiopatologia dos AVCs, como fibrilação atrial (Gretarsdottir, Thorleifsson et al. 2008; Gudbjartsson, Holm et al. 2009), dislipidemia (Karvanen, Silander et al. 2009) e hipertensão (Karvanen, Silander et al. 2009), o que ressalta a heterogeneidade e consequente complexidade da isquemia cerebral. A associação de

APOE-e4 com infartos cerebrais também foi estudada isoladamente, porém os trabalhos careciam de poder estatístico para tal investigação. (Betard, Robitaille et al. 1994; Slioter, Tang et al. 1997; Traykov, Rigaud et al. 1999; Schneider, Bienias et al. 2005)

Existem diferenças étnicas importantes no risco cardiovascular. Estudo epidemiológico no Canadá em 2010 com 164 mil pessoas revelou diferenças marcantes entre brancos e negros em quase todos os fatores de risco e dados demográficos. Brancos apresentaram maior nível socioeconômico, maior residência rural e maior prevalência de tabagismo, enquanto negros apresentaram maiores prevalências de diabetes, hipertensão, sedentarismo e etilismo. Obesidade tinha prevalências diferentes dependendo do gênero: mulheres negras eram mais obesas que brancas, enquanto homens brancos eram mais obesos que negros. (Chiu, Austin et al. 2010) Em estudos nos Estados Unidos, negros com AVC apresentavam maior prevalência de diabetes e hipertensão que brancos, mas também receberam cuidados médicos de pior qualidade após o evento. (Bravata, Wells et al. 2005; Schwamm, Reeves et al. 2010) É interessante notar que a diferença étnica de risco para AVC tornou-se insignificante após correção para nível sócio-econômico. (Bravata, Wells et al. 2005)

Em outras regiões do globo, inúmeros grupos étnicos foram comparados. Estudo na Nova Zelândia mostrou que pessoas de origem asiática ou do Pacífico (nativos da Oceania) tinham risco maior para AVCs isquêmicos e hemorrágicos que caucasianos, mesmo controlando para nível socioeconômico. (Feigin, Carter et al. 2006) Em Israel, judeus com AVC apresentaram menor prevalência de diabetes que árabes. (Telman, Kouperberg et al. 2010) Em nenhum estudo, no entanto, houve caracterização genética da ancestralidade.

Demência de Corpúsculos de Lewy

DCL foi descrita em 1961 por Okazaki e colegas. (Okazaki, Lipkin et al. 1961) DCL tem critérios clínicos de consenso, que incluem perda cognitiva progressiva (critério principal) associado a flutuação da cognição (episódios de “desligamento”), alucinações visuais recorrentes e parkinsonismo não relacionado a neurolépticos. Outras alterações incluem distúrbio comportamental do sono REM, sensibilidade grave a neurolépticos e perda de neurônios dopaminérgicos em PET ou SPECT. (Lippa, Smith et al. 1994; McKeith, Galasko et al. 1996; Connor, Salmon et al. 1998; Shimomura, Mori et al. 1998; McKeith, Dickson et al. 2005)

Os critérios apresentam valor preditivo positivo moderado (60-80%), mas com baixa sensibilidade (20-30%). (Litvan, MacIntyre et al. 1998) Outro estudo demonstrou valores mais otimistas, porém somente quando patologia de DA estava ausente, o que é incomum. (Verghese, Crystal et al. 1999) Novas técnicas, como análise de sinucleína no líquido (Kasuga, Tokutake et al. 2010) e ressonância com aquisição DTI (Kantarci, Avula et al. 2010) podem melhorar estes índices, porém ainda não há estudos prospectivos com comprovação anátomo-patológica.

Neuropatologia da Demência de Corpúsculos de Lewy

Os corpúsculos de Lewy são inclusões neuronais intracitoplasmáticas cujo conteúdo principal é a sinucleína alfa, a mesma da doença de Parkinson. Na DCL, os corpúsculos estão espalhados no núcleo dorsal motor do vago, núcleos bulbares gigantocelulares, locus ceruleus, núcleo da rafe, tegumento mesencefálico e hipotálamo. Os corpúsculos neocorticais são menos eosinofílicos e menos definidos, sendo assim melhor detectados por anticorpos antissinucleína. Os córtices límbico e temporal são particularmente vulneráveis. (Lennox, Lowe et al. 1989; Kuzuhara and Yoshimura 1993; Braak and Braak 2000; Dickson 2002; Braak, Del Tredici et al. 2003; Braak, Ghebremedhin et al. 2004)

Genética da Demência de Corpúsculos de Lewy

História familiar de demência é mais comum em familiares de afetados com DCL que em controles. (Woodruff, Graff-Radford et al. 2006) Algumas famílias já foram descritas com formas mendelianas de DCL, nas quais alguns familiares apresentaram-se clinicamente como doença de Parkinson e outros com DCL. (Scott, Grubber et al. 2000; Singleton, Farrer et al. 2003; Zarranz, Alegre et al. 2004; Bogaerts, Engelborghs et al. 2007) Em algumas destas famílias, o gene identificado foi o da própria sinucleína alfa. (Singleton, Farrer et al. 2003; Zarranz, Alegre et al. 2004) Em outras famílias, estudos de ligação indicaram sobreposição com o locus PARK8, o gene LRRK2. (Scott, Grubber et al. 2000; Paisan-Ruiz, Jain et al. 2004) Mais recentemente, estudos mostraram associação de DCL com mutações do gene GBA, que também está envolvido na doença de Parkinson e de Gaucher. (Clark, Katsaklis et al. 2009; Sidransky, Nalls et al. 2009) A sobreposição patológica, clínica e genética da doença de Parkinson com DCL sugere que estas são expressões variáveis do mesmo processo fisiopatológico. (Gasser 2009) Os diversos GWAS de Parkinson devem contribuir também para melhor compreensão de DCL. (Simon-Sanchez, Scholz et al. 2008; Latourelle, Pankratz et al. 2009; Pankratz, Wilk et al. 2009; Sidransky, Nalls et al. 2009; Edwards, Scott et al. 2010)

Capítulo III. Objetivos

Os objetivos desta tese são:

1. Analisar uma amostra populacional de pessoas falecidas da cidade de São Paulo utilizando marcadores genéticos de ancestralidade autossômicos, identificando os componentes ancestrais individuais e da população, permitindo maior compreensão da sua potencial estratificação.
2. Comparar índices de ancestralidade com alterações anátomo-patológicas encontradas nos encéfalos dos sujeitos e identificar as causas de demência que diferem entre subgrupos.

Capítulo IV. Materiais e Métodos

Amostragem

As amostras de tecido humano foram obtidas pelo projeto Banco de Encéfalos do Grupo de Estudos do Envelhecimento Humano da Faculdade de Medicina da USP, aprovado no CEP do Hospital das Clínicas da FMUSP sob número 254/04. Os cérebros e outros tecidos foram doados após consentimento informado de familiares de pessoas falecidas e cujos corpos foram submetidos a necrópsias no Serviço de Verificação de Óbitos da Capital. Os critérios de exclusão eram idade menor que 50 anos no momento do óbito, causas cerebrais diretas de morte (AVC isquêmico, hemorrágico ou traumatismo craniano), casos em que entrevistados não tivessem conhecimento do status funcional do paciente (contato mínimo de uma vez por semana) e tecido em estado de degradação que impossibilitasse a análise neuropatológica. O DNA foi obtido, quando possível, do sangue ventricular ou alternativamente, do tecido cerebral (cerebelo e córtex occipital) e extraído por técnicas padrão. (Miller, Dykes et al. 1988)

Os familiares dos pacientes foram entrevistados por equipe de enfermagem especificamente treinada, utilizando questionários padronizados, conforme publicação prévia. (Grinberg, Ferretti et al. 2007; Ravid and Grinberg 2008) Para esta tese, foram utilizados os dados demográficos gerais (sexo, idade, escolaridade, nível sócio-econômico ABIPME e raça), avaliação cognitiva pelos questionários CDR e IQCODE e questionários de fatores de doenças prévias (hipertensão, diabetes, dislipidemia, doença arterial crônica, insuficiência cardíaca e arritmia) e fatores de risco ambientais para doença cardiovascular (tabagismo, etilismo e sedentarismo).

A investigação anátomo-patológica consistiu de avaliação macroscópica de tecido cerebral e

vasos maiores, com identificação de aterosclerose e infartos cerebrais. Os cortes histológicos foram avaliados por técnicas padrão hematoxilina-eosina, Gallyas (prata) e imuno-histoquímica para presença de placas neuríticas (quantificados pela escala CERAD neuropatológica), emaranhados de tau (quantificados pela escala de Braak) e corpúsculos de Lewy. Outras alterações neuropatológicas, como acúmulos de ubiquitina, grãos argirofílicos, siderocalcinose, esclerose hipocampal e angiopatia amiloide não foram incluídas no estudo devido à raridade.

No total, foram analisados 547 pacientes do banco para marcadores de ancestralidade, sendo 202 pacientes com os dados completos para o estudo epidemiológico de demência.

Genotipagem

As amostras foram genotipadas em duplicata para os polimorfismos de APOE (e2, e3 e e4), que constituem um haplótipo formado pelos SNPs rs429358 e rs7412, pela técnica de RealTime PCR, conforme descrito previamente. As amostras foram também genotipadas para 90 SNPs por espectrometria de massa no sistema Sequenom MassArray, no Instituto Broad de Harvard e MIT, Estados Unidos. Estes SNPs (tabela 1) foram previamente descritos como marcadores de ancestralidade por Nassir e colegas. (Nassir, Kosoy et al. 2009)

Tabela 1: SNPs usados como marcadores de ancestralidade.

Cromosso				Cromosso			
SNP	mo	Alelo 1	Alelo 2	SNP	mo	Alelo 1	Alelo 2
rs7554936	1	C	T	rs4717865	7	A	G
rs316873	1	C	T	rs32314	7	C	T
rs3118378	1	A	G	rs6464211	7	C	T
rs2986742	1	C	T	rs3943253	8	A	G
rs1040404	1	A	G	rs1471939	8	C	T
rs12130799	1	A	G	rs7844723	8	C	T

rs4908343	1	A	G	rs12544346	8	A	G
rs647325	1	A	G	rs10108270	8	A	C
rs1325502	1	A	G	rs2306040	9	C	T
rs3737576	1	C	T	rs10513300	9	C	T
rs2627037	2	A	G	rs2073821	9	C	T
rs260690	2	A	C	rs4746136	10	A	G
rs7421394	2	A	G	rs4918842	10	C	T
rs13400937	2	G	T	rs10839880	11	C	T
rs798443	2	A	G	rs1837606	11	C	T
rs4666200	2	A	G	rs948028	11	A	C
rs4670767	2	G	T	rs2946788	11	G	T
rs10496971	2	G	T	rs11227699	11	A	G
rs9845457	3	A	G	rs2416791	12	A	G
rs6548616	3	C	T	rs214678	12	C	T
rs12629908	3	A	G	rs772262	12	A	G
rs10510228	3	A	G	rs9522149	13	C	T
rs1513181	3	A	G	rs9530435	13	C	T
rs9809104	3	C	T	rs7997709	13	C	T
rs2030763	3	A	G	rs9319336	13	C	T
rs734873	3	A	G	rs3784230	14	A	G
rs2702414	4	A	G	rs1760921	14	C	T
rs7657799	4	G	T	rs2357442	14	A	C
rs1369093	4	C	T	rs200354	14	G	T
rs385194	4	A	G	rs1950993	14	G	T
rs6451722	5	A	G	rs8035124	15	A	C
rs12657828	5	A	G	rs12439433	15	A	G
rs316598	5	C	T	rs4781011	16	G	T
rs6422347	5	C	T	rs4984913	16	A	G
rs1871428	6	A	G	rs2125345	17	C	T
rs4463276	6	A	G	rs11652805	17	C	T
rs1040045	6	A	G	rs10512572	17	A	G
rs2504853	6	C	T	rs874299	18	C	T
rs7745461	6	A	G	rs7238445	18	A	G
rs2397060	6	C	T	rs8113143	19	A	C
rs731257	7	A	G	rs3745099	19	A	G
rs705308	7	A	C	rs3907047	20	C	T
rs7803075	7	A	G	rs4821004	22	C	T
rs10236187	7	A	C	rs5768007	22	C	T
rs2330442	7	A	G	rs1296819	22	A	C

Análise de Ancestralidade

Os genótipos dos 90 SNPs foram obtidas das 210 pessoas de 4 populações estudadas na

primeira fase do HapMap: YRI (Nigeria), CEU (Estados Unidos), CHB (China) e JPT (Japão). Estas foram combinadas com as amostras de 1043 pessoas de 51 populações estudadas no Human Genome Diversity Panel (HGDP). Todas as amostras estão disponíveis livremente nos sítios dos projetos na internet. (HapMap 2005; Li, Absher et al. 2008; Nassir, Kosoy et al. 2009)

Os 1253 controles mundiais, em conjunto às amostras que apresentaram boa qualidade-confiabilidade na fase de genotipagem (547 amostras do Banco de Cérebros e 35 amostras providas de Quilombos no sul do estado de São Paulo cedidas em colaboração com Profa. Dra. Celia Regina Mingronni Netto) foram analisados no programa Structure versão 2.3.3, divididos em 3 séries de aproximadamente 200 amostras brasileiras (além dos controles mundiais). Os parâmetros de análise foram 100.000 burn-ins e 200.000 iterações para $k = 3, 4$ e 6 , todos com presunção de mistura populacional (ADMIXTURE) e sem utilização de origem amostral para classificação (LOCPRIOR = 0). Todas as análises estatísticas e gráficos foram gerados utilizando o programa SPSS versão 18.0, Linguagem R versão 2.11 e OpenOffice Calc versão 3.2.

Capítulo V. Manuscrito I:

A Major Semitic Genetic Contribution to the Brazilian Population

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Abstract

According to historical evidence, a large percentage of Portuguese immigrants to Brazil in colonial times (1500-1800) were “New Christians” fleeing persecution by the Inquisition. (1, 2) We have genotyped 547 inhabitants of São Paulo, Brazil for ancestry-informative markers that have previously been shown to distinguish individuals with Semitic and European ancestry. Surprisingly, Central-South Asian (CSA) ancestry corresponds to 29%, larger than the African cluster (19%). The percentage of CSA ancestry within the Caucasian cluster (44%) was comparable to Semitic groups of the Human Genome Diversity Project. CSA ancestry was also more frequent in Brazilian subjects with higher

African and Amerindian ancestry (colonial/pre-colonial populations), as well as genetically isolated descendants of African slaves ($p < 0.001$ for each), pointing towards a colonial arrival to Brazil. Our results suggest a major Semitic contribution to the Brazilian gene pool, with implications on complex disease genetics studies.

Resumo em Português

Segundo evidências históricas, uma grande porcentagem de imigrantes portugueses que vieram ao Brasil no período colonial (1500-1800) eram “cristãos-novos” fugindo da perseguição pela Inquisição. (1, 2) Nós determinamos o genótipo de 547 pessoas de São Paulo, Brasil de marcadores de ancestralidade que distinguem origem semítica de europeia. Surpreendentemente, ancestralidade centro-sul asiática (CSA) corresponde a 29%, maior que o grupo africano (19%). A porcentagem de CSA dentro da ancestralidade caucasiana (europeia + CSA) é 44%, comparável a grupos semitas do Human Genome Diversity Project. Ancestralidade CSA também é mais frequente em indivíduos com maior ancestralidade africana e ameríndia, que são as populações do período colonial e pré-colonial, além de aumentada em descendentes parcialmente isolados de africanos ($p < 0,001$ para cada). Isto aponta para uma origem colonial da ancestralidade semítica. Nossos resultados sugerem uma grande contribuição semítica para a genética da população brasileira, com implicações no estudo de doenças complexas.

Main Text

The current populations of the Americas, particularly Brazil, result from the admixture of native Amerindians, African slaves, and European immigrants. Until 1808, Portugal was the main source of immigrants to Brazil. Historical evidence suggests that a large percentage of these were “New Christians” fleeing persecution by the Inquisition. (1,2) These Jewish and Moor converts subsequently

lost most of their cultural heritage making it difficult to accurately assess their contribution to the current population. Studies in Portugal and Brazil have pointed towards a high demographic impact of these groups using Y-chromosome markers (up to 20% Jewish ancestry), but autosomal ancestry-informative markers (AIMs) used to date did not have the power to separate European from Middle Eastern and Central/South Asian (CSA) ancestry. (3,4) After 1808, over 5 million people migrated from Europe and the Middle East to Brazil, but less than 5% were Jews or Arabs.

We have examined a population-based sample of 547 inhabitants of São Paulo in Brazil using 90 AIMs that have previously been shown able to distinguish individuals with Semitic and European ancestry. (5) The São Paulo population, when separated into 4 clusters, shows a high level of admixture that is 69.6% Eurasian, 20.0% African, 5.7% Asian, and 4.7% Amerindian, similar to previous reports. (figure 1, k = 3, 4) (6) Remarkably, when we separated these subjects into 6 clusters, the Eurasian cluster is broken into a European cluster of 41.8% and a CSA cluster of 29.3%. (figure 1, k = 6) This CSA contribution (out of the total Eurasian ancestry) to the Brazilian genetic pool comparable to the Druze and Palestinians. (figure S1, S2)

To differentiate between a colonial and a recent (1808 until today) Semitic contribution, we analyzed the estimated percentage of the CSA ancestry within the Caucasian cluster (European + CSA ancestry) as compared to African and Amerindian ancestry in each individual. The older the Semitic presence in Brazil, the higher the probability of admixture with female African slaves and Amerindians, who were the majority of the colonial population. (7) Both African ($p < 0.001$, Spearman's rank correlation test) and Amerindian ancestry ($p < 0.001$, Spearman's rank correlation test) positively correlated with CSA percentage, indicating a colonial origin. We then genotyped 35 samples of inhabitants of “Quilombos”, communities of descendents of runaway African slaves (founded between 1750 and 1889) that still live in partial genetic isolation in southeastern Brazil. CSA percentage (of total Caucasian ancestry) was significantly higher in the “Quilombo” group when compared to the São

Paulo population (66% versus 42%, $p < 0.001$, two-sample t-test).

Our results show strong genetic evidence of a major contribution of Jewish and Arab descendants to the colonization of Brazil. Furthermore, our data indicate that AIM panels that cannot differentiate between CSA and European ancestry to control for population stratification in association studies may be ineffective in Brazil, and possibly in other populations in the Americas as well. Future studies in the northeastern region of Brazil, a frequent destination of “New Christian” immigration in the colonial period, may yield even higher genetic contributions.

Supplement Materials and Methods

Samples were obtained from the Brazilian Aging Brain Study Group of University of São Paulo Medical School, collected between 2004 and 2009, of subjects over 50 years old at death. The population base includes all inhabitants of the city of São Paulo, approximately 11 million people (5.6% of the population of Brazil) and study samples do not significantly deviate from census data for age, sex, race, years of schooling, or socioeconomic levels. All tissue donations were made by next-of-kin after providing informed consent and the study was approved by the institutional review boards of participating institutions.

Samples were genotyped for 90 SNPs previously described from an ancestry-informative marker panel reported. (5) The AIMS were genotyped using Sequenom MassArray from the Genotyping Facility of the Broad Institute of MIT and Harvard. Samples with more than 90% call rate were included in the analysis. SNPs with >10% no-call rate were excluded from the analysis.

We estimated ancestry by modeling ancestral populations ($k = 3, 4$, and 6) with admixture in Structure version 2.3.3 (100,000 burn-ins, 200,000 iterations, $LOCPRIOR = 0$), alongside samples from the Human Genome Diversity Panel and the HapMap (Phase I) project, both publicly available.

(8-10)

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Figure 1: Population structure analysis of worldwide populations (HGDP and HapMap subjects), subjects from São Paulo, and from “Quilombos”. Number of clusters on left side (k). São Paulo population is sorted by decreasing CS Asian ancestry, except for first 10 subjects with self-declared East Asian ancestry.

Figure S1: Population structure analysis of worldwide populations, São Paulo population, and subjects from “Quilombos”, averaged per group, $k = 6$.

Figure S2: Percentage of European ancestry (of total Eurasian ancestry) for each population, plotted by distance from the Levant, negative values for locations to the North or West (225 to 45 degrees), positive values for locations to the South or East (45 to 225 degrees). Distance from London, UK was assigned to HapMap CEU (Utah residents). Arrow indicates ancestry of the São Paulo population.

Capítulo VI. Manuscrito II:

African Ancestry protects against Alzheimer's Disease-related neuropathology

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ABSTRACT

Background

Previous studies in dementia epidemiology have reported higher Alzheimer's disease rates in subjects of African descent when compared to Caucasians. We conducted a population-based study to determine whether genetically-determined African ancestry is associated with neuropathological changes commonly associated with dementia.

Methods

We studied 202 brains obtained in the brain bank of the Brazilian Aging Brain Study Group of the University of Sao Paulo between 2004 and 2009 for presence of neuritic plaques, neurofibrillary tangles, small vessel disease, brain infarcts, and Lewy bodies. African ancestry was determined through the use of ancestry-informative markers. We also adjusted the results for multiple environmental risk factors and APOE genotype.

Results

Contrary to previous studies, subjects with African ancestry ($n = 112$, 55.4%) showed lower prevalence of neuritic plaques in the univariate analysis (OR 0.72, 95% CI 0.55-0.95, $p = 0.01$) and when adjusted for age, sex, APOE genotype, and environmental risk factors (OR 0.43, 95% CI 0.21-0.89, $p = 0.02$). There were no differences for the presence of other neuropathological alterations. This suggests that unknown variants more frequent in the “African” genome reduce the accumulation of β -amyloid or increase its clearance. The results are robust and are not altered when studying only those who self-defined themselves as Whites, when adjusting for APOE4 status only, or when adjusting for age and sex only.

Conclusion

Here we show for the first time, using genetically-determined ancestry markers, that African ancestry is

highly protective of Alzheimer's disease neuropathology.

RESUMO

Introdução

Estudo prévios em epidemiologia de demências relataram prevalência de doença de Alzheimer mais alta em indivíduos descendentes de africanos quando comparados aos caucasianos. Nós realizamos um estudo populacional para determinar se ancestralidade africana geneticamente determinada está associada à alterações neuropatológicas comunmente associadas à demência.

Métodos

Nós estudamos 202 cérebros obtidos entre 2004 e 2009 no banco de encéfalos do Grupo de Estudos de Envelhecimento Cerebral da Faculdade de Medicina da USP, identificando a presença de placas neuríticas, emaranhados neurofibrilares, arterioloesclerose, infartos cerebrais e corpúsculos de Lewy. Ancestralidade africana foi determinada usando marcadores genéticos de ancestralidade. Nós também ajustamos os resultados para múltiplos fatores de risco e genótipo de APOE.

Resultados

Ao contrário de estudos prévios, indivíduos com ancestralidade africana (n = 112, 55,4%) apresentaram menor prevalência de placas neuríticas na análise univariada (OR 0,72, 95% CI 0,55-0,95, p = 0,01) e quando ajustado para idade, sexo, genótipo de APOE e fatores de risco ambientais (OR 0,43, 95% CI 0,21-0,89, p = 0,02). Não houve diferenças estatisticamente significativas para outras alterações neuropatológicas. Isto sugere que variantes desconhecidas, mais frequentes no genoma “Africano”, reduzem o acúmulo de β -amylóide ou aumentam sua degradação. Os resultados são robustos e não se alteram quando restringimos a comparação aos que se auto-declaram brancos, nem quando ajustamos para genótipo de APOE4, ou quando ajustamos para idade e sexo somente.

Conclusão

Pela primeira vez, nós demonstramos com marcadores genéticos de ancestralidade, que ancestralidade africana é protetora para a neuropatologia da doença de Alzheimer.

INTRODUCTION

Self-declared race and skin color are often used as surrogates for genetic ancestry despite being poor biological classifiers, especially in countries with admixed populations where significant overlaps between groups exist.¹ Furthermore, racial categorization is modifiable by environmental factors such as sunlight exposure, socioeconomic level, physical appearance, and cultural aspects.²⁻⁴ The marked epidemiological differences in health status between racial groups in countries such as Brazil and the United States are likely a combination of genetic and environmental factors, particularly socioeconomic levels.^{5,6}

Dementia is a complex phenotype caused by frequently overlapping neuropathological processes such as neuritic plaques and neurofibrillary tangles (Alzheimer's Disease), small vessel disease and/or brain infarcts (Vascular Dementia), and synuclein deposits (Lewy Body Disease & Parkinson's Disease), as well as other rarer alterations.⁷ The clinical diagnosis of dementia is further influenced by the educational level, language and cultural aspects. Several studies have shown that African Americans are more frequently diagnosed with dementia (in general) and Alzheimer's disease than Caucasians.⁸⁻¹² These differences may be caused by genetic variants or the environment.¹³⁻¹⁶ Few studies have focused on neuropathological pos-mortem diagnosis and none on ancestry-informative marker- (AIM) determined genetic ancestry.¹⁷⁻¹⁹

The quantitative assessment of ancestry using AIMS has been previously demonstrated to be useful in breast cancer epidemiology and lung-function prediction.^{20,21} The population of Brazil is highly admixed, with major historic contributions from European immigrants, African slaves, and

native Amerindians.²²⁻²⁴ The genetic structure of the population of Brazil is approximately 80% European, 15% African, and 5% Amerindian, with significant variation between regions.^{25,26}

METHODS

Study Population

Brain samples from the Brazilian Aging Brain Study Group of University of São Paulo Medical School, collected from 2004 to 2009 were studied.²⁷ Exclusion criteria included age at death of less than 50 years, causes of death or tissue condition that impeded neuropathological analysis, informants without knowledge of the functional status of subjects (minimum 1 visit/week), and violent/criminal deaths. Tissue donations were obtained in the municipal São Paulo Autopsy Service, in Brazil. The population base includes all inhabitants of the city of São Paulo, approximately 11 million people (5.6% of the population of Brazil) and study samples do not significantly deviate from census data for age, sex, race, years of schooling, or socioeconomic levels. (data not shown) All tissue donations were made by next-of-kin after providing informed consent and the study was approved by the institutional review boards of all participating institutions. Knowledgeable informants were interviewed by nurses trained specifically for the questionnaires, including cognitive evaluation (IQCODE and CDR) and demographics.^{28,29}

Neuropathological Assessment

All neuropathological diagnoses were carried out by two trained specialists (LTG and MPA). Brains were inspected macroscopically, and lesions of Alzheimer's disease (neuritic plaques and neurofibrillary tangles), Lewy bodies, and small-vessel disease (microinfarcts, lacunes, and small vessel disease) were scored according to accepted criteria.³⁰⁻³² Immunohistochemistry was done with antibodies against β -amyloid (4G8, 1/5000, Signet Laboratories, Dedham, MA), phospho-tau (PHF-1,

1/1000, gift of Peter Davies, New York City, NY), and α -synuclein (EQV-1, 1/10 000, gift of Kenji Ueda, Tokyo, Japan). If TDP-43opathy was suspected, immunostaining for TDP-43 (1/500, ProteinTech Group, Chicago, IL) was performed. All sections were submitted to antigenic retrieval. The reactions were detected using the Vectastain Elite ABC Kit method (Vector Laboratories, Burlingame, CA, USA). Neuritic plaques were classified as absent, mild, moderate, or frequent. Neurofibrillary tangles were classified according to the Braak score of 0-VI.³¹

Ancestry Estimation and Genotyping

Samples were genotyped for 90 SNPs previously described from an ancestry-informative marker panel reported to efficiently separate Caucasian, African, Amerindian, and Asian populations.³³ The AIMs were genotyped using Sequenom MassArray from the Genotyping Facility of the Broad Institute of MIT and Harvard. Samples with more than 90% call rate were included in the analysis. SNPs with >5% no-call rate were excluded from the analysis. APOE genotype (SNPs rs429358 and rs7412) was determined by RealTime PCR, in duplicates, as previously described.³⁴

We estimated ancestry by modeling four ancestral populations ($k = 4$) with admixture in Structure version 2.3.3 (100,000 burn-ins, 200,000 iterations, LOCPRIOR = 0), alongside samples from the Human Genome Diversity Panel and the HapMap (Phase I) project, both publicly available.³⁵⁻

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Statistical Analysis

Neuropathological variables (neuritic plaques, small vessel disease, infarcts, Lewy bodies) were dichotomized between presence and absence, and neurofibrillary tangles were divided between Braak score 0-III and IV-VI. Ancestry was also dichotomized at 2% African ancestry and race between whites and non-whites (black or brown). Subjects/relatives of self-declared Asian origin ($n = 6$) were excluded

from analysis. No subjects/relatives self-identified as Amerindian.

Logistic regression was used to model the effect of African ancestry (or alternatively non-white race) on the presence of neuropathology. Each pathology was considered a separate outcome. Analysis was carried out with adjustment for age at death, sex, years of schooling, socioeconomic level, APOE genotype, and family-reported cardiovascular risk factors (hypertension, diabetes, hypercholesterolemia, heart failure, arrhythmia, smoking, alcohol consumption, and sedentary lifestyle). A subgroup analysis using ancestry restricted to whites (75% of the samples) was also done. Significant findings were considered when $p < 0.05$.

RESULTS

Ancestry and Race

The study consisted of 202 brain samples with complete genotyping, neuropathology, and close relatives' interviews. There were 112 individuals (55.4%) with African ancestry (table 1). Non-white race and genetically-determined African ancestry were highly correlated ($p < 0.01$). Some self-declared whites were over 70% African, while a few non-whites had more than 99% European ancestry. (figure 1) Table 1 shows demographic characteristics for subjects with significant ($>2\%$) African ancestry and those without. African ancestry was associated with lower educational ($p < 0.05$) and socioeconomic levels ($p < 0.01$). There were no differences in cognitive status between groups, as measured by IQCODE or CDR scale.

Ancestry and Neuropathology

The relative prevalence of each neuropathological change is shown in figure 2. It includes comparisons of African versus non-African ancestry in all subjects, African versus non-African ancestry in self-declared whites, and whites versus non-whites.

The prevalence of Alzheimer's disease neuritic plaques was significantly lower in subjects with African ancestry (0.72, 95% CI 0.55-0.95, $p = 0.01$). African ancestry also correlated with less pathology in the subgroup analysis restricted to self-declared whites (0.63, 95 CI 0.42-0.95, $p = 0.02$). The prevalence of Alzheimer's related neurofibrillary tangles showed a trend towards lower pathology in subjects with African-ancestry when all subjects were analyzed (0.64, 95% CI 0.33-1.21, $p = 0.16$) and when analysis was restricted to white race (0.50, 95% CI 0.22-1.10, $p = 0.11$). The prevalence of small vessel disease, brain infarcts, and Lewy bodies was higher in subjects with African compared to non-African ancestry, but none were statistically significant. Self-declared race showed no statistically significant differences for any of the neuropathological end-points. (table 2, figure 2)

Adjustment for possible confounding factors did not alter the findings. (figure 3) Neuritic plaques were less prevalent in subjects with African ancestry when adjusted for age and sex (OR 0.47, 95% CI 0.25-0.89, $p = 0.02$), when adjusting for age, sex, and APOE4 status (OR 0.35, 95% CI 0.17-0.70, $p < 0.01$), and when adjusting for all factors including socioeconomic level, educational level, and cardiovascular risk factors (OR 0.43, 95% CI 0.21-0.89, $p = 0.02$). (table 3, figure 3)

DISCUSSION

The Brazilian population is highly admixed, with >90% of its ancestry derived from African slaves and European immigrants, which makes it ideal for ancestry-related studies. Moreover, Brazilian law mandates that autopsies be performed in all persons without a death certificate, which provides a large recruitment base for population studies in neuropathology, without bias towards demented persons. The University of São Paulo alone performs more than 14,000 autopsies per year, encompassing the full range of demographic variation of the city. The centering of all samples in a single institution also greatly reduced inter-rater variation in both interviews and pathology.

Contrary to previous studies, our results show that African ancestry is highly protective of

Alzheimer's disease neuropathology (neuritic plaques), with an adjusted odds ratio of 0.43. This suggests that unknown variants more frequent in the “African” genome reduce the accumulation of β -amyloid or increase its clearance, when compared to the “European” genome. The results are robust and are not altered when studying only those who self-defined themselves as whites, when adjusting for APOE4 status only, or when adjusting for age and sex only.

Previous studies with the United States population reported a significantly higher prevalence of dementia in African Americans when compared to Caucasians.⁸⁻¹² These differences could be due to cultural differences in the performance on cognitive screening tests such as the Mini Mental Status Examination, genetic differences between races, environmental differences, or likely a combination of factors.¹³⁻¹⁶ In a study comparing dementia in Nigerians and African Americans, the former had significantly lower disease rates. Our data suggests that these results might be explained not only by environmental differences, but also by the European admixture present in African Americans.³⁸ Further studies are needed to confirm this.

Cardiovascular disease risk and stroke also vary between races, but statistical significance often disappears when adjustments for socioeconomic levels are applied.^{14,39,40} Lower educational and socioeconomic levels in those with higher African ancestry may create important differences in disease susceptibility which is independent of genetics, but confounds the analysis.

To our knowledge, few studies have compared autopsy-diagnosed cases in different races and none used ancestry-informative markers.¹⁷⁻¹⁹ The growing clinical use of AIMS was recently shown by Kumar and colleagues as a tool for improved lung-function prediction in African Americans, although this should only be considered an intermediary step towards use of specific disease-related variants in personalized medicine.^{21,41} Furthermore, dementia is a complex clinical phenotype that may be caused by widely diverse pathologies, and post-mortem diagnosis remains the gold-standard. The specific neuropathology of Alzheimer's Disease, Lewy Body Dementia, and Vascular Dementia may have

different underlying biological and genetic causes, and should therefore be studied separately.

A few notes of caution regarding the present study should be pointed out. First, although we adjusted for multiple environmental and social factors as well as APOE genotype, other untested environmental factors may be confounding our ancestry results. Second, African populations are highly variable (more so than Europeans), and therefore we cannot state from our data that this effect is applicable to all African populations.¹³ It is unknown if there are population subgroups within Africa with different risk profiles for neuropathological alterations.

In conclusion, our study shows, for the first time, that Alzheimer's neuropathological findings depend on the ancestral genetic background. It clearly demonstrates that the presence of neuritic plaques are reduced in persons with African ancestry in a population-based sample, independently of known confounding factors. This should serve as a basis for future genetics studies of Alzheimer's disease, as well as alert against over-interpreting epidemiological studies using race and clinically-diagnosed dementia.

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Figure 1 : Percent of African ancestry classified by self-declared race in Brazil.

Figure 2: Odds ratio of neuropathological alterations, classified by genetic ancestry, genetic ancestry in self-declared whites, and by race.

Figure 3: Odds ratio of presence of neuritic plaques, comparing African to Non-African ancestry, adjusted for age and sex; age, sex, and APOE4 status; and all factors.

Table 1: Study Characteristics

Table 1 (Study Characteristics)	African Ancestry	Non-African Ancestry	p-value
Number	112	90	
Age at Death (mean +/- SD)	74.5 +/- 11.9	76.9 +/- 11.4	P = 0.18
Gender (% female)	57.3%	60.0%	P = 0.68
Socio-economic Level (mean ABIPEME score +/- SD)	15.5 +/- 9.4	20.3 +/- 9.0	P < 0.001
Educational Status (mean years of schooling +/- SD)	3.7 +/- 3.7	4.8 +/- 3.8	P = 0.03
Race (% Self-Declared White)	58.0%	95.5%	P < 0.001
Cognitive Status (mean IQCODE +/- SD)	3.44 +/- 0.72	3.40 +/- 0.68	P = 0.81
APOE4 Status (% positive)	26.8%	20.0%	P = 0.26

Table 2: Odds ratio of neuropathological alterations, classified by genetic ancestry, genetic ancestry in self-declared whites, and by race.

Table 2 (Risk of Pathology Present)	OR	Lower	Upper	p-value	
Ancestry (African vs Non-African)	0,72	0,55	0,95	0,01	Neuritic Plaques
Ancestry (African vs Non-African) - Whites only	0,63	0,42	0,95	0,02	
Self-Declared Race (Black and Mixed vs White)	0,73	0,44	1,22	0,22	
Ancestry (African vs Non-African)	0,64	0,33	1,21	0,16	Neurofibrillary Tangles
Ancestry (African vs Non-African) - Whites only	0,50	0,22	1,10	0,11	
Self-Declared Race (Black and Mixed vs White)	0,92	0,44	1,90	0,78	
Ancestry (African vs Non-African)	1,24	0,95	1,63	0,18	Small Vessel Disease
Ancestry (African vs Non-African) - Whites only	1,20	0,76	1,86	0,44	
Self-Declared Race (Black and Mixed vs White)	1,63	0,99	2,70	0,08	
Ancestry (African vs Non-African)	1,05	0,76	1,45	0,78	Infarcts
Ancestry (African vs Non-African) - Whites only	1,09	0,69	1,73	0,75	
Self-Declared Race (Black and Mixed vs White)	0,81	0,40	1,65	0,59	
Ancestry (African vs Non-African)	1,36	0,97	1,89	0,14	Lewy Bodies
Ancestry (African vs Non-African) - Whites only	1,43	0,83	2,47	0,26	
Self-Declared Race (Black and Mixed vs White)	1,36	0,63	2,89	0,45	

Capítulo VII. Discussão e Conclusões

Os objetivos desta tese de doutorado eram dois: 1) a análise de uma amostra populacional de pessoas falecidas da cidade de São Paulo utilizando marcadores genéticos de ancestralidade autossômicos, identificando os componentes ancestrais individuais e da população, permitindo maior compreensão da sua potencial estratificação e 2) a comparação de estimativas de ancestralidade com alterações anátomo-patológicas encontradas nos encéfalos dos sujeitos, com a identificação das causas de demência que diferem entre subgrupos.

No manuscrito I (capítulo V) estudamos uma amostra representativa (547 indivíduos) da cidade de São Paulo, obtida no Serviço de Verificação de Óbito da Capital. Os marcadores genéticos estudados tem poder de distinguir ancestralidade europeia de oriente médio/centro-sul asiático (Paquistão, Índia). (Nassir, Kosoy et al. 2009) As análises que separaram os indivíduos em 3 e 4 grupos resultaram em estimativas similares às publicadas previamente. (Lins, Vieira et al. 2009; Pena, Bastos-Rodrigues et al. 2009) No entanto, quando a população foi subdividida em 6 grupos, um padrão surpreendente emergiu: a divisão dos caucasianos em 56% ancestralidade europeia e 44% centro-sul asiática (total centro-sul asiática é 29%, maior que ancestralidade africana de 19%). (figura 1 do manuscrito I) Esta proporção de 44% é similar a grupos de drusos e palestinos do Human Genome Diversity Project.

A origem desta substancial contribuição semítica genética para nossa população provavelmente resulta da herança árabe-judáica da península ibérica (Read 1975), amplificada pela grande migração de “cristãos-novos” para o Brasil no período colonial até a abertura dos portos em 1808. (Grinberg 2005) Estes imigrantes procuravam oportunidades financeiras e liberdade religiosa longe do alcance da Inquisição, que nunca se instalou de forma definitiva no Brasil. (Novinsky 1972; Novinsky and Biblioteca Nacional (Portugal) 1987; Novinsky 1992) Os dados apoiam fortemente a origem semítica

mais antiga e não recente, não somente porque judeus e árabes constituíram menos de 5% dos 5 milhões de imigrantes após 1808, mas por evidências genéticas também. A porcentagem de origem centro-sul asiática (do total de origem europeia + centro-sul asiática) é uma medida que segrega-se de maneira independente da ancestralidade africana ou indígena. Os ameríndios e escravos africanos estavam presentes no Brasil antes do grande influxo de europeus nos últimos 200 anos. Ao demonstrarmos que há correlação positiva ($p < 0,001$ para ambos) entre ancestralidade africana e porcentagem de centro-sul asiática, assim como ancestralidade ameríndia e a mesma porcentagem, confirmamos esta origem colonial.

Os Quilombos, comunidades parcialmente isoladas de escravos fugitivos e indígenas locais, se formaram a partir de 1750. Na comparação da porcentagem de ancestralidade centro-sul asiática, também obtemos diferenças estatisticamente significativas (66% nos quilombos, 44% nos paulistanos, $p < 0,001$), mais uma vez confirmando a presença antiga da genética semítica na nossa população. Estes resultados genéticos são novos, vão de encontro aos dados históricos e tem implicações científicas importantes.

O uso de marcadores de ancestralidade para controle de estratificação populacional, conforme expus na introdução, é um método em crescente uso. Na população brasileira, marcadores de ancestralidade que não separem europeus de centro-sul asiáticos certamente não corrigirão adequadamente a estratificação. Isto é relevante não somente para São Paulo, mas para o resto do Brasil e possivelmente toda a América.

No manuscrito II (capítulo VI), comparamos a ancestralidade africana às principais alterações anátomo-patológicas encontradas em 202 amostras do Serviço de Verificação de Óbito. O principal achado do estudo é a proteção conferida pela ancestralidade africana para placas neuríticas, que está relacionada à gênese da doença de Alzheimer. Em estudos epidemiológicos de demência nos Estados Unidos, conforme citei na introdução, o critério racial foi (e é) utilizado como suposto equivalente à

ancestralidade. Os resultados sugerem uma propensão à doença de Alzheimer nos negros. No entanto, os problemas comuns a todos estes estudos são o uso de raça ao invés de ancestralidade medida geneticamente, o uso de diagnósticos clínicos ao invés de diagnósticos anátomo-patológicos, e o desenho dos estudos em forma de caso-controle, todos estes corrigidos nesta tese.

Ademais, comparamos os resultados obtidos usando estimativas de ancestralidade no grupo completo e no subgrupo auto-declarado “branco”. Também realizamos as análises estatísticas de forma univariada e multivariada, corrigindo para todos os principais fatores de risco conhecidos (idade, sexo, genótipo de APOE, nível socio-econômico e educacional, fatores de risco cardiovasculares como diabetes, hipertensão, dislipidemia, sedentarismo, etilismo, tabagismo e outros). Todos os resultados referentes à principal alteração de Alzheimer (do ponto de vista fisiopatológico) foram consistentes e estatisticamente significativos ($p < 0,05$).

Os resultados do manuscrito II sugerem que devemos ter cautela na interpretação de estudos epidemiológicos raciais nos Estados Unidos (e em qualquer lugar). Importante estudo relatou que a prevalência de demência na Nigéria foi relatada como sendo menor que em Indianapolis, nos EUA. Deste estudo conclui-se que diferenças ambientais causaram as diferenças. (Ogunniyi, Baiyewu et al. 2000) Alternativamente, com nossos resultados podemos sugerir uma hipótese alternativa, pois os negros americanos não estão somente em um ambiente diferente, mas tem 20-30% de origem européia, o que aumenta o risco genético para Alzheimer. Por fim, os resultados tem claras implicações no desenho de estudos de genética de demências no Brasil, reforçando a necessidade de controle robusto da estratificação populacional.

Podemos concluir desta tese que o uso de marcadores genéticos de ancestralidade (mas não raça) é de grande utilidade tanto para compreensão de fenômenos históricos e antropológicos, assim como para o estudo de doenças complexas. As técnicas de sequenciamento de alta capacidade e baixo custo (o lendário “*\$1000 Genome*”) que estão surgindo deverão suplantam o uso de marcadores de

ancestralidade usados isoladamente. No meio tempo, entretanto, servem de grande utilidade.

Ouvi desde a infância que o importante em ciência são as perguntas interessantes que fazemos.

Termino a tese com algumas levantadas por seus resultados:

1. Qual a composição da ancestralidade semítica em outras regiões do Brasil, da América Latina e da Península Ibérica?
2. Existem outras heranças genéticas ainda crípticas no Brasil?
3. A dieta do Mediterrâneo é aceita como protetora cardiovascular. Isto pode ser pela ancestralidade semítica? Ela influencia os processos neuropatológicos ou doenças cardiovasculares?
4. Por que os negros americanos tem mais demência que os brancos – por ambiente, genética, viés do diagnóstico clínico ou uma combinação destes fatores?

Capítulo VIII. Abstract

Race/ethnicity is poor surrogate for estimating ancestry. Genetic testing using ancestry-informative markers are a significant improvement, especially in admixed populations such as the Brazilian population. We have genotyped 547 inhabitants of São Paulo, Brazil for 90 ancestry-informative markers that have previously been shown to distinguish individuals with Semitic and European ancestry. Central-South Asian (CSA) ancestry emerged as the second largest cluster within our population (29%). Further comparisons indicated that this semitic contribution to the Brazilian gene pool is likely derived from Portuguese “New Christians” during colonial times.

We then investigated whether genetically-determined African ancestry is associated with neuropathological changes commonly associated with dementia, as suggested by studies in African Americans. We studied 202 brains obtained in the brain bank of the Brazilian Aging Brain Study Group of the University of Sao Paulo between 2004 and 2009 for presence of neuritic plaques, neurofibrillary tangles, small vessel disease, brain infarcts, and Lewy bodies. We also adjusted the results for multiple environmental risk factors and APOE genotype.

Contrary to previous studies, subjects with African ancestry showed lower prevalence of neuritic plaques in the univariate and multivariate analysis. The results are robust and are not altered when studying only those who self-defined themselves as Whites, when adjusting for APOE4 status only, or when adjusting for age and sex only. We therefore showed for the first time, using genetically-determined ancestry markers, that African ancestry is highly protective of Alzheimer's disease neuropathology.

Our use of genetically-determined ancestry has led to results that have direct implications on the study of the genetics of complex diseases.

Capítulo VIII. Resumo Geral

Raça e etnia são substitutos ruins para ancestralidade genética. O uso de marcadores genéticos de ancestralidade melhoram o quadro significativamente, especialmente em populações miscigenadas como a brasileira. Nós determinamos o genótipo de 547 pessoas de São Paulo, Brasil de 90 marcadores de ancestralidade que distinguem origem semítica de européia. Ancestralidade centro-sul asiática (CSA) correspondeu a 29% do total, o segundo maior grupo. Outras análises indicam que esta contribuição genética semítica é derivada dos “cristãos-novos” durante o período colonial brasileiro.

Nós então investigamos se ancestralidade africana geneticamente determinada está associada a alterações neuropatológicas comumente ligada à demência, conforme sugerido por estudos de negros nos Estados Unidos. Nós estudamos 202 cérebros obtidos entre 2004 e 2009 no banco de encéfalos do Grupo de Estudos de Envelhecimento Cerebral da Faculdade de Medicina da USP, identificando a presença de placas neuríticas, emaranhados neurofibrilares, arterioesclerose, infartos cerebrais e corpúsculos de Lewy. Nós também ajustamos os resultados para múltiplos fatores de risco e genótipo de APOE.

Ao contrário de estudos prévios, indivíduos com ancestralidade africana apresentaram menor prevalência de placas neuríticas nas análises univariada e multivariadas. Os resultados são robustos e não se alteram quando restringimos a comparação aos que se auto-declaram brancos, nem quando ajustamos para genótipo de APOE4, ou quando ajustamos para idade e sexo somente. Pela primeira vez nós demonstramos com marcadores genéticos de ancestralidade, que ancestralidade africana é protetora para a neuropatologia da doença de Alzheimer.

Nosso uso de ancestralidade determinada geneticamente tem implicações diretas no estudo da genética de doenças complexas.

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Anexo

NEUROLOGY

A family with McLeod syndrome and calpainopathy with clinically overlapping diseases

A. Starling, D. Schlesinger, F. Kok, M. Rita Passos-Bueno, M. Vainzof and M. Zatz

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A family with McLeod syndrome and calpainopathy with clinically overlapping diseases

Abstract—The authors describe a family with six patients with muscular dystrophy with a variable course. One is a compound heterozygote for CAPN3 mutations (calpainopathy) and the others have a single CAPN3 mutation. Linkage analysis and sequencing revealed a XK gene mutation (McLeod syndrome). This illustrates the variable phenotype of XK mutations and suggests the possibility that CAPN3 heterozygotes may have their condition caused by nonallelic mutations in other unrelated genes.

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Limb girdle muscular dystrophy (LGMD) is a genetically heterogeneous disorder characterized by progressive weakness of proximal girdles. Seventeen genes associated with LGMD have been mapped, 10 autosomal recessive (AR)¹ and seven autosomal dominant (AD).² The prevalent form in most populations is LGMD2A (calpainopathy), caused by mutations in the calpain-3 (CAPN3) gene.^{1,3}

McLeod syndrome is an X-linked neuroacanthocytosis syndrome with hematologic, neuromuscular, and CNS manifestations with onset usually after the third decade of life that also presents with elevated creatine kinase (CK) levels and subclinical myopathy, although rarely a severe myopathy as well. Hematologic features include acanthocytosis, compensated hemolysis, absent Kx red blood cell (RBC) antigen, and weak antigenicity to Kell RBC antigens.^{4,5}

We ascertained a white Brazilian family with six affected men (five brothers and a first-degree once-removed cousin) who presented an overlapping phenotype between LGMD and McLeod syndrome.

Methods. The proband, IV-1, a 16-year-old boy, was ascertained due to severe proximal weakness in the lower and upper limbs. Onset was at age 10, with rapid progression and confinement to a wheelchair at 17. He had a clinically normal younger brother (IV-2, age 11) and five affected cousins, related through their father (II-1). The parents (I-1 and I-2) were clinically normal and nonconsanguineous.

His youngest affected cousin, a 26-year-old man (III-10, figure)

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had severe proximal weakness mainly in upper limbs with onset at age 24. When last seen, at age 31, he was unable to raise his arms and walked with difficulty. EMG performed in patient III-10 revealed a myopathic pattern. Four of his brothers (III-4, III-5, III-7, and III-9) had elevated CK levels. Three of them (III-4, III-7, and III-9) had lower limb weakness. Their mother (II-2) had no clinical symptoms. For details of the patient's clinical features and CK levels, see table E-1 on the *Neurology* Web site at www.neurology.org.

After informed consent, DNA was isolated from blood by standard methods. Four hundred microsatellite markers spaced at 10-cM intervals, from ABI PRISM linkage-mapping set version 2.5, were analyzed in an ABI 3700 DNA capillary sequencer using GENESCAN and GENOTYPER software (Applied Biosystems). Markers located in chromosome 15 (CAPN3 gene location) and on the X chromosome (XK gene) were typed in all family members.

Three exons and splicing sites of the XK gene were amplified (Patients III-1, III-7, and III-10) as previously described.⁴ The 17 exons and splicing sites of the calpain-3 gene were amplified (for Patients III-7, III-10, IV-1, and IV-2) according to Richard et al.⁶ Amplicons were sequenced in the MegaBACE Sequencer and analyzed through Bioedit software.

Muscle biopsy specimens were analyzed with standard stains, and protein expression of dystrophin, sarcoglycans α , β , γ , and δ , calpain-3, dysferlin, and telethonin were done using immunofluorescence (IF) and Western blot (WB), as previously described.⁷

Results. *Muscle biopsy.* Proband IV-1 displayed a dystrophic histologic pattern including variation in fiber size, internal nuclei, and discrete perimysial proliferation of connective tissue. Muscle protein analysis showed a normal pattern for dysferlin, the four sarcoglycans, and telethonin but revealed a decrease in the amount of the 94-kd calpain-3 band on WB (data not shown).

The proband's cousins (III-5 and III-7) displayed a variable myopathic histologic pattern. The oldest patient (III-5) showed more severe alterations, great variability in fiber size, internal nuclei, degeneration, and endomysial proliferation of connective tissue. Patient III-7 showed milder alterations, with scattered ghost isolated fibers. In both of them, ATPases in three different pHs showed a preserved proportion of fiber types, but with some groups of type I and II fibers (see figure E-1 on the *Neurology* Web site at www.neurology.org). Mild neurogenic alterations were also present in III-5 and III-7. Muscle protein analysis showed a normal pattern for all the LGMD-analyzed proteins (calpain-3, dysferlin, the four sarcoglycans, and telethonin) as well as for dystrophin.

DNA and linkage analysis. Screening of the CAPN3 gene confirmed that the Proband IV-1 is a compound heterozygote for two allelic mutations: one null mutation (the Basque common mutation 2362-2363AG>TCATCT) inher-

Figure. Filled upper left corner denotes CAPN3 254ΔK mutation, filled upper right corner denotes CAPN3 Basque mutation, filled lower left corner denotes clinical disease, and filled lower right corner denotes XK gene mutation.

ited from his father and an in-frame 254ΔK deletion inherited from his mother; both disease-causing mutations.⁸

Subsequently, we found that all five affected brothers (III-4, III-5, III-7, III-9, and III-10) carried the same 254ΔK deletion in one allele of the CAPN3 gene (inherited from their father), but no mutation was found in the maternal allele. Linkage analysis excluded the possibility that the second mutation lay in the CAPN3 gene and suggested a candidate gene at Xp21 (maximum lod score of 2.10 at $\theta = 0$ for marker DXS8018).

The XK gene was considered a good candidate since patients with mutations in this gene have elevated CK levels and may present with muscle weakness. Indeed, the sequencing of this gene revealed an insertion of nucleotide C at codon 151 of this gene, leading to a premature stop codon at 198, in the five affected brothers, but not in the proband IV-1.

Clinical reevaluation. We reassessed these patients 5 years after the first clinical visit. Besides muscle weakness, already observed in the first neurologic examination, Patient III-10 displayed involuntary choreic movements. In addition, we collected a new blood sample from him and his affected brother (III-4) to look for the presence of acanthocytes, which was confirmed in a low percentage of cells in both patients (data not shown).

Discussion. McLeod syndrome is a rare disorder with approximately 150 patients reported worldwide. On the other hand, LGMD2A is the most prevalent form of LGMD in many populations, accounting for more than 30% of cases. The mutation found in the XK gene in the present family was previously reported in a 55-year-old Japanese man with hyperCKmia, mild proximal muscle weakness, chorea, and hyporeflexia.⁹

Families with affected men displaying X-linked inheritance and a normal dystrophin pattern have been previously reported.¹⁰ Despite no identified abnormality in the dystrophin gene or muscle protein,

these patients were diagnosed as probable Xp21 Becker muscular dystrophy (BMD) because the dystrophin and the XK genes lie in the same Xp21 interval. McLeod syndrome should be included in the differential diagnosis of men diagnosed as having BMD or LGMD with elevated serum CK, but no identified mutation or muscle protein abnormalities. Detailed neurologic examination (seeking areflexia in particular), careful blood smear analysis for acanthocytes, and determination of Kx and Kell antigens should be performed to exclude McLeod syndrome.

Several independent studies reported that in approximately 10% of the patients with LGMD, only one mutation was identified in the CAPN3 gene or in other associated AR LGMD.^{3,8} It has often been suggested that the second mutation may lie in the non-coding region of the other allele. We are not aware of other reported patients carrying one mutation in the CAPN3 gene in whom the disease was caused by another unrelated mutation.

Molecular analysis in the present family showed that two affected relatives with a similar phenotype (LGMD) have different genetic disorders, calpainopathy and McLeod syndrome, while brothers carrying the same mutation in the XK gene and heterozygous for CAPN3 presented with widely variable clinical courses. It is possible that the combination of an XK mutation and one CAPN3 mutation causes a disease with an LGMD phenotype, although testing this hypothesis was not possible in this family because all affected men carried both mutations.

The present study suggests that in AR disorders in which the second mutated allele is not identified, it may be important to search for mutations in other neuromuscular disease genes.

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Case report

Mutation analysis in the *FKRP* gene provides an explanation for a rare cause of intrafamilial clinical variability in LGMD2I

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Abstract

We report a limb-girdle muscular dystrophy 2I family with three affected sisters and a highly variable clinical course. *FKRP* gene sequencing showed that all three sisters carried a nonsense paternal mutation (*W225X*). The two oldest sisters with a severe phenotype carried two maternal mutations *V79M* and *P89A*. However, the youngest sister with a milder course carried the paternal and only the *V79M* maternal mutation, due to an intragenic recombination.

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1. Introduction

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of progressive muscle disorders with a primary or predominant involvement of the shoulder-girdle or pelvic muscles. There are at least 17 different genetically defined subtypes of LGMD, most autosomal recessive (AR), characterized by normal intelligence and great clinical variability [1].

The *fukutin-related protein (FKRP)* gene is associated to LGMD2I and it is composed of four exons containing a 1488-bp open reading frame that encodes a 495-amino-acid protein [2]. The *FKRP* is a type II transmembrane protein Golgi-resident thought to be a glycosyltransferase or phosphoryl-ligand transferase [3].

Mutations in this gene cause also congenital muscular dystrophy type 1C (MDC1C), characterized by severe weakness presenting at birth or in the first few weeks of life [2]. LGMD2I has a variable course, ranging from

severe Duchenne-like forms with early onset and rapid progression, to mild forms with late onset and mild phenotype or no visible weakness [2,4]. In both disorders, weakness and wasting of shoulder-girdle muscles, primary restrictive respiratory and cardiac involvement has been reported [2]. Understanding the spectrum of severity associated with *FKRP* mutations remains a great challenge.

Here, we report a family with three affected sisters with a variable course, who were followed in our center for more than 20 years. The two oldest ones had a typical Duchenne-like course while the youngest shows a milder phenotype. Screening of mutations in the *FKRP* gene as well as linkage analysis revealed that the two more affected sisters carried an additional pathogenic mutation, which was not present in the third one.

2. Subjects and methods

2.1. Subjects

The three sisters were first seen when they were 2, 4 and 6 years old. The parents were not consanguineous

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and there were no other cases in the family (Fig. 1A). When first examined, the youngest sister was asymptomatic, but the two older sisters had hypertrophic calves and performed Gower's maneuver to raise up from the floor. By ages 11 and 12 they were confined to a wheelchair and died of cardiopulmonary failure at ages 14 and 15, respectively. The youngest sister, who is currently 27 years old, has a milder course and was able to walk short distances with support until the age of 24. All three had very high serum CK levels (on average 50-fold above normal) assessed on different occasions.

2.2. Methods

DNA was extracted from whole blood using standard procedures following informed consent. Mutation analysis was performed in the three sisters and both parents, as previously reported, amplifying the entire *FKRP* coding region of genomic DNA and subsequent sequencing [4]. Linkage analysis was performed using microsatellite

markers D19S420, D19S571, D19S201, and D19S902 (Fig. 1B).

3. Results

Sequencing analysis, performed first in the youngest sister (the only one who is still alive), revealed that she is a compound heterozygote for one stop codon (*W225X*), inherited from the father, and one missense change (*V79M*), from the mother as previously reported [4]. Unexpectedly a third missense mutation (*P89A*) was identified in the two oldest sisters (Fig. 2). This additional mutation, which is 30 bp downstream of *V79M*, was also present in the mother. The *P89A* is a novel mutation which was not found in 300 normal control chromosomes.

In an attempt to explain the absence of the *P89A* mutation in the third sister we performed haplotype analysis using four flanking markers. It revealed that the three sisters inherited the same paternal allele with a null mutation and the same maternal allele. However,

Fig. 1. Pedigree and haplotype analysis of the family studied. (A) Pedigree. (B) Localization and order of the markers at the chromosome 19. The three sisters have a common haplotype. However, the maternal allele in the youngest sister shows a recombination event between the markers *D19S902* and *D19S201* where the *FKRP* is located.

Fig. 2. Sequencing chromatograms showing the two mutations identified at the maternal allele: at the left side the 235G>A and at the right side the 265C>G. (A) Youngest sister (II-3); (B) sister II-2; (C) sister II-1; (D) mother I-2; (E) father I-1.

the youngest sister received a maternal allele with only one missense mutation due to a recombination event that occurred between the two maternal missense mutations. The markers *D19S571* and *D19S902* showed that the telomeric region of the *FKRP* gene is not shared among the three sisters (Fig. 1B).

4. Discussion

LGMD2I, one of the most prevalent form of AR-LGMD in several European countries, has been associated with a broad clinical variability ranging from severe MDC to milder or even asymptomatic cases [5]. Patients with two missense mutations usually show a milder progression course while compound heterozygotes who carry a null mutation in one allele and a missense in the other one usually have a more severe DMD-like course [6]. We found another Brazilian family with two affected sisters who are compound heterozygotes for the common mutation (*L276I*) and another mutation (*P89L*), in the same codon as the *P89A* mutation. The youngest has an apparently less severe phenotype than the oldest but none have a typical Duchenne-like course. No additional mutation in the *FKRP* gene was found but the *L276I* mutation has been reported to cause clinical variability [6]. Matsumoto et al. [7] reported a Japanese

MDC1C patient, who also carries a mutation in codon 89 (*P89R*) in trans with a 2-bp deletion, which causes a premature stop codon, which has been associated to a severe phenotype [3].

The occurrence of two pathogenic mutations in the same allele has been reported previously for other conditions [8], but apparently not for LGMD2I. It might provide an explanation for the more severe course in the two older sisters, possibly due to the presence of a less functional maternal allele than in the youngest one. Although mutations in the *FKRP* gene have been associated with a variable phenotype it is noteworthy that the two sisters who carried two mutations in cis had a very similar severe course. In addition, the observation of an intragenic recombination which was identified in the present family due to the presence of two mutations, which are only 30 bp apart, was unexpected.

Understanding intrafamilial clinical variability in Mendelian disorders has been a great challenge. Different mechanisms such as genetic modifiers [9], different genetic background [10], or epigenetic factors have been proposed. For LGMD2I the pathogenesis appears to be linked to a downregulation of α -dystroglycan and clinical severity with depletion of α -dystroglycan and secondary reduction in laminin- α_2 [3]. The occurrence of an intragenic recombination may be an additional

mechanism to explain such variability in a small subgroup. Although the existence of two pathogenic mutations in the same allele is rare, clinical variability caused by the association or not of a pathogenic mutation and a relatively common polymorphism have been reported for other conditions [9]. These results emphasize the importance of sequencing the entire gene even after the identification of a pathogenic mutation in sibs with discordant phenotypes.

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Mutations in the *KIAA0196* Gene at the *SPG8* Locus Cause Hereditary Spastic Paraplegia

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Hereditary spastic paraplegia (HSP) is a progressive upper-motor neurodegenerative disease. The eighth HSP locus, *SPG8*, is on chromosome 8p24.13. The three families previously linked to the *SPG8* locus present with relatively severe, pure spastic paraplegia. We have identified three mutations in the *KIAA0196* gene in six families that map to the *SPG8* locus. One mutation, V626F, segregated in three large North American families with European ancestry and in one British family. An L619F mutation was found in a Brazilian family. The third mutation, N471D, was identified in a smaller family of European origin and lies in a spectrin domain. None of these mutations were identified in 500 control individuals. Both the L619 and V626 residues are strictly conserved across species and likely have a notable effect on the structure of the protein product strumpellin. Rescue studies with human mRNA injected in zebrafish treated with morpholino oligonucleotides to knock down the endogenous protein showed that mutations at these two residues impaired the normal function of the *KIAA0196* gene. However, the function of the 1,159-aa strumpellin protein is relatively unknown. The identification and characterization of the *KIAA0196* gene will enable further insight into the pathogenesis of HSP.

Hereditary spastic paraplegia (HSP) has a worldwide prevalence of 1–18 in 100,000^{1–3} and is characterized by central-motor-system deficits leading to lower-limb spastic paraparesis.^{4–6} This is due to a “dying back” phenomenon whereby upper motor neurons degenerate progressively, commencing with the longest axons.^{7,8} HSP can be classified into pure and complicated forms.⁵ In pure HSP, lower-limb spasticity is the only major symptom. Alternatively, in complicated HSP, this spasticity can be accompanied by other neurological or nonneurological symptoms, such as ataxia, dementia, mental retardation, deafness, epilepsy, ichthyosis, retinopathy, ocular neuropathy, and extrapyramidal disturbances.^{5,9} There is clinical heterogeneity within families, where age at onset and severity can differ markedly; between families that map to the same locus; and certainly between families that map to separate loci. This heterogeneity complicates genotype-phenotype correlations for HSP.

HSP is also extremely genetically heterogeneous. From >30 loci mapped (*SPG1–33*), 11 genes have been identified. This disease can be transmitted in a dominant (13 loci), a recessive (15 loci), or an X-linked manner (4 loci).^{9–11} By far, the most common locus for the disease is *SPG4* (MIM 604277), with mutations in the microtubule-severing protein spastin accounting for ~40% of dominant HSP cases.^{12,13}

Families that map to *SPG8* are considered to have one

of the more aggressive subtypes of HSP, with disease onset occurring for patients as early as their 20s or 30s. It was first identified in a white family as a 6.2-cM region between markers *D8S1804* and *D8S1774*.¹⁴ The family had 15 patients affected with spasticity, hyperreflexia, extensor plantar reflexes, lower-limb weakness, decreased vibration sensation, and limited muscle wasting. The candidate region was further reduced to 3.4 cM because of a lower recombinant in a second family, which narrows the interval between markers *D8S1804* and *D8S1179*.¹⁵ This family, as well as a third Brazilian family linked to *SPG8*, also presented with pure adult-onset HSP.¹⁶ For two of the families, a muscle biopsy was performed^{14,16}; however, no gross histological or histochemical abnormalities were observed. Ragged red fibers have been observed in muscle biopsies of patients with HSP who have paraplegin mutations.¹⁷

In the present study, we identified four additional families that are linked to the *SPG8* locus. Genes were screened in an expanded candidate *SPG8* locus defined by these four families, along with the British and Brazilian families described above.^{15,16} This led to the identification of three point mutations in the *KIAA0196* gene encoding the strumpellin protein product.

Material and Methods

Subjects

Protocols were approved by the Ethics Committee of the Centre Hospitalier de l'Université de Montréal. Patients gave informed

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Table 1. Primers and Amplification Conditions for *KIAA0196*

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

consent, after which patient information and blood was collected. DNA was extracted from peripheral blood through use of standard protocols.

Genotyping and Locus Exclusion

PCR-amplified fragments incorporating α -³⁵S-2-deoxyadenosine 5-triphosphate were resolved on 6% denaturing polyacrylamide gels. Alleles were run alongside an M13mp18 sequence ladder and were scored on the basis of allele sizes and frequencies from the Fondation Jean Dausset-CEPH database. LOD-score calculations and multipoint analysis were performed using the MLINK program of the LINKMAP software package.¹⁸

Mutation Screening

The 28 exons of *KIAA0196* were screened by automated sequencing, including at least 50 bp of each intronic region. Primers were designed using the PrimerSelect program (Lasergene) and were synthesized by Invitrogen Canada. Primer sequences and amplification conditions for each exon are listed in table 1.

Variants were first tested in 12 control individuals by sequencing, followed by allele-specific oligomerization (ASO).^{19,20} In brief, 4 μ l of PCR product was hybridized onto Hybond-N+ Nylon membranes (Amersham Biosciences) by use of a dot-blot apparatus. P³²-labeled probes specific to the mutation or normal sequence were hybridized and then visualized on autoradiographic film after overnight exposure. ASO primers for exon 11 are 5'-ACTAGAAAACCTTCAAGCT-3' (normal) and 5'-ACTAGAAGACCTTCAAGCT-3' (mutated). For exon 14, ASO primers of 5'-GGAGAGTTGGTATC-3' (normal) and 5'-GGAGAGTTTCGTATC-3' (mutated) were used. Exon 15 ASO primers were 5'-CACTGAAGGTTTG-3' (normal) and 5'-CACTGAAGTTTTTG-3' (mutated).

Protein-Sequence Alignment

Cluster analysis was performed using the Probcons (v. 1.09) program. Proteins from aligned species included *Homo sapiens* (Q12768), *Canis familiaris* (GenBank accession number XP_532327), *Pan troglodytes* (GenBank accession number XP_519952), *Drosophila melanogaster* (GenBank accession number CG12272), *Caenorhabditis elegans* (GenBank accession number CE13235), *Xenopus tropicalis* (GenBank accession number MGC89323), *Rattus norvegicus* (GenBank accession number XP_343250), *Danio rerio* (GenBank accession number BC045490), *Gallus gallus* (GenBank accession number XP_418441), *Dictyostelium discoideum* (GenBank accession number EAL63144), and *Mus musculus* (GenBank accession number NP_705776.2).

Homology Modeling

The size of the strumpellin protein (1,159 aa) made it prohibitive to obtain a template for the entire protein. Instead, 200 aa (amino acids 501–725) around the two mutations were selected and inputted in the Phyre program version 2.0 (Phyre Protein Fold Recognition Server). The template with the highest score was selected—namely, 1dn1b from the Neuronal-Sec1 syntaxin 1a complex. The SwissProt database viewer (v. 3.7)²¹ was used to visualize

the model, with concentration on the α -helix in which the two mutations lie and on a second α -helix in nearby 3D space. Peptides incorporating one or the other identified point mutation were visualized in the same manner.

Expression Studies

Northern-blot and RT-PCR analyses.—The *KIAA0196* cDNA pBluescript clone was kindly provided by the Kazusa DNA Research Institute. A 1-kb probe specific to the C-terminal region of strumpellin was generated by digesting the *KIAA0196* pBluescript vector with *Xho*I and *Not*I. Thirty micrograms of total RNA per sample was loaded. RNA was extracted from various regions of the brain of a control individual. An RT-PCR was performed using Moloney murine leukemia virus–reverse transcriptase (Invitrogen). Primers in exons 10 (forward) and 15 (reverse) of *KIAA0196* were used. Glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified as a control.

Constructs

Each mutation was introduced into the *KIAA0196* pBluescript clone by site-directed mutagenesis through use of the primers 5'-CTGGAGAGTTCGTATCCTATGTG-3' for the exon 14 variant and 5'-CCTATGTGAGAAAATTTTGCAGATC-3' for the exon 15 variant, along with primers of their complementary sequence. Wild-type and mutant *KIAA0196* cDNAs were cloned, upstream of Myc and His tags, into a pCS2 vector and were transcribed in vitro by use of the SP6 mMESAGE mMachine kit (Ambion) for zebrafish studies. The protein expression from each of these constructs was validated after their transient expression in cell (HeLa) culture and subsequent western-blot analysis with an anti-Myc antibody. A band at the appropriate height (~134 kDa for strumpellin) was observed.

Zebrafish Knockdown Studies

Morpholino injections.—Wild-type zebrafish were raised and mated as described elsewhere.²² Antisense morpholinos (AMOs) were purchased from Genetools. The morpholino sequences were designed against the zebrafish strumpellin ortholog BC045490. The oligonucleotide CTCTGCCAGAAAATCAC(CAT)GATG (*KIAA MO*) binds to the ATG of the *KIAA0196* gene, which prevents its translation, and CTCTcCCAcAAAATgAg(CAT)cATG (*CTL MO*) is a 5-bp mismatch control. AMO injections were performed as described elsewhere, at a concentration of 0.8 mM.²³ The rescue injections were performed as mentioned above, with morpholino and mRNA concentrations of 0.8 mM and 50 ng/ μ l, respectively.

Immunohistochemistry.—Standard protocols were used for immunohistochemistry.²² In brief, 3-d-old embryos were fixed in 4% paraformaldehyde, were washed, and were blocked at room temperature. Primary antibody (anti-acetylated tubulin; 1:50 [Sigma]) was added overnight. After extensive washing, the embryos were incubated with the fluorescently labeled secondary antibody Alexa 568 (Molecular Probes). Imaging was performed on an Ultra-View LCI confocal microscope (Perkin Elmer) with use of Methamorph Imaging software (Universal Imaging). The statistical significance between the different conditions was calculated using a χ^2 test.

Figure 1. Pedigrees for families with *KIAA0196* mutations. *A*, Family FSP24. *B*, Family FSP29. *C*, Family FSP34. *D*, Family FSP91. Blackened boxes represent affected individuals, and a diagonal line through the symbol means the individual is deceased. A vertical blackened bar indicates an individual with an unconfirmed phenotype. Sex of each individual has been masked to preserve confidentiality. Individuals marked “P” represent proximal recombinants; “D” represents the distal recombinant. An asterisk (*) indicates that DNA and clinical information have been collected for the particular individual. The age at onset of affected individuals is listed below each symbol, although this information is not available for each patient. All studied affected patients are heterozygous for a c.1956C→T mutation (pedigrees A, B, and C) or a c.A1491G mutation (pedigree D) in *KIAA0196*.

Results

Clinical Information and Family Details

Family FSP24 with the *SPG8* mutation is from British Columbia. It is composed of 13 members affected with a spastic gait and lower-limb stiffness (fig. 1A); genetic information is available for 10 of them. Symptoms were first observed in individuals between ages 35 and 53 years. Intrafamilial phenotypic heterogeneity exists, as shown by the symptoms presented and the range of disease severity in patients. Deep-tendon reflexes were brisk or increased, and decreased vibration sensation was also noted in three patients. Occasional bladder-control problems were also observed. Walking aids were required for some individuals, whereas one is confined to a wheelchair. Together, these features are consistent with a pure, uncomplicated HSP similar to that described for other families linked to

the *SPG8* locus. Family FSP29 is of European descent and resides in the United States. There are 31 affected individuals in the family, and genetic information is available for 10 of them (fig. 1B). Age at onset was quite variable, with symptom onset ranging in patients from their 20s to their 60s. The family was negative for mutations in the spastin gene.

Linkage Analysis

Two large families that map to the *SPG8* locus were identified. In family FSP24, seven markers spanning the candidate region from markers *D8S586* to *D8S1128* were genotyped in the 18 individuals studied (fig. 1A). A disease haplotype segregated with the disease in all 10 affected individuals (table 2). A recombination event occurred in one individual (fig. 1A) between markers *D8S586* and

Table 2. Haplotype Comparison between SPG8-Linked Families

Marker	Position (Mb)	Alleles Number for Family		
		FSP24	FSP29	FSP34
<i>D8S586</i>	121.2	1	11	11
<i>D8S1804</i>	124.8	5	3	3
<i>D8S1832</i>	125.4	2	2	Not typed
<i>D8S1179</i>	125.9	3	9	9
<i>D8S1774</i>	127.5	3	5	4
<i>D8S1128</i>	128.5	7	5	1

NOTE.—Flanking markers in the candidate region are *D8S1832* and *D8S1774* for family FSP29. The *KIAA0196* L619F mutation was at position 126.1 Mb for all three families. The allele for rs2293890 (126.4 Mb) was G for family FSP24 and was C for both families FSP29 and FSP34.

D8S1804, which defined the proximal border of the locus in this family. A lower recombinant was neither identified nor searched for, since the haplotype extended beyond the limits of the *SPG8* locus. The maximum LOD score for this family was 3.43 at $\theta = 0$, by use of CEPH allele frequencies for the marker *D8S1804*, along with a maximum multipoint of 4.20 at marker *D8S1799*.

The same seven markers tested in family FSP24 were genotyped for family FSP29. A disease haplotype was established for all 10 studied affected individuals; it included many informative recombination events. The proximal recombinant occurred between markers *D8S1799* and *D8S1832* in three affected individuals (fig. 1B), and the distal recombinant was between markers *D8S1774* and *D8S1128* for another affected individual (fig. 1B). This yielded a candidate interval of 3.15 Mb. The maximum LOD score for this family was 5.62 ($\theta = 0$) for marker *D8S1179* when CEPH allele frequencies were used. Multipoint analysis was also conducted for this family in this region, which yielded a maximum LOD score of 6.73, 0.5 cM centromeric to the *D8S1128* marker.

Gene Screening

The previously published *SPG8* locus spanned 3.4 cM (1.04 Mb) between markers *D8S1804* and *D8S1179* on chromosome 8q23-8q24. We screened nine known genes surrounding this candidate region, as annotated in the University of California–Santa Cruz Genome Browser (UCSC) May 2004 update, along with many clustered ESTs and mRNAs that aligned to the locus, without detecting a mutation. Therefore, we opted to redefine the candidate region on the basis of the critical interval determined by an upper recombinant in our FSP29 family at marker *D8S1832*, and a lower recombinant at *D8S1774* was based on published data (fig. 2A).¹⁴ This increased the size of the region to 5.43 cM (3.15 Mb), a region that contains three additional known genes (fig. 2B). These additional genes were screened, and three mutations were identified in the *KIAA0196* gene (fig. 2C).

Mutation Analysis

A valine→phenylalanine mutation was identified in amino acid 626 for families FSP24 and FSP29 (p.V626F) (fig. 3A). All studied affected individuals from each family were screened and were positive for this mutation. The same mutation was also found to segregate in a British family.¹⁵ This G→T nucleotide change is at position 1956 of the mRNA (GenBank accession number NM_014846.2). A total of 500 ethnically matched control individuals (400 from North America and 100 from CEPH) were negative for this mutation, on screening by a combination of ASO and sequencing. No unaffected members or spouse control individuals in any family were positive for the mutations.

A second mutation was identified in the Brazilian family¹⁶ in exon 14, a G→C transition at position 1937 of the mRNA (fig. 3B). This leucine→phenylalanine change (p.L619F) is only 7 aa away from the V626F mutation. It was not found, with use of ASO, in 500 controls.

The *KIAA0196* gene was screened in probands from 24 additional dominant HSP-affected families that are negative for mutations in both spastin and atlastin, resulting

Figure 2. Region spanning the *SPG8* locus. **A**, Markers defining the borders of each described family with the *SPG8* mutation and the scaled marker positions on chromosome 8q24.13. **B**, Candidate region used to search for the *SPG8* gene between markers *D8S1804* and *D8S1774*. Genes in the region are shown in their observed orientation. **C**, The 28-exon *KIAA0196* gene, drawn to scale, with the location of three mutations in exons 11, 14, and 15 highlighted.

Figure 3. Mutation analysis of the *KIAA0196* gene. *A–C*, Sequence trace of a patient with HSP above the sequence trace of a control individual. Exon 15 (*A*), 14 (*B*), and 11 (*C*) heterozygous point mutations are indicated. *D*, Multiple-sequence alignment for strumpellin homologues surrounding the two coding changes (boxed). The Probcons (v.1.09) program was used for cluster analysis. *E*, RT-PCR of multiple brain regions performed using a *KIAA0196*-specific probe. *F*, Northern blot of the *KIAA0196* transcript performed using 30 μ g of total RNA and a 1-kb C-terminal probe.

in the identification of two more families with missense mutations in the *KIAA0196* gene. Thus, the frequency of mutations in our *SPG3A*- and *SPG4*-negative autosomal dominant cohort is ~8% (2 of 24). FSP34 has the same p.V626F change in its three affected studied family members. This family is originally from Great Britain and resides in Canada (fig. 1C). Haplotype analysis of this family with markers *D8S1804*, *D8S1179*, *D8S1774*, and *D8S1128* indicated that there is allele sharing between this family and family FSP29, which suggests an ancestral haplotype (table 2). An additional mutation was found in three affected siblings of another North American family of European origin, family FSP91 (fig. 1D). This c.A1491G transition results in an asparagine→aspartate amino acid change (p.N471D) and is not present in the 500 controls tested (fig. 3C).

Mutated amino acids at positions 619 and 626 are strictly conserved across all 11 examined species, from human to the social amoeba, *D. discoideum* (fig. 3D). Indeed, the entire region surrounding these two mutations appears to be functionally relevant for the protein, since 73 consecutive aa (amino acids 576–649) are 100% identical in the human, dog, chicken, mouse, rat, and orangutan. Despite this high level of conservation, this region is an unknown domain, on the basis of searches of the National Center for Biotechnology Information (NCBI)

Conserved Domain Database, NCBI BLAST, and the Sanger Institute's Pfam database. Position 471 is conserved across all species except *D. melanogaster* (with a glutamine residue) and *X. tropicalis* (with a histidine residue).

The exon 15 mutation is in the very first nucleotide of the exon, which leads to the speculation that the splicing of this exon might be compromised in our study families. Splice-site prediction programs, including NetGene2, suggested that the strength of the splice-site acceptor may be reduced by 33% in the mutant form. However, both normal and mutant alleles were observed in cDNA analysis, with use of several pairs of primers, of patient lymphoblasts. The *KIAA0196* gene was expressed ubiquitously, including all regions of the brain that were examined by RT-PCR (fig. 3E). There were no alternative splice isoforms detected in control brain samples or patient whole-blood samples by RT-PCR and northern-blot analysis (fig. 3E and 3F). For the full *KIAA0196* gene, all spliced ESTs and mRNAs from the UCSC browser, May 2004 draft, were analyzed for potential alternative splice products. One alternative first exon often appears; however, of the 356 entries, only 2 (AK223628 and DA202680) contain exons that are skipped. Thus, overall, the gene is not frequently spliced, and the two spliced entries may represent spurious transcripts.

Figure 4. Three-dimensional modeling of strumpellin, with d1dn1b as a template, through use of SwissProt database viewer. Two helices from the 1,159-aa protein are shown, including amino acids 614–634 in one α -helix and amino acids 662–672 from a nearby α -helix in the antiparallel direction. *A*, Residues L619 and V626, in the same orientation in an α -helix opposite a second helix in an antiparallel direction. Only residue side chains that are closest in physical space are shown. *B*, The L619F mutation adds a bulky phenylalanine side group that likely exceeds the space available between the two α -helices. *C*, V626F mutation. The ϵ carbon of the F626 aromatic ring impinges on Q666 and may force apart the two α -helices.

KIAA0196 Profile

The *KIAA0196* gene spans 59.7-kb pairs of genomic DNA, is 28 exons long, and codes for a protein of 1,159 aa (strumpellin). The European Bioinformatics Institute's InterProScan program predicts a spectrin-repeat-containing domain in amino acids 434–518. Thus, the mutation at position 471 may abrogate the binding of the spectrin domain with other spectrin-repeat-containing proteins. In examination of the secondary structure by use of PSIPRED,²⁴ 74% of the protein is considered to be α -helical. The program further predicts an α -helix in the protein from amino acids 606–644, which encompasses the two other mutations that have been identified.

Homology Modeling

Given the high proportion of *KIAA0196* considered to be α -helical, it is not surprising that the optimal homology-modeling candidates are similar in secondary-structure composition. This is true for 1dn1b, a stat-like t-SNARE protein neuronal-Sec1 syntaxin 1a complex. This is the most appropriate model for strumpellin, according to the Phyre program (Phyre Protein Fold Recognition Server). The two mutated residues lie within an α -helix from amino acids

619–628 that is in close 3D proximity to another α -helix from residues 665–670 (fig. 4A). A mutation in either Val-626 or Leu-619 to a phenylalanine residue would appear to have significant structural implications, given the change in bulkiness between the residues. In addition, Tyr-622 points in the same direction from the α -helix residue. To have two amino acids with aromatic rings in such a physical proximity could force apart the alignment of the two α -helices or induce alterations in the α -helix backbone. The N471D mutation was identified well after the two other mutations and so was not tested in homology modeling or in subsequent zebrafish-rescue experiments.

Zebrafish-Rescue Experiments

To validate the functional phenotype of the *SPG8* mutations in vivo, we developed a zebrafish model. Morpholino oligonucleotide knockdown of the KIAA0196 protein ortholog in zebrafish (*KIAA MO*) resulted in an enlarged

Figure 5. Zebrafish knockdown and rescue of *KIAA0196* function. *A*, Gross morphological features of normal wild-type zebrafish, depicted at 3 dpf. *B*, Injection of a 5-bp mismatch morpholino (CTL MO), which results in no obvious disease phenotype. *C* and *D*, KIAA MO-injected fish with a severely curly tail (*C*) or with a slightly curly tail (*D*). Their heart cavities are also enlarged, which is commonly seen in injected fish. *E* and *F*, Fish, injected with both KIAA MO and normal human *KIAA0196* mRNA, with partially developed curly tail (*F*) or no effect at all (*E*), depending on the injected quantity. *G* and *H*, Disease phenotype not alleviated when the KIAA MO is injected with the mutant forms ($\times 14$ [panel *G*] and $\times 15$ [panel *H*]) of the human mRNA. These fish resemble the KIAA MO fish (*C* and *D*).

Table 3. Phenotype Profile from Zebrafish Morpholino Oligonucleotide Knockdown

Condition	Percentage (No.) of Zebrafish				Total
	Normal	Slightly Curly Tail	Severely Curly Tail	Dead	
<i>KIAA0196</i> morpholino	19.1 (34)	28.1 (50)	37.1 (66)	15.7 (28)	178
Control morpholino	56.1 (83)	24.3 (36)	7.4 (11)	12.2 (18)	148
Wild-type rescue	63.2 (127)	19.4 (39)	8.0 (16)	9.5 (9)	201
Mutant × 14 rescue	16.0 (32)	37.0 (74)	36.0 (72)	11.0 (22)	200
Mutant × 15 rescue	13.2 (29)	37.4 (82)	30.1 (66)	19.2 (42)	219

heart cavity, along with a curly-tail phenotype that severely impaired the ability of the fish to swim properly. The overall phenotype ranged in severity and was classified in three major groups: normal, slightly curly, and severely curly. This phenotype was clearly visible after dechorionating by 1 d post fertilization (dpf). At 3 dpf, wild-type zebrafish are ~5 mm long, with a straight tail (fig. 5A). Fish injected with a mismatch-control morpholino (CTL MO) were initially used to titer a KIAA MO-specific nontoxic injection dose (fig. 5B). Injection of the KIAA MO resulted in 66 (37%) of 178 fish with a severely curly tail and 50 (28%) of 178 fish with a slightly curly tail (table 3 and fig. 5C and 5D). The KIAA MO fish had a significantly different distribution of phenotypic groups compared with those with CTL MO injections ($P < .001$). When wild-type human *KIAA0196* mRNA was coinjected with KIAA MO, the curly-tail phenotype was rescued to levels comparable to CTL MO injections ($P = .51$) (fig. 5E and 5F). This suggests that, in zebrafish, human *KIAA0196* mRNA can compensate for the loss of endogenous zebrafish mRNA. Conversely, coinjection of human *KIAA0196* mRNA incorporating either the exon 14 or exon 15 mutation failed to significantly rescue the phenotype (fig. 5G and 5H). Injection of mutant exon 14 or exon 15 mRNA alone (without morpholinos) did not lead to a curly-tail phenotype or influence lethality in zebrafish, which suggests that the two mutations do not exert a dominant negative effect. Approximately 200 embryos were injected per experimental condition (table 3). The difference in distribution between KIAA MO injection alone and KIAA MO coinjection with wild-type mRNA was significant ($P < .001$). Similarly, coinjection of wild-type mRNA versus either exon 14 or exon 15 mutant mRNA was significantly different, with a P value $< .001$. There was no statistical difference between the coinjection of the exon 14 mutant and the exon 15 mutant ($P = .10$). On histochemical analysis of the embryos by use of an antiacetylated tubulin stain for growing axons, we found that the motor neurons in the spinal cord did not develop normally (fig. 6). Motor-neuron axons in fish injected with KIAA MO alone or with the mutant mRNAs were shorter and showed abnormal branching. The structure of interneurons in the spinal cord was also different. The absence of the *KIAA0196* gene or mutations in this gene during early development thus seemed to hamper axonal outgrowth.

Discussion

HSP is one of the most genetically heterogeneous diseases, caused by mutations in at least 31 different genes. This means that $>0.1\%$ of genes in the human genome can be mutated and result in one predominant neurological outcome: the degeneration of upper-motor-neuron axons. Four families in this study share the same p.V626F mutation, which suggests that the altered nucleotide is a mutation hotspot. For one of these families,¹⁵ the *KIAA0196* gene lies just beyond its distal candidate boundary. However, the flanking marker likely represents either a marker mutation or genotyping error in this family, since the p.V626F mutation is present in the supposed recombinant as well as all other affected individuals.

An *SPG8* mutation causes a pure form of HSP with relatively little interfamilial variability in phenotype. Interestingly, two missense mutations were identified in highly conserved amino acids in a predicted α -helix. The helix consists of a heptameric repeat, with hydrophobic residues aligning in inaccessible regions at the center of the helix. The hydrophobic lysine and valine amino acids are 7 aa apart in the protein sequence; thus, it is expected that they would be buried in the helix, close in 3D space (fig. 5A). When replaced by a bulky phenylalanine residue at either position, the stability of the α -helix could very well be disrupted.

The one known domain in strumpellin is a spectrin repeat that consists of three α -helices of a characteristic length wrapped in a left-handed coiled coil.²⁵ These spectrin repeats appear in the spectrin/dystrophin/ α -actinin family. The spectrin proteins have multiple copies (15–20) of this repeat, which can then form multimers in the cell. Spectrin also associates with the cell membrane via spectrin repeats in the ankyrin protein. Likewise, four spectrin repeats are found in α -actinin beside two N-terminal calponin homology domains that anchor the complex to actin.²⁶ This effectively connects the cell membrane with the actin-cytoskeletal network. The stability and structure of this network also provide appropriate routes for intracellular vesicular transport, a mechanism already linked to other mutated HSP genes. Proteins with three or fewer spectrin repeats can be considered to have transient association with the spectrin network. The single repeat in strumpellin is more likely to be involved in docking with

Figure 6. Immunohistochemical analysis of zebrafish with the *KIAA0196* knockdown phenotype. *A*, Motor neurons in the ventral roots of wild-type zebrafish, segmented and oriented at 3 dpf. The spinal cord consists of the cell bodies of motor neurons and interneuron bundles. The picture was taken near the gut of the fish. *B*, Mismatch control has a motor-neuron distribution similar to the wild type. *C*, *E*, and *F*, Zebrafish injected with KIAA MO (*C*) and fish coinjected with mutant mRNA ($\times 14$ [*panel E*] and $\times 15$ [*panel F*]), showing shorter, branching motor neurons that are not oriented. *D*, Wild-type *KIAA0196* mRNA coinjections with KIAA MO, which partially rescue the motor-neuron phenotype. The axons are longer and oriented.

one of the cytoskeletal spectrin repeats, which could help in protein localization or signal transduction. It will be interesting to determine with which protein(s) strumpellin interacts through its spectrin domain and, particularly, how the mutation identified at the core of this domain influences this potential interaction.

Proteins with a spectrin repeat have been identified in other neurological disorders—most notably, dystrophin, mutated in myotonic dystrophy (MIM 300377).²⁷ The repeat also has been found in a form of cerebellar ataxia (MIM 117210).²⁸ β -III spectrin itself is found to be mutated in spinocerebellar ataxia 5.²⁹ Whereas none of the genes mutated in HSP have a spectrin domain, L1CAM (*SPG1*) has an indirect association.^{9,30} L1CAM is a single-pass transmembrane protein with a glycosylated extracellular component that facilitates the outgrowth and migration of neurons in the corticospinal tract. The intracellular C-terminus, however, binds to the spectrin-repeat-containing protein ankyrin that links the cell membrane to intracellular spectrin. Thus, strumpellin, with its spectrin domain, may also be involved in this process.

The only detail known so far about the human *KIAA0196* gene is that it has previously been implicated in prostate cancer.³¹ An increase in gene-copy number was assayed by real-time quantitative PCR and FISH, which determined >10-fold overexpression of the gene in PC-3 prostate cancer lines and in approximately one-third of advanced prostate cancers examined.³¹ How this relates to a spastic paraplegia phenotype is not clear.

Analysis of other species has provided some insight into a potential function for *KIAA0196*. A 118-kDa homologue of the strumpellin protein was identified as part of a TATA-

binding protein-related factor 2 (TRF2) complex in a *Drosophila* nuclear extract.³² Eighteen proteins were pulled down, along with TRF2, in this complex, including NURF and SWI, with functions for chromatin remodeling and transcription activation. TRF2 is selective for promoters lacking TATA or CAAT boxes. One protein of the complex is DREF, which binds to DRE elements common in controlling genes involved in cell-cycle regulation and cell proliferation.^{33,34}

With little known about the function of *KIAA0196*, we decided to test the functionality of the missense changes with a zebrafish knockdown model. Interestingly, the KIAA MO-injected fish showed a severe tail phenotype characterized by abnormal motor-neuron outgrowth in the spinal cord. The knocked-down zebrafish resembles other mutants affecting midline development.³⁵ Injection of wild-type human *KIAA0196* mRNA concurrently with zebrafish KIAA MO knockdown rescued the phenotype to values not significantly different from a control morpholino injection. However, coinjecting human *KIAA0196* mRNA containing either the exon 14 or the exon 15 mutation yielded a phenotype comparable to injection of KIAA MO alone. These experiments demonstrate the importance of the *KIAA0196* gene in early development of zebrafish and suggest that the two missense mutations impair the normal function of strumpellin. Further characterization of the *KIAA0196* knockdown phenotype is necessary to better understand the role of this gene in early fish development—more precisely, in motor-neuron outgrowth. This phenotype is remarkably similar to what was recently observed in *SPG4* morpholino knockdown experiments in the zebrafish³⁶: motor-neuron-axon out-

growth is impaired, and embryos also have a curly tail. This is another instance in which a dominant adult-onset disease in humans displays an embryonic phenotype in the zebrafish. It can be hypothesized that a complete knockdown of either *SPG4* or *SPG8* yields an embryonic phenotype, whereas one copy of the gene is enough in humans to remain at a subphenotypic state until adult stages. Importantly, in both instances, it is the motor neurons that are impaired.

The identification of *KIAA0196* as the gene mutated in *SPG8* adds another component to the various genes already identified for the disease. Two missense mutations in a conserved part of the protein have been identified, including one mutation common to four families. Additional work will aid in clarifying the function of the protein and how it relates to other proteins implicated in HSP and the overall disease pathogenesis. As more of the genes involved in HSP emerge, the responsible pathways and mechanisms of toxicity will be better understood. This will also help in elucidating the pathophysiology of a related disease—amyotrophic lateral sclerosis—in which the reduced life span of patients complicates the cloning of genes by linkage analysis.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Conserved Domain Database, <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>

Fondation Jean Dausset–CEPH, <http://www.cephb.fr/>

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *C. familiaris* [accession number XP_532327], *P. troglodytes* [accession number XP_519952], *D. melanogaster* [accession number CG12272], *C. elegans* [accession number CE13235], *X. tropicalis* [accession number MGC89323], *R. norvegicus* [accession number XP_343250], *D. rerio* [accession number BC045490], *G. gallus* [accession number XP_418441], *D. discoideum* [accession number EAL63144], *M. musculus* [accession number NP_705776.2], and *H. sapiens* mRNA [accession number NM_014846.2])

InterPro, <http://www.ebi.ac.uk/interpro/> (for InterProScan from the European Bioinformatics Institute)

NCBI BLAST, <http://www.ncbi.nlm.nih.gov/blast/>

NetGene2, <http://www.cbs.dtu.dk/services/NetGene2/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *SPG4*, myotonic dystrophy, and cerebellar ataxia)

Pfam, <http://www.sanger.ac.uk/Software/Pfam/>

Phyre Protein Fold Recognition Server, <http://www.sbg.bio.ic.ac.uk/~phyre/>

Probcons, <http://probcons.stanford.edu/>

UCSC Genome Browser, <http://www.genome.ucsc.edu/>

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MUTATION IN BRIEF

Screening of ARHSP-TCC Patients Expands the Spectrum of *SPG11* Mutations and Includes a Large Scale Gene Deletion

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Autosomal recessive spastic paraplegia with thinning of corpus callosum (ARHSP-TCC) is a complex form of HSP initially described in Japan but subsequently reported to have a worldwide distribution with a particular high frequency in multiple families from the Mediterranean basin. We recently showed that ARHSP-TCC is commonly associated with mutations in *SPG11/KIAA1840* on chromosome 15q. We have now screened a collection of new patients mainly originating from Italy and Brazil, in order to further ascertain the spectrum of mutations in *SPG11*, enlarge the ethnic origin of *SPG11* patients, determine the relative frequency at the level of single Countries (i.e., Italy), and establish whether there is one or more common mutation. In 25 index cases we identified 32 mutations; 22 are novel, including 9 nonsense, 3 small deletions, 4 insertions, 1 in/del, 1 small duplication, 1 missense, 2 splice-site, and for the first time a large genomic rearrangement. This brings the total number of *SPG11* mutated patients in the SPATAX collection to 111 cases in 44 families and in 17 isolated cases, from 16 Countries, all assessed using homogeneous clinical criteria. While expanding the spectrum of mutations in *SPG11*, this larger series also corroborated the notion that even within apparently homogeneous population a molecular diagnosis cannot be achieved without full gene sequencing. © 2008 Wiley-Liss, Inc.

KEY WORDS: ARHSP; TCC; *SPG11*; mutation screening.

INTRODUCTION

Hereditary spastic paraplegias (HSPs), also known as Strümpell-Lorrain disease, are a heterogeneous group of inherited disorders in which the main clinical feature is progressive spasticity in the lower limbs due to pyramidal tract dysfunction (Harding 1983). Brisk tendon reflexes with bilateral Babinski sign, muscle weakness and urinary urgency are also present at clinical examination and are likely the result of a 'dying back' degeneration of the corticospinal tracts. Scholastically, HSPs are classified as pure or complex depending on whether spasticity in the lower limbs occurs in isolation or it is associated with other neurological and extraneurological signs (Harding 1983; Tallaksen et al. 2001; Depienne et al. 2007). However, an ever widening clinical spectrum, the recognition of subtle differences between apparently stereotypical neurological disorders, and the large underlying genetic heterogeneity call for a more locus-driven classification in HSP (Fink 2006; Stevanin et al. 2008b).

Autosomal recessive spastic paraplegia with thinning of the corpus callosum (ARHSP-TCC; MIM# 604360) is a frequent and complex form of HSP initially described in Japan (Nakamura et al. 1995). Since the initial reports linking Mediterranean and Asian ARHSP-TCC families to the *SPG11* locus on chromosome 15q (Martinez-Murillo et al. 1999; Shibasaki et al. 2000), several groups, including our teams, proved that this phenotype has a worldwide distribution (Casali et al. 2004; Lossos et al. 2006; Olmez et al. 2006; Winner et al. 2006; França et al. 2007; Stevanin et al. 2007, 2008a) and that it is particularly prevalent in the Mediterranean basin (Casali et al. 2004; Stevanin et al. 2006). More recently, we showed that ARHSP-TCC at this locus is associated with mutations in *SPG11/KIAA1840* (MIM# 610844), a gene with an open reading frame of 7,787 nucleotides that comprises 40 exons and which spans a genomic region of approximately 100 Kb (Stevanin et al. 2007). *SPG11* is predicted to encode a 2,443 amino-acid-long protein, which has no homology with known proteins although a high degree of conservation is present across vertebrates. Its involvement in ARHSP-TCC was subsequently confirmed by other groups (Del Bo et al. 2007; Hehr et al. 2007; Paisan-Ruiz et al. 2008; Lee et al. 2008; Zhang et al. 2008).

We have recently reported the clinical and molecular genetic features of the largest group of *SPG11* patients, mainly coming from Western Europe and North-Africa, collected within SPATAX, an European and Mediterranean network for spinocerebellar degenerations (Stevanin et al. 2008a). We have now screened a collection of new patients referred to a single centre, and mainly originating from Italy and Brazil, in order to further ascertain the spectrum of mutations in *SPG11*, enlarge the ethnic origin of *SPG11* patients, determine the relative frequency at the level of single Countries (i.e., Italy), and establish whether there is one or more common mutation. We identified 22 new mutations and analyzed the associated phenotypes.

METHODS

Patients

Thirty-one kindred (57 patients) with an autosomal recessive inheritance and 20 isolated cases with no apparent family history of the disease were selected in our shared databases and were classified in four diagnostic categories: i) spastic paraplegia with mental retardation (MR) or cognitive impairment and thinning of the corpus callosum (TCC) demonstrated by brain MRI in at least one affected member (36 in 20 families and 8 isolated cases), ii) spastic paraplegia with TCC in at least one affected member (7 in 4 families and 10 isolated cases), iii) spastic paraplegia with cognitive impairment but without TCC in all affected members (7 in 4 families and 2 isolated cases), iv) “apparently” pure spastic paraplegia without TCC at MRI (7 cases in 3 families).

Clinical and paraclinical evaluations followed a protocol established by the SPATAX network (coordinator: Dr A. Durr) that included: full medical history and physical examination, estimation of the age and the symptoms at onset in the patient, disease duration and severity according to the Spastic Paraplegia Rating scale (Schule et al. 2006), presence/absence of additional neurological symptoms/signs, electromyographic (EMG) and nerve conduction velocity (NCV) studies, and brain and spinal cord MRI whenever possible. Neuropsychological evaluations were performed in the vast majority of cases by measuring their IQ, the Mini Mental State Evaluation (MMSE), and the Wechsler Memory Scale (Wechsler 1987). According to the DSM-IV criteria (2000), mental retardation was considered when the patient had an IQ $\leq$ 70 before the age of 18 years.

Having received the patients’ (or their parents’) written informed consent and the authorization of our local Ethical Committees, we purified genomic DNA from peripheral blood samples for subsequent genetic analyses. In all the patients, we had previously ruled out other causes of acquired spastic paraplegia such as multiple sclerosis, adrenoleukodystrophy, and mitochondrial diseases, as described (Casali et al. 2004).

Fifteen of the 31 families were Italian, eight were Brazilian, two were Turkish and one each was from Malta, Germany (with Turkish origin), Austria, The Netherlands, Montenegro, and Slovenia. Seventeen families were non-consanguineous and 14 consanguineous. Most of the 20 isolated patients were Italian (n=14), three Brazilian whereas the remaining originated from other European countries. In sporadic cases, mutations or rearrangements in the *SPG4* gene and mutations in the *SPG7* and *SPG21* genes had also been excluded (data not shown).

Mutation detection

The coding sequence and splice site boundaries of the 40 exons of the *SPG11/KIAA1840* gene (GenBank reference sequence NM_025137.3) were amplified by PCR and sequenced on both strands using the Big Dye chemistry (Applied Biosystems, Foster City, CA) as previously described (Stevanin et al. 2007). In non mutated cases, in addition to the coding sequence, 1166 bp upstream the first ATG and 425 bp downstream the last termination codon of the *SPG11* gene were amplified with a classical protocol and sequenced using four primers pairs (Supp. Table S1). The sequence products were run on an automated ABI 3730 sequencer and the results were analyzed with SeqScape 2.5 software (Applied Biosystems). Alternatively, PCR products generated using primers and PCR conditions indicated in Supp. Table S2 were processed for denaturing High Performance Liquid Chromatography (d-HPLC). To this end, the untreated PCR products (5–15 μ l) from the patients and a control DNA — previously deemed free of sequence variants — were mixed, denatured at 94°C for 5 minutes and renatured overnight at room temperature to allow the formation of heteroduplex prior to d-HPLC analysis. Samples were then analyzed using the Transgenomic WAVE DHPLC system (Transgenomic Inc, Omaha, NE) and results were interpreted essentially as reported (Patrono et al. 2005).

Segregation of mutations detected in this study was verified by direct sequencing or d-HPLC, or both in all available family members whenever possible. In addition, the relative frequencies of missense variants were determined on unrelated healthy controls (100 Italians and 50 European Caucasians), for a total of 300 chromosomes, by d-HPLC, ad hoc designed PCR-restriction fragment length polymorphisms (data not shown) or direct sequencing. Synonymous, missense and splice site variations were systematically evaluated for modifications of exonic splicing enhancers (www.rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi) or consensus splicing sequences in order to determine the splice site score

(rulai.cshl.edu/new_alt_exon_db2/HTML/score.html and www.fruitfly.org/seq_tools/splice.html). Multiple alignments with *spatacsin* orthologs were performed using ClustalW (www.ebi.ac.uk/clustalw/) to evaluate the degree of conservation of missense variants.

Nomenclature of mutations and variants followed the guidelines of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>) and referred to the cDNA sequence (GenBank reference sequence version NM_025137.3) with the A of the translation initiation codon as +1.

Haplotyping

In order to identify possible shared haplotypes between families carrying the same mutations, we genotyped the *SPG11* flanking microsatellites *D15S781*, *D15S537*, *D15S516*, and *D15S659*. We also used two new flanking markers, one of which lies in *SPG11* (primers listed in Supp. Table S1). PCR-amplified fragments were pooled with GeneScan500Liz marker, sized on an ABI 3730 automated sequencer and analyzed with GeneMapper 4 software (Applied Biosystems), according to the manufacturer's recommendations. Haplotypes were manually reconstructed considering the minimal number of recombinations.

Other methods

Cultured skin fibroblast polyA⁺ RNA was purified and reversely transcribed using the 1st Strand cDNA Synthesis Kit (Roche, Hamburg, Germany) according to the manufacturer's random primer protocol, and the consequences of the exon 24 c.4046T>A/p.F1349I mutation on splicing in patient F33[COL] was examined by RT-PCR using primers designed in exons 16 and 30.

To determine the segregation of the large-scale rearrangement in family F-NL02 we PCR-amplified a genomic fragment using oligonucleotide primers Spg11-30delF (5'-3') AAT GTA TTG GGT TGC TTT CCT G and Spg11-35R (5'-3') CCC TCC ATT TTC CCA AGA GT, High Fidelity Taq Polymerase (Roche Diagnostic, Milan, Italy) and PCR conditions consisting of 25 cycles of 15 s at 94°C, 30 s at 57°C, and 8 min at 68°C with an increase of 5 s/cycle after the first 10 cycles.

RESULTS

SPG11 mutation screening

SPG11 mutation screening has been undertaken in at least one index case in 31 families with a neurological picture highly resembling the clinical features of ARHSP-TCC (Shibasaki et al. 2000; Casali et al. 2004; Lossos et al. 2006; Stevanin et al. 2006; Winner et al. 2006) and in whom linkage to chromosome 15q had not been established *a priori*. We also included in the study a subset of isolated cases (n=20). In a total of 25 of these patients (16 familial index cases and 9 sporadic), we detected 32 different sequence changes in the coding regions or consensus splice sites of the *SPG11* gene; 22 of these are novel mutations whereas 10 have previously been reported (Table 1).

Table 1. List of *SPG11* mutations identified in this study (GenBank reference sequence NM_025137.3).

Mutation Type	Nucleotide/Amino acid position	Exon	Homo- or heterozygous state	Family (n index cases)	Origin	Reference
Nonsense	c.268G>T/p.E90X	2	Heterozygous	F-BRA12175 (1)	BR	This study
	c.1679C>G/p.S560X	8	Heterozygous	F16[SB] (1)	IT	This study
	c.1951C>T/p.R651X	10	Heterozygous	MP; F28[VAC] (3)	IT	This study
	c.2697G>A/p.W899X	15	Homozygous	F10; TK-SH (4)	TR	This study
	c.5470C>T/p.R1824X	30	Heterozygous	F8 (1)	DE	This study
	c.5870C>G/p.S1957X	31	Heterozygous	F16[SB] (1)	IT	This study
	c.5970C>G/p.Y1990X	31	Homozygous	FA (2)	IT	This study
	c.6091C>T/p.R2031X	32	Homozygous	F1 (3)	TR/DE	Lee et al. 2008
	c.6100C>T/p.R2034X	32	Homozygous	F-BRA21325 (2)	BR	Stevanin et al. 2007
	c.6856C>T/p.R2286X	38	Heterozygous	F14[CAL] (1)	IT	This study
Insertion/deletion	c.7023C>A/p.Y2341X	39	Homozygous	F-BRA21215 (2)	BR	This study
	c.408_428del21/p.E136_I142del	2	Heterozygous	F35[TER] (1)	IT	This study
	c.529_533del5/p.I177_F178del>S177fsX180	3	Heterozygous	F17[BRA]; F-BRA12175 (2)	BR	Stevanin et al. 2007
	c.733_734del2/p.M245VfsX247	4	Hetero- and Homozygous	F-BRA12162; F-BRA19070; F35[TER] (5)	BR; IT	Stevanin et al. 2007
	c.1203delA/p.K401fsX415	6	Heterozygous	F17[SP]; FB (3)	IT	Stevanin et al. 2007
	c.1697_1712del16insTACTCCCAT/p.D566VfsX595	8	Heterozygous	DKD (2)	IT	This study
	c.2355_2356del2/p.K785SfsX796	13	Homozygous	F34[GARG] (1)	IT	This study
	c.2716delC/p.Q906SfsX920	15	Homozygous	F9 (2)	BR	This study
	c.2849_2850insT/p.L950FfsX953	16	Heterozygous	MP (2)	IT	This study
	c.3075_3076insA/p.E1026RfsX1029	17	Heterozygous	F8; F33[COL] (2)	DE; IT	Hehr et al. 2007
	c.3664_3665insT/p.K1222fsX1236	21	Heterozygous	F17[SP] (1)	IT	This study
	c.3741_3742insA/p.P1248TfsX1264	22	Heterozygous	F17[BRA] (1)	BR	This study
	c.4307_4308del2/p.Q1436RfsX1442	25	Heterozygous	F16[BRA] (1)	BR	Stevanin et al. 2008a
	c.5255delT/p.F1752SfsX1837	30	Heterozygous	SP (2)	IT	Hehr et al. 2007
	c.5987_5990dupCTCT/p.C1996fsX1999	31	Heterozygous	DKD (2)	IT	This study
	c.5986_5987insT/p.C1996LfsX1999	31	Heterozygous	SP (2)	IT	Stevanin et al. 2008a
	c.5992insT/p.Y1998fsX1999	31	Heterozygous	FB (2)	IT	This study
	c.5898+5493_6509-491del/p.T1966fsX1968	31-34	Homozygous	F-NL02 (1)	NL	This study
Splice-site	c.2444G>T/p.R815M, r.?	13	Heterozygous	F28[VAC] (1)	IT	This study
	c.2444+1G>C, r.?	Intron 13	Heterozygous	F16[BRA] (1)	BR	This study
	c.2833A>G/p.R945GfsX950, r.2834_2835ins2834+1_2834+65	15	Heterozygous	F14[CAL] (1)	IT	Stevanin et al. 2008a
Missense	c.4046T>A/p.F1349I	24	Heterozygous	F33[COL] (1)	IT	This study

New mutations are indicated in bold. Origin of patients adopts the ISO indication for Country code.

Figure 1 shows a sample of the mutations detected in this study. In 24 cases, including 9 sporadic patients, the segregation was verified within the family throughout the analysis of 60 additional samples, of whom 15 were affected and 45 healthy relatives. Overall, 40 patients showed to have *SPG11* mutations on both alleles.

Figure 1. A sample of the new *SPG11* mutations identified in this study. A selection of the electropherograms flanking novel mutations in the *SPG11* gene is shown. Their segregation in the patients' family is also indicated. The novel mutations are indicated by arrows. Square symbols are men, the circles are women. The filled symbols are affected individuals. Stars indicate sampled subjects. M = mutation; + = wild type.

The index cases in 12 kindred presented with a homozygous mutation, whereas two heterozygous variants were detected in four families. Not too surprisingly, we only found compound heterozygosity in the 9 remaining isolated cases, all non-consanguineous. The majority of the mutations (Table 1) were predicted to be pathogenic because they lead to an early stop codon or frameshift, producing a premature termination of translation, and included nonsense mutations ($n = 11$), small deletions ($= 8$), small insertions ($= 6$), small duplication ($= 1$). It is of note that in one family (DKD) we detected a new micro-rearrangement (c.1697_1712del16insTACTCCCAT/p.D566VfsX595) at the compound heterozygous state. Also, we detected the first homozygous large-scale rearrangement sized about 9 Kb (c.5898+5493_6509-491del) in a Dutch family (F-NL02) predictably resulting in premature protein truncation at residue 1968 (p.T1966fsX1968) (Figure 2). The mutation deleted the sequences of exons 31-34 and was flanked by a 42-bp repeat sequence (5'-ggtggctcacgcctgtaatcccagcacttggaggccgagg -3'). Moreover, three changes (c.2444G>T; c.2444+1G>C; c.2833A>G) – which were ruled out in 150 controls and expected to involve nucleotides predicted to alter the correct splicing of the *SPG11* mRNA – were detected on one allele in three families. In the Italian family F28[VAC] the c.2444G>T mutation affected the last nucleotide of exon 13, predicting

a missense change (p.R815M), but also altering the 5'-splicing donor site of intron 13 (splice score of +0.2 versus +3.7). In the Brazilian kindred F16[BRA], mutation c.2444+1G>C affected the first base of the splice donor site and likely led to abolition of splicing at this site (splice score of -7 versus +3.7). Although strongly predicted *in silico*, effects on splicing could not be experimentally tested in living cells. In addition, the c.2833A>G variation detected in the Italian family F14[CAL] affected the last nucleotide of exon 15, predicted a missense change (p.R945G) and caused the reduction of the splice score from +4.9, for the wild type, to +2.7. When this same mutation was detected in an ARHSP-TCC Israeli kindred (Stevanin et al. 2008a), analyses of mRNA from patients have shown alteration of *SPG11* mRNA splicing with retention of 65-bp of intron 15 and subsequent early stop codon (r.2834+1_2834+65ins/ p.R945GfsX950). Interestingly, a missense mutation was detected in a single Italian patient F33[COL] who harbored the c.4046T>A/p.F1349I mutation in compound heterozygosity with the c.3076_3077insA/p.E1026RfsX1029. Residue F1349 is a well-conserved amino acid in other vertebrates and was not found mutated in 300 control chromosomes of European ancestry. When tested in cultured skin fibroblasts from the patient, the c.4046T>A/p.F1349I did not affect mRNA stability or correct splicing (not shown).

Figure 2. A novel c.5898+5493_6509-491del large-scale rearrangement encompassing exons 31-34 of the *SPG11* gene. Top panel. Polymerase Chain Reaction (PCR) detection of the novel c.5898+5493_6509-491del large-scale rearrangement in *SPG11* found in family F-NL02. C, control; P, patient F-NL02; MW, DNA 100-bp molecular marker size. Bottom panel. Using PCR conditions outlined in the text (see methods), a ~9.0 Kb fragment is amplified in a normal control (C) whereas a 720-bp fragment is detected in the propositus of kindred F-NL02 (P) as the result of the homozygous large scale deletion. Both the healthy parents (F, father and M, mother) and the brother (B) carried the heterozygous deletion. MW, DNA 1-Kb molecular size marker.

Molecular analyses identified families harboring the same mutation and sharing the same haplotype although they bear different surnames and formally deny relationships. As an example, we detected the homozygous c.2697G>A (p.W899X) in two presumably unrelated Turkish families (F10 and TK-SH) and the homozygous c.733_734del2 (p.M245VfsX247) variant in two seemingly unrelated Brazilian kindred (F-BRA12162 and F-BRA19070) (see

Table 2). However, we cannot exclude the existence of a distant common ancestor. The c.733_734del2 mutation has already been reported in four families, two of which are shown in Table 2, in association with different haplotypes (Stevanin et al. 2007; Del Bo et al. 2007; Hehr et al. 2007; Stevanin et al. 2008a). Moreover, we found the c.1951C>T (p.R651X) in two apparently unrelated families who originated from the same geographical region in central Italy and shared partial common haplotype, although different from the previously reported patient from Romania (Stevanin et al. 2008a). We also observed mutations recurring in several ethnicities. As an example, we found the frequent homozygous c.6100C>T (p.R2034X), already detected in northern African families (Stevanin et al. 2007; Stevanin et al. 2008a; Boukhris et al. 2008a), in one Brazilian kindred with partially common haplotypes (see Table 2). Similarly, the c.529_533del5 (p.I177_F178del>S177fsX) mutation, already reported in three Portuguese families (Stevanin et al. 2007; Stevanin et al. 2008a), was also detected in an additional Brazilian family again segregating with a similar disease-bearing chromosome.

Besides pathogenic changes in *SPG11*, we detected two reported homozygous SNPs (dbSNP: rs3759875 and rs3759873), two frequent heterozygous polymorphisms (p.F463S, and p.Y2341Y) (Stevanin et al. 2008a), and one new variant (p.K1273R); these variants were also detected in about 3% of normal alleles.

No mutations in coding exons and flanking introns were detected in 15 familial index cases, and in 11 isolated patients. Sequencing of 1166 bp upstream the first ATG and 425 bp downstream the last termination codon did not reveal mutations, too. These cases were not informative enough to formally exclude the involvement of *SPG11* through microsatellite genotyping, however.

Clinical characteristics of the new *SPG11* patients

Clinical information was available for all cases in which we could ascertain the presence of two mutant alleles. Table 3 reports clinical findings in the 40 individuals who proved to carry pathogenic variants on both alleles.

Familial and sporadic cases did not present with distinguishable clinical profiles. Also, the phenotype of patients harboring the missense mutation was similar to that of the patients having variants predicting early protein truncation. The disease followed a broadly similar course and affected siblings tended to show the same pattern of symptom evolution and rate of disease progression. Age at onset of the disease ranged from 5 to 23 with a mean of 14.0 ± 4.1 years and it was mostly characterized by difficult walking (75%) with leg stiffness, gait abnormalities and in some cases frequent falls whereas learning disabilities were the presenting symptom in 13% of the cases. In the remaining cases, both deterioration of gait and cognitive delay occurred at the same time or their onset could not be recorded accurately. In a single patient (DKD-H83) the disease manifested initially with upper limbs tremor at age 17 years but pyramidal tract signs and cognitive delay followed within two years. After a mean disease duration of 7.9 ± 5.5 years, all affected members of the families presented with a mild to severe spasticity of the legs with weakness in most. Deep tendon reflexes were brisk in the legs and, after less than 10 years, also in the arms. Most of the patients had mental impairment (87%) with objective evidence in 20 patients with IQ ranging from 53 to 69. Few cases initially showed a low-normal IQ but scored poorly at subsequent testing.

The MRI scans were not recorded in three cases with disease duration of two, four and 12 years. However, their similarly affected sibs had shown typical neuroimaging features of TCC. Neuroradiological evidence of TCC was present in 34/37 (91.8%) patients with scans recorded whereas white matter hyperintensities were detected in 57%. Cortical or cerebellar atrophy, or both, were noted in seven patients (19%). Two familial cases only presented with moderate white matter signal abnormalities at the frontal horns of the lateral ventricles. Only one patient had a normal MRI, but after a disease duration of less than one year.

Less frequent additional neurological findings consisted of cerebellar ataxia in 32% and extrapyramidal features in 16% of the cases (Table 3), usually in late stages of the disease. Signs of axonal neuropathy were detected in 15/34 patients (44%). Few patients showed neurogenic changes resembling lower motor neuron involvement. A slow electroencephalogram was also recorded in two cases. A single patient showed reduced visual acuity at night but neither electroretinogram nor detailed funduscopy were performed; another case carried a psychiatric diagnosis of bipolar disorder with psychosis and hallucinations. Concurrent Down syndrome was also observed in one case (Table 3).

Table 2. Haplotypes of six close markers segregating with the recurrent mutations in the *SPG11* gene in this study and in previous reports.

Family Origin	F10 Turkey	TK-SH Turkey	Family Origin	F-BRA12162 Brazil	F-BRA19070 Brazil	FSP117* France	FSP870# Tunisia
<i>D15S781</i>	185	185	<i>D15S781</i>	187	187	185	187
<i>D15S537</i>	180	180	<i>D15S537</i>	160	160	172	180
<i>Chr15 :42720928</i> [^]	280	280	<i>SPG11 exon 4</i>	<i>c.733_734del2</i>	<i>c.733_734del2</i>	<i>c.733_734del2</i>	<i>c.733_734del2</i>
<i>SPG11 exon 15</i>	<i>c.2697G>A</i>	<i>c.2697G>A</i>	<i>Chr15 :42720928</i> [^]	272	272	278/280	286
<i>Chr15 :42800751</i> [□]	198	198	<i>Chr15 :42800751</i> [□]	198	198	196/198	196
<i>D15S516</i>	195	195	<i>D15S516</i>	195	195	195	195
<i>D15S659</i>	199	199	<i>D15S659</i>	175	179	179	195

Family Origin	F28[VAC] Italy	MP Italy	FSP683# Romania
<i>D15S781</i>	185	185	187/191
<i>D15S537</i>	172/176	172/176	164/184
<i>Chr15 :42720928</i> [^]	278/280	278/280	278/280
<i>SPG11 exon 10</i>	<i>c.1951C>G</i>	<i>c.1951C>G</i>	<i>c.1951C>G</i>
<i>Chr15 :42800751</i> [□]	198	198	194/198
<i>D15S516</i>	195	195	193/195
<i>D15S659</i>	199/179	195/183	199/179

Family Origin	FSP732* Algeria	FSP446* Morocco	FSP881# Tunisia	FSP221* Algeria	FSP792# Morocco	FS845# Morocco	F-BRA21325 Brazil	FSP400# Algeria
<i>D15S781</i>	185	185	185	185	185	185	185	185
<i>D15S537</i>	176	180	180	176	176	176	176	180
<i>Chr15 :42720928</i> [^]	282	280	280	280	280	280	280	280
<i>SPG11 exon 32</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>	<i>c.6100C>T</i>
<i>Chr15 :42800751</i> [□]	198	198	198	198	196	196	196	196
<i>D15S516</i>	191	191	191	191	191	191	191	191
<i>D15S659</i>	175	179	179	179	179	179	191	195

Family Origin	FSP831# Portugal	FSP754* Portugal	FSP386* Portugal	F17[BRA] Brazil	FSP386* Portugal
<i>D15S781</i>	185	185	185	185	185
<i>D15S537</i>	172	172	172	ND	172
<i>SPG11 exon 3</i>	<i>c.529_533del5</i>	<i>c.529_533del5</i>	<i>c.529_533del5</i>	<i>c.529_533del5</i>	<i>c.529_533del5</i>
<i>Chr15 :42720928</i> [^]	286	286	286	282/286	286
<i>Chr15 :42800751</i> [□]	196	196	196	196	194
<i>D15S516</i>	195	195	195	195	193
<i>D15S659</i>	203	203	195	195	195

*Stevanin et al. 2007; Del Bo et al. 2007, #Stevanin et al. 2008a, ND= not done; ^ = Intragenic marker; □ = 3' UTR marker. Conserved regions are highlighted in grey. Genotypes are indicated in base pairs.

Table 3. Clinical features of 40 patients with mutations in the *SPG11* gene.

M= male, F= female, ND= not done, UL= upper limbs, LL= lower limbs, IQ= intellectual quotient, MMSE= Mini Mental State Evaluation, ++= increased; += present; -= absent; PWM, periventricular white matter; TCC= thin corpus callosum.

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-BRA12175	BR	12/F	7	Difficulties at school	Mild	Mild/No	+	++	MUTE	-	Delay	TCC, PWM	Normal	
F16[SB]	IT	25	10	Difficulties in concentration and walking	Moderate	Moderate spasticity, Walking difficulties	++	+++	+	+	Mental deterioration	TCC, PWM	Neuropathic changes	Slight tremor finger to nose
F17[BRA]	BR	17/F	15	Spasticity	Mild	Mild/No	+	+++	+	-	Retarded	TCC, PWM	Normal	
MP-1	IT	26/M	18	Spasticity	Severe	Severe/ Moderate	Normal	++	++	-	Mental retardation/ Progressive cognitive deterioration	TCC, PWM, Cortical atrophy	Axonal Neuropathy	
MP-2	IT	21/F	20	Spasticity	Moderate	Moderate/ Moderate	++	++	++	-	Moderate	TCC, PWM	Axonal Neuropathy	Urinary urgency
F28[VAC]	IT	18F	14	Feet deformities and stiff legs	Moderate	Moderate/ Mild	-	++	+	-	No cognitive deficit	TCC, PWM	Normal	Severe pes cavus
F10-1	TK	12/M	10	Falls	Severe	Severe/ Moderate	++	++	++	+	Mental retardation/ Progressive cognitive deterioration	TCC, PWM, Cortical atrophy	Axonal Neuropathy	
F10-2	TK	16/M	14	Tremor	Moderate	Severe/ Moderate	+	++	+	-	Cognitive delay	ND	ND	Slow EEG
TK-SH-1	TK	27	8	Deterioration of gait	Moderate, at 22 yrs still able to walk with crutches	Moderate LLspasticity, Feet drop due to weakness of ant. tibial muscle	++	+++	++	+	Some cognitive decline	Mildly increased signal of periventricular and deep cerebral white matter, "Flames" at the frontal horns, Mild cerebral atrophy, TCC	Axonal Neuropathy	Dysarthria, Decreased manual dexterity
TK-SH-2	TK	22	10	Deterioration of gait	Mostly wheelchair dependent	Moderate LLspasticity, Feet drop due to weakness of tibial muscle	++	+++	Equivocal	+	Cognitive decline	ibidem	Axonal Neuropathy (mild)	Down syndrome, Progressive serious dysarthria, Decreased manual dexterity

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F8	DE	24/F	10	Deterioration in school performance (10yrs), Gait disturbance (14 yrs)	Progressive deterioration, now severely disabled	Standing with support, No walking	+++	+++	+	-	Initial normal cognitive development, Mental deterioration starting at 10 yrs	TCC, PWM, Cortical atrophy	NC studies normal, EMG of ant. tibial muscle showed mild neurogenic changes at 17 yrs	Foot drop
FA-1	IT	17/F	15	Spasticity	Mild	Moderate/ No	+	+++	++	-	Mental retardation, Progressive cognitive deterioration	TCC	ND	Visual problems at night
FA-2	IT	12/F	12	Delayed	Mild	Mild/No	+	++	++	-	Delayed learning and memory	Normal	ND	
F1-1	TK-DE	22/F	10	Walking difficulties	Moderately impaired	+++	++	+++	+	-	Severe, she can hardly add 4 + 5	TCC	ND	
F1-2	TK-DE	24/M	15	Walking difficulties	Now: can hardly walk	+++	++	+++	-	-	Now communication via language is hardly possible, but he can add 4 + 5	TCC	Nerve conduct. study compatible with mild axonal loss	Nerve biopsy 2000: mild axonal loss, CK mildly elevated
F1-3	TK-DE	17/F	16	Pain in her legs	Gait only mildly impaired	+	+	+++	+	-	Mildly impaired	TCC	Normal NCV, Normal MAP-amplitudes, Normal needle EMG	CK normal
F-BRA21325	BR	21/F	17	Walking difficulties	Use of walker	Severe	++	+++	+	-	Mental retardation	TCC, PWM	Normal	
F-BRA21326	BR	18/M	15	Walking difficulties	Moderately impaired	Moderate	++	+++	+	-	Mental retardation	TCC	Normal	
F14[CAL]	IT	29/F	17	Hypospina, Difficulties in walking	Severe	Severe, unable to walk unaided	+++	+++	+	+	Mental deterioration	TCC, PWM	Axonal Neuropathy	
F-BRA21215	BR	27/F	15	Gait abnormalities	Wheelchair at 24 yrs	Severe	+	+++	MUTE	-	Mental retardation	TCC	Normal	Dysarthria
F-BRA21216	BR	21/F	17	Gait abnormalities	Moderate	++	+	+++	+	-	Mental retardation	TCC	ND	Pes cavus
F35[TER]	IT	46	15	Spasticity	Wheelchair at 23 yrs	Severe	+	+++	+	+	Mental retardation	TCC	Neurogenic	Severe psychosis, Allucinations

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-BRA12162	BR	24/M	8	Deterioration in school performance	Deterioration, now severely disabled	Standing with support, no walking	+++	+++	+	-	Mental retardation	TCC	ND	
F-BRA12163	BR	22/F	10	Walking difficulties	Moderately impaired	+++	++	+++	+	-	Mental retardation	ND	ND	
F-BRA19070	BR	32/M	16	Spasticity	Wheelchair at 27 yrs	Severe	-	+++	+	-	Mental retardation	TCC	Normal	
F-BRA19069	BR	33/F	22	Stiff legs	Cane at 32	Moderate	-	+++	+	-	Mental retardation	TCC	ND	
F-BRA19271	BR	27/F	23	Gait abnormalities	Cane at 26	Moderate	-	+++	+	-	Mental retardation	ND	ND	
F17[SP]	IT	29/M	6	Mild learning disabilities	Mild	Moderate	+++	+++	+	-	Mental retardation/ Progressive cognitive deterioration	TCC, PWM	Normal	Urinary urgency
FB-1	IT	28/F	17	Feet deformities and stiff legs	Severe	Moderate/ Mild	+	-	++	+	Mental retardation/ Progressive cognitive deterioration	TCC, Cerebellar atrophy, Cortical atrophy	Axonal Neuropathy	Dysarthria, Severe pes cavus
FB-2	IT	24/F	19	Falls	Severe	Moderate/ Mild	+	-	++	-	Mental retardation/ Progressive cognitive deterioration	TCC	Axonal Neuropathy	
DKD-H82	IT	30	13	Stiffness while walking	Severe	Marked/severe unable to walk	+++	+++	+	+	Mental deterioration (2007 severe)	Striking TCC, PWC	Neurogenic changes	
DKD-H83	IT	26	17	Hand tremor and difficulties in walking	Moderate	Marked walking difficulties	+++	++	+	+	Mental deterioration	ibidem	Neurogenic changes	Slight adiadokinesia at finger to nose test
F34[GARG]	IT	29/F	22	Spasticity	Mild	Moderate	-	++	+	-	-	PWM	Normal	
F9-1	BR	22/M	14	Gait disturbances	Severe	Severe/ Moderate	+	++	++	-	Mild decline	TCC	Normal	Slow eeg, Dysathria
F9-2	BR	20/M	12	Pain in LL, Gait disturbances	Severe	Severe/ Moderate	++	++	++	-	Mild decline	TCC	ND	Pes cavus, Tremor upper limbs
F33[COL]	IT	27/F	15	Learning slow	Mild	Moderate	+	++	+	-	Cognitive delay	TCC, PWM	Neurogenic	Dysarthria
F16[BRA]	BR	23/F	17	Stiff legs	Mild	++	+	+++	+	-	Mental deterioration	TCC, PWM	Normal	Dysarthria
SP-H28	IT	20/F	15	Learning difficulties	Moderate	+++	++	+++	+	+(distal)	Mild decline	TCC, PWM	Normal	Pes varus, Fragmented smooth pursuit
SP-H29	IT	13/M	12	Walking difficulties	Moderate	++	+	-	+	-	-	PWM	Axonal Neuropathy	Pes varus, Fragmented smooth pursuit

Patient ID code	Origin (ISO cod)	Age at examination (years)/sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-NL02	NL	15	5	Deterioration of gait	Moderate, at 15yrs still able to walk	Moderate LL spasticity	++	+++	++	-	-	Mildly increased signal of periventricular and deep cerebral WM, "Flames" at the frontal horns, Mild cerebral atrophy, Thin anterior CC	Normal	Mild Dysarthria

DISCUSSION

In a large cohort of 31 families with HSP-TCC and in 20 sporadic cases we identified *SPG11* disease alleles in 25/51 index patients (49%). Broadening the spectrum of mutations in spatacsin, we identified 32 variations, of which 22 are novel, distributed all over the gene. Eleven nonsense mutations, eight small deletions, six small insertions, one small duplication and three mutations with effects on splicing were identified. Moreover, we detected one in/del and one large scale deletion sized about 9.0 Kb. The causative nature of the missense p.F1349I variant remains to be proved because single heterozygous deletions can have escaped detection in the carrier patient. Nonetheless, the p.F1349I, which affects a conserved amino acid and was associated with a truncated allele in trans, was absent in 300 control chromosomes. Moreover, this mutation is predicted to be intolerant (PolyPhen — www.genetics.bwh.harvard.edu/pph/ — and SIFT www.blocks.fhcrc.org/sift/SIFT.html) and could lead to loss of function of the protein.

Together with the already reported changes (Stevanin, et al. 2007; Del Bo et al. 2007; Hehr et al. 2007; Stevanin, et al. 2008; Boukhris et al. 2008a; Paisan-Ruiz et al. 2008; Zhang et al. 2008; Lee et al. 2008; Erichsen et al. 2008), disease-associated mutations in *SPG11* are now 67 and almost invariably alter the correct formation of a complete protein product in all except one case. If formed, the protein would be rapidly degraded or significantly shortened. Consequently, no significant domain-related clinical differences could be observed. As an example, the c.529_533del5 mutation lies in the first transmembrane domain whereas the c.3741_3742insA in the third transmembrane domain and the large deletion removes the Myb domain. These mutations presented with comparable age at onset, disease severity and progressive neurological impairment. The practical consequence of the large number of different mutations identified, in terms of the diagnosis of ARHSP-TCC, is that a quick, DNA-based test seems unfeasible. We can speculate that a protein-based diagnostic assay of the type being developed in other forms of spinocerebellar degenerations [i.e., *ALS2* (Eymard-Pierre et al. 2006), ataxia-telangiectasia syndrome (Sutton et al. 2004) or ataxia with oculomotor apraxia type 1 (Ferrarini et al. 2007)] will be more effective.

Our study enlarges the ethnic origin of *SPG11* patients. Like in previous studies (Hehr et al. 2007; Stevanin et al. 2008a), no population-related mutation seems to emerge in both Italian and Brazilian cases and frequent compound heterozygosity is observed which in turn might imply higher than expected carrier frequencies. Even within apparently homogeneous populations, a molecular diagnosis cannot be achieved without full gene sequencing. For example, in a total of 29 Italian index cases with compatible phenotypes (15 familial and 14 apparently sporadic patients), we detected 12 subjects — half of whom had no positive family history — harboring pathogenic variants in *SPG11* (41%) and 21 different mutations broadly distributed along the coding exons. A similar ample allelic heterogeneity has been reported in Tunisian (Boukhris et al. 2008a), Pakistani (Paisan-Ruiz et al. 2008) and Turkish families (Hehr et al. 2007). Interestingly, few mutations seem to be recurrent in patients' population originating from the Mediterranean basin, indicating founder effects with subsequent migrations. The haplotypes segregating with the c.529_533del5 *SPG11* mutation are particularly well conserved in the Brazilian and Portuguese cases, but the telomeric and centromeric portions of the haplotype segregating with the c.6100C>T mutation in patients from North Africa and Brazil are more divergent and might indicate a more ancient mutational event. However, a mutational hot spot cannot be excluded. Additional mutated families are required to date these events.

When clinical features are driving molecular testing, roughly one case in two of familial complicated spastic paraplegia harbors *SPG11* mutations (Table 4). Although we (Stevanin et al. 2007; Stevanin et al. 2008a; Boukhris et al. 2008a, and this study) and others (Hehr et al. 2007; Paisan-Ruiz et al. 2008) have at times noticed that intrafamilial differences are possible, and similar mutations not necessarily lead to similar onset and disease course (Table 3), *SPG11* patients usually develop a progressive motor and cognitive deterioration from school years, but earlier learning problems and attention deficits are likely to occur. Combining evidence from this study and our previous reports (Stevanin et al. 2007; Stevanin et al. 2008a) it is clear that the presence of motor deterioration with signs of pyramidal dysfunction of the legs in young students who perform badly in junior-high school appears sufficient to propose a molecular testing after appropriate clinical and imaging evaluation. Practically all *SPG11* mutations are associated with a widely similar disease course with frank spastic paraplegia manifesting within the second-third decade of life. At the same time, cognitive deterioration worsens and produces in late disease stages frank dementia. The lower rate of progression observed in this study, when compared to previous analyses (Stevanin et al. 2008a), might relate to ethnic differences among patients or to a different clinical setting, being mostly pediatric in our study and more related to adult neurology centers in the previous one.

Table 4. Frequency of *SPG11* mutations according to the clinical categories outlined in the Methods.

Phenotypes	Group I: HSP + MR + TCC	Group II: HSP + TCC	Group III: HSP + MR, no TCC	Group IV: Apparently pure HSP	Sum
Number of index cases					
Familial	20	4	4	3	31
Sporadic	8	10	2	0	20
Mutation detected					
Familial	14/20 [§] (70%)	1/4 [§] (25%)	0/4	1/3 (33%)	16/31 (52%)
Sporadic	8/8 (100%)	1/10 (10%)	0/2	0	9/20 (45%)

[§]Refer to index cases in the families.

Neuroimaging appears even more helpful in deciding on performing genetic analyses. Thin rostral corpus callosum with a “beaked” shape and mostly sparing the splenium, frequently associated with “flames” at the frontal horns involving periventricular and deep cerebral white matter, with or without mild cerebral atrophy, appear the distinctive neuroradiological features of this disorder. In advanced disease stages, atrophy of higher cortical regions such as the prefrontal cortex matches thinning of the anterior corpus callosum (Figure 3). In a small set of individuals presenting without TCC, mutations in *SPG11* are found (8% in this study; 4.5% in Stevanin et al. 2008a) after a disease duration of less than 5 years. In such cases, however, either the presence of a sib with a full syndrome (clinical and neuroradiological) or evidence of learning disabilities and white matter lesions raise the clinical suspicion and we cannot exclude that follow-up MRI might show a “curved” shaped, thinner anterior corpus callosum.

2001

2008

Figure 3. Axial and sagittal brain MRI images after a 7-year follow up in patient SP-H28 harboring the heterozygous p.F1752SfsX1837 and p.C1996LfsX1999 mutations in *SPG11* showed worsening of TCC, “flames” at ventricular horns, and progression of cortical atrophy.

When clinical and neuroradiological features are combined, they also remain a useful criterion for deciding to screen isolated patients. In our cohort, 9/20 of cases (45%) with complicated HSP without a positive family history harbored mutations in *SPG11*. Eight patients had a combination of HSP+TCC and cognitive impairment (Table 4). This higher than expected positive mutation rate is different from what has been observed by others (1/25, 4%) (Paisan-Ruiz et al. 2008) but might depend on the use of more stringent selection criteria or might relate to different patients' ethnicities. In general, however, it is hard to speculate on the rate of *SPG11* mutations when patients under screening have less canonical phenotypes, such as HSP + mental retardation without TCC or pure spastic paraplegia with onset in early adulthood and no family history because we did not test enough patients meeting these criteria. Analyses in this latter group of patients, by far the most frequent in the clinical practice, require more cost-effective modalities of molecular screening after a more complete clinical examination and a dedicated *SPG11* testing.

About half of patients, both sporadic and familial, did not show mutation in *SPG11* notwithstanding our in depth mutation screening. The clinical presentation and the MRI features in such cases did not grossly differ from patients having both alleles mutated. These findings reinforce previous notions on genetic heterogeneity in ARHSP-TCC (Casali et al. 2004; Boukhris et al. 2008a). Although the presence of non-conventional variants remains possible, mutations in other genes are also to be expected. For instance, it has been shown that a subgroup of ARHSP-TCC with clinical features highly similar to *SPG11* cases (Elleuch et al. 2007) are instead linked to the *SPG15* locus on chromosome 14q (about one in four in Boukhris et al. 2008a; Boukhris et al. 2008b) or mutated in the corresponding gene (Hanein et al. 2008). The future identification of other HSP-TCC related genes will permit better correlations with neuroimaging and clinical phenotype.

In summary, *SPG11* mutations appear the most frequent genetic determinant of autosomal recessive HSP worldwide with cases described on European, Asian and North-African genetic background. At least in Italy, *SPG11* seems to account equally for sporadic and familial cases and it currently denotes the most common etiology in ARHSP, with or without TCC, mirroring what *SPG4* represents for the autosomal dominant forms. Our results suggest that *SPG11* should be tested in patients, even with no family history, if progressive motor degeneration and early onset mental decline are associated with distinctive brain MRI since onset or after a few years of disease duration.

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Supp. Table S1. Oligonucleotide primers and annealing temperatures (T-°C) used to amplify the 5'- and 3'-untranslated regions (UTR) of the *SPG11* gene and two novel intragenic dinucleotide markers.

Region flanking <i>SPG11</i>	FORWARD SEQUENCE (5'-3')	REVERSE SEQUENCE (5'-3')	T°C
5'UTR-1	CAGGCGTCAAAGAAAGCACT	TAGCAAAGCCACATCTGCC	60°
5'UTR-2	TAAGCTTAGCTGGGGCTGTG	CCTTGCCAACATAGGGAGAC	60°
5'UTR-3	AGGATAGCCTTCATTACAGGTTT	TTCTGAGGACAGAGATGACCA	60°
3'UTR	TGGATGAACAATCATCTAAAATCAA	TCATAAACTTGTGTTCGTATGCC	60°
Intragenic <i>SPG11</i> markers			
chr15:42720928	GAATACATCCACCACACAAAC	GTGCTGGCAAGAACATCAAG	60°
chr15:42800751	CATATTATACCACACTTACC	TTTGAAAGTCAAGGTGAGC	60°

Supp. Table S2. Oligonucleotide primers, annealing temperature (Ta-°C PCR), anticipated product size in base pairs (bp) and DHPLC conditions (T-°C DHPLC) used to analyze the 40 exons of the *SPG11* gene.

Exon	bp	Ta-°C PCR	5'- 3' Primers Forward/Reverse	T-°C DHPLC
1	308	60	agtcagggtccggcgaaaagt / ccaactctccctcagcactt	54.8
2	323	62	accagggtcaactaaactgttctct / tatgctgaaagaccacctgtaga	55.3
3	305	58	ccagttgtaaaattgtgacc / tcaatcaacacttctaccac	55.1
4	320	62	gttaggcatacttacaactggc / cgaggatattttaacctcttatca	54.8 - 52.8
5	330	60	caggagcagtagtaacacaa / aaagggtacagcgtcagcat	54.9
6	450	58	ctgtgacaggtgttaagtta / atctaatacaagacagtctc	54
7	275	58	tagtactgaagtattgagta / ttaagtaattgttctgggca	50.6
8	450	60	ctgccccagattgcataat / tccaaaaagtacgtaaaatccca	54.1
9	342	62	gcaggttaataagcctgcagaa / ccccttctctagctgctatt	54.1
10	331	62	cacacacacaaattggcaca / aacctttgccaacattctga	54.1
11	293	57	gttacataaatgtataatccctg / cattttaagactttatggattac	54.6
12	210	62	tgttcaaaatagttccattacaaa / ttcttccaaggttttctcca	52.5
13	289	62	tttgcaaaagtgtctgatttt / tgcaggctcagttccacata	51.5
14	246	62	ggaatgatgcctttttctcc / tctcacactgccttctgga	55.8
15	345	60	cacagcgagatctgtctca / cctcactgtaagatgatgcc	55-58
16	309	62	tgtgggcatgatttggctca / acctgctcaaggacaaatgc	55.2
17	239	62	aatcatgcctgagcaaaat / ccagtgactgatccaaagca	53.9
18	324	58	ccctcttaaggagaaaaacac / cagccttatcctctgctctt	53-50
19	299	60	gctaactctgtttcacagg / cctggctgaactctgataga	55
20	311	62	tggaaaaggggagcagacta / tgcgaactattttctcttgg	52.3
21	303	58	ttccatgtgcaaatctgaaatta / tcccaaaagtgcctgggattac	58
22	383	62	gaggaggccacaaatcacat / gccttagacctcgtcacacc	55.8
23	356	62	tgctcaggttttgactttttctc / ttctactgatggcaagatgc	53.1
24	267	60	accacccccacctctaattc / ctacacaacagaaagaatgc	57.1
25	361	60	ccagctgaaactgaaagtgg / ctgggtacttacttcaggct	56.6
26	293	60	tgtacatttgccaggtaatcca / cttaagctctggaaagaagtg	56
27	330	62	cactgtgccctgccttatta / tctgcctgagtaaccgagtg	53.6
28	329	62	tcccagatttgagggttttg / tgcattttaatttctaactacc	53.5
29	330	56	gctgtagtggcattttattg / cctgggtgacagagcaagac	56
30a	323	62	gagggtgggaggtatctcttg / gatgtgttcagagcagccaa	58-55
30b	304	62	taagctggaggagctggaga / ttgtgtccccttaacttgg	58-55
31	318	62	caggagcttcaagcagaga / tgacttgccaatgtccaaaa	56.9
32	323	60	cctggcttcaaaagtggcc / aagcacaacatccaaatcctt	58.8
33	349	62	agctgcagagctccataagc / taggcatecagagcaggaa	57.6
34	336	62	gaggttgacagtgggcagcca / gccagccaactctcaagta	56.9
35	312	62	ggcatctgaaagcaaccact / cctccattttccaagagt	54
36	376	62	caacaggaaagcacacatgc / gtgtggctgtgacctcactc	52
37	313	62	aacatggctgggatgtttct / ttctggttggcctatgatg	57.6
38	315	62	ggggtgaataaccgtgtgag / acctctgggttccatgagtg	56.9
39	380	62	aatgccaacacacacctga / ctcaaaagcagaggcaaggag	53.2
40a	390	62	agactgctctctgcaactcc / ccgggattgttcaactttage	54.4
40b	321	58	cagtatcttaacctgtacat / ccgggattgttcaactttage	54.2

AUTOSOMAL RECESSIVE ATAXIAS

20 types, and counting

Emília Katiane Embiruçu¹, Marcília Lima Martyn¹, David Schlesinger², Fernando Kok³

Abstract – More than 140 years after the first description of Friedreich ataxia, autosomal recessive ataxias have become one of the more complex fields in Neurogenetics. Currently this group of diseases contains more than 20 clinical entities and an even larger number of associated genes. Some disorders are very rare, restricted to isolated populations, and others are found worldwide. An expressive number of recessive ataxias are treatable, and responsibility for an accurate diagnosis is high. The purpose of this review is to update the practitioner on clinical and pathophysiological aspects of these disorders and to present an algorithm to guide the diagnosis.

KEY WORDS: autosomal recessive ataxias, cerebellar ataxia, cerebellum, Friedreich ataxia.

Ataxias autossômicas recessivas: 20 tipos e muito mais

Resumo – Mais de 140 anos após a primeira descrição da ataxia de Friedreich, as ataxias autossômicas recessivas se transformaram em um dos mais complexos campos da Neurogenética. Atualmente, este grupo de doenças é composto por mais de 20 entidades clínicas e possui um número ainda maior de genes associados. Algumas doenças são muito raras, tendo sido observadas apenas em populações isoladas, enquanto que outras são encontradas no mundo todo. Um número expressivo de ataxias é tratável, e a responsabilidade em se fazer um diagnóstico correto é alta. A finalidade desta revisão é a de atualizar o neurologista a respeito dos principais aspectos clínicos e fisiopatológicos destas doenças e de apresentar um algoritmo para auxiliar a sua investigação e o seu diagnóstico.

PALAVRAS-CHAVE: ataxias autossômicas recessivas, cerebelo, ataxia cerebelar, ataxia de Friedreich.

More than 20 different clinical types of autosomal recessive ataxias (ARA) are currently recognized. They are clinically characterized by balance abnormalities, incoordination, kinetic and postural tremor, and dysarthria¹. Typically, symptoms start before 25 years of age, and cerebellum, brainstem, and spinocerebellar tracts are involved². Peripheral neuropathy, ophthalmological abnormalities, and non-neurological signs and symptoms might also be present¹. Friedreich ataxia, the most common ARA, was first described in 1863 and is seen worldwide^{1,2}. In the last few years, several other conditions have also been recognized and their loci and genes identified.

Pathophysiology is quite variable and the defective gene product might be involved with: (A) Cerebellar and/or brain stem development; (B) Mitochondrial energy generation; (C) Intermediate metabolism; (D) DNA repair; and (E) Cerebellar integrity maintenance. Several classifications have been proposed so far, using clinical, neuroimaging, genetic and pathophysiologic data². In this review, a pathophysiological classification is used (Table 1).

We should be particularly aware of treatable forms of ARA, which includes Refsum disease, ataxia with vitamin E deficiency, coenzyme Q10 deficiency, cerebrotendinous xanthomatosis, and abetalipoproteinemia.

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Table 1. Classification and molecular aspects of the autosomal recessive ataxias

	Gene (Locus)	Protein	Protein function
Congenital			
Cayman ataxia	<i>ATCAY</i> (19p13.3)	Caytaxin	Synapse between granulates and Purkinje cells (?)
Joubert syndrome	<i>AHI1</i> (16q23.3)	Joubertin	Cerebellar estruturation; cilia estruturation and functions
	<i>NPHP1</i> (2q13)	Nefrocistin-1	
	<i>CEP290</i> (12q21.34)	Nefrocistin-6	
	<i>TMEM67</i> (8q21.1-q22.1)	Meckelin	
	<i>RPGRIP1L</i> (16q12.2)	Protein phantom	
Cerebellar hypoplasia associated VLDL receptor	<i>VLDLR</i> (9p24.2-3)	VLDL Receptor	Signalling neuroblast migartion
Mitochondrial			
Friedreich ataxia	<i>FRDA</i> (9q13)	Frataxin	Mitochondrial iron metabbolism
Coenzyme Q10 deficiency with cerebellar ataxia	<i>PDSS1</i> (10p12.1) and <i>PDSS2</i> (6q21)	Prenyldiphosphate synthase subunit 1 e 2	Coenzyme Q10 biosynthesis
	<i>COQ2</i> (4q21-q22)	OH-benzoate polyiprenyl transferase	Coenzyme Q10 biosynthesis
	<i>ADCK3</i> (CABC1) (1q42.2)	ADCK3 (Mitochondrial protein)	Coenzyme Q10 biosynthesis
Ataxia with mutation in polymerase gamma	<i>POLG</i> (15q22-26)	DNA polymerase γ	Mitochondrial DNA maintenance
Infantile onset spinocerebellar ataxia	<i>C10orf2</i> (10q24)	Twinkle	Mitochondrial DNA repair and maintenance
Metabolic			
Ataxia with vitamin E deficiency	α - <i>TTP</i> (8q13.1-13.3)	α -tocopherol transfer protein	α -tocopherol incorporation in VLDL
Abetalipoproteinemia	<i>MTP</i> (4q22-24)	Microsomal tryiglyceride transfer protein	Lipoprotein metabolism
Refsum disease	<i>PHYH</i> (10pter-11.2)	Phytanoyl-CoA hydroxylase	Fatty acid α -oxidation
	<i>PEX7</i> (6q21-22.2)	Peroxisomal biogenesis factor-7	Peroxisomal protein importation
Cerebrotendinous xanthomatosis	<i>CYP27</i> (2q33-ter)	Sterol 27-hydroxylase	Bile acid synthesis
DNA repair defects			
Ataxia telangiectasia	<i>ATM</i> (11q22.3)	Ataxia telangiectasia mutated	DNA double-strand break repair
Ataxia-telangiectasia- like disorder	<i>MRE11A</i> (11q21)	Meiotic recombination 11	DNA double strand break repair
Ataxia with oculomotor apraxia type 1	<i>APTX</i> (9p13)	Aprataxin	DNA single strand break repair
Ataxia with oculomotor apraxia type 2	<i>SETX</i> (9q34)	Senataxin	DNA and RNA repair
Spinocerebellar ataxia with axonal neuropathy	<i>TDPI</i> (14q31-32)	Tyrosyl DNA phosphodiesterase I	DNA repair
Degenerative			
Spastic ataxia of Charlevoix Saguenay	<i>SACS</i> (13q11)	Sacsin	Chaperone-mediated protein foling
Marinesco-Sjögren syndrome	<i>SIL1</i> (5q31)	BiP associated protein	Stabilization and folding of newly synthesized polypeptides

Legend: (?), possibly.

ATAXIA CAUSED BY CEREBELLAR AND/OR BRAINSTEM MALFORMATION

In this group, neuroimaging studies are able to identify cerebellar and/or brainstem malformation and clinically it is characterized by non progressive cerebellar ataxia. Three conditions are discussed in this section: Cayman ataxia, Joubert syndrome and Cerebellar hypoplasia associated to VLDL receptor.

Cayman ataxia

Cayman ataxia (CA) is a condition characterized by variable developmental delay, early onset hypotonia and non-progressive axial cerebellar ataxia, associated to nystagmus, intention tremor, and dysarthria³. MRI presents with cerebellar hypoplasia³. The CA has only been found in Grand Cayman Island, where heterozygote frequency is supposed to be of 18%³. CA is caused by mutation at *ATCAY*, which codes for caytaxin, a protein involved with glutamate synthesis and also with synaptogenesis of cerebellar granular neurons and Purkinje cells⁴. Interestingly, *ATCAY* contains a CRAL-TRIO domain that binds small lipophilic molecules, similar to the alpha-tocopherol transport protein that causes ataxia with vitamin E deficiency³.

Joubert syndrome

Joubert syndrome (JS) is a rare genetically heterogeneous inherited disorder with an estimated prevalence in the United States of 1 in 100,000⁵. JS is characterized by congenital ataxia, hypotonia, developmental delay, and at least one of the following features: neonatal respiratory disturbances and abnormal eye movements (nystagmus or oculomotor apraxia). In some cases, Leber congenital amaurosis, pigmentary retinopathy, renal and hepatic abnormalities can also be found. A combination of midline cerebellar vermis hypoplasia, deepened interpeduncular fossa, and thick, elongated superior cerebellar peduncles gives the axial view of the midbrain the appearance known as *molar tooth sign* (Fig 1), an obligate finding for JS diagnosis⁵⁻⁷.

Recently, Valente, Brancati and Dallapiccola⁶ proposed a clinical classification of JS in which the molar tooth sign was considered an obligatory criterion. They were able to recognize six subgroups of JS: (1) Pure Joubert syndrome; (2) JS with retinal abnormality; (3) JS with renal disorders; (4) CORS (cerebello-oculo-renal syndrome), or JS with retinal abnormality and kidney involvement; (5) COACH (cerebellar vermis hypoplasia/aplasia, oligophrenia, ataxia, ocular coloboma, and hepatic fibrosis) or JS with mental retardation, ocular coloboma and liver disorder; and (6) Oro-facio-digital syndrome type VI, or JS with orofacial abnormality and polydactyly.

Seven loci and five genes have been identified so far

Fig 1. Molar tooth sign (arrow) in axial RMI images, for a patient with Joubert syndrome.

(Table 2)⁶. It is believed that other loci and genes will be recognized in the future, as mutations in known genes account for only a small fraction of patients. There is no clear correlation between genetic and clinical in JS, nonetheless, *AHI1* mutations are usually associated with pure JS and approximately 50% of individuals with cerebello-oculo-renal syndrome have *CEP290* mutations^{6,7}. In large series, mutations in *AHI1* are found in 10 to 15% of cases, and of *CEP290* in 10%⁵.

Cerebellar hypoplasia associated with VLDL receptor

Cerebellar hypoplasia associated with very low density lipoprotein (VLDL) receptor (CHVR) is clinically characterized by severe developmental delay, hypotonia, global ataxia, flat feet, strabismus, and moderate to severe mental retardation^{8,9}. Epilepsy and short stature might occasionally be seen⁸. MRI discloses a symmetric cerebellar hypoplasia, mostly of its inferior segment, with variable brainstem and corpus callosum hypoplasia, and plain cortical gyri^{8,9}. This form of non-progressive cerebellar ataxia was first reported as disequilibrium syndrome among North-Americans Hutterites⁸. CHVR is caused by mutation in the gene that encodes VLDL receptor (*VLDLR*)⁹. This transmembrane protein is part of reelin signaling pathway, which guides neuroblast migration in the developing cerebellum and cerebral cortex⁹.

Table 2. Joubert syndrome identified loci, genes and their products.

Locus	Location	Gene	Protein
JBTS1	9q34.3	Not known	Not known
JBTS2	11p12-q13.3	Not known	Not known
JBTS3	6q23.3	<i>AHI1</i>	Joublerin
JBTS4	2q13	<i>NPHP1</i>	Nephrocystin-1
JBTS5	12q21.34	<i>CEP290</i> (<i>NPHP6</i>)	Nephrocystin-6
JBTS6	8q21.1-q22.1	<i>TMEM67</i>	Meckelin
JBTS7	16q12.2	<i>RPGRIPL</i>	Protein phantom

ATAXIAS CAUSED BY DEFICIENCY OF MITOCHONDRIAL ENERGY GENERATION

Ataxias caused by deficiency of mitochondrial energy generation includes Friedreich ataxia, Ataxia with CoQ10 deficiency, Mitochondrial recessive ataxic syndrome (MIRAS) and Infantile-onset spinal cerebellar ataxia (IOSCA).

Friedreich ataxia

Friedreich ataxia (FA) is the most common recessive ataxia worldwide, with an estimated prevalence in Caucasian population of 1:30,000 to 1:50,000¹⁰ and a carrier frequency of 1:85¹. Its onset is usually in the second decade of life, but can vary from 2 to 25 years of age. Clinical manifestations are characterized by a combination of sensory and cerebellar symptoms, and gait instability is usually the first recognized abnormality. Relentlessly progressive ataxia is characteristic, and after 10 to 15 years of onset, patients are usually wheelchair bound. Dysarthria is also an early and incapacitating symptom, leading to an almost incomprehensible speech. Vibratory and positional sense is affected, and Romberg sign is usually positive. Deep tendon reflexes are absent, but extensor plantar reflex (Babinski sign) is usually present. Abnormal eye movements and defective fixation are also observed. Cognitive function is preserved, but communication abilities can be affected. Systemic abnormalities as hypertrophic cardiomyopathy, cardiac conduction defects and diabetes can occur. As disease progresses, pes cavus and scoliosis are almost always present. Although there are significant variations in the onset and rate of disease progression, the mean age of death has been reported to be approximately 38 years, with a range as wide as 5 to 70 years. Death usually is secondary to progressive cardiomyopathy¹⁰.

Brain MRI in FA is normal; using the multigradient echo sequence it is sometimes possible to detect iron deposits in dentate nuclei of cerebellum. Spinal cord MRI can disclose mild atrophy of its cervical segment, which is explained by the large loss of primary sensory neurons in

the dorsal root ganglia, early in the course of the disease. Nerve conduction studies characteristically show axonal sensory neuropathy¹⁰. Atypical forms of FA, as late onset or with maintained reflexes, have been proposed, but it is now clear that they are also caused by mutations in the same gene.

FA is caused by mutations in the *FRDA* gene, which encodes frataxin, a protein involved in mitochondrial iron regulation. Loss of mitochondrial iron-sulfur centers, impairment of mitochondrial respiratory chain, increased mitochondrial iron and increased oxidative damage are observed when frataxin is deficient. Almost all patients are homozygotes for a GAA expansion which occurs in intron 1 of *FRDA* gene. Normal individuals have up to 40 GAA repeats, and in patients this number can vary from 70 or 90 to over 1,700 repeats. Presence of biallelic expansion confirms diagnosis, independent of clinical phenotype^{10,11}. Close to 2% of patients are compound heterozygotes, with a combination of a GAA expansion in one allele and a point mutation in the other¹.

Coenzyme Q10 (CoQ10), and its synthetic analog idebenone, vitamin E and, more recently the iron chelator deferiprone have been used for treating FA, with some promising but still very preliminary results^{12,13}. Deferiprone, an atypical iron chelator, may decrease accumulation of toxic iron in the mitochondria in patients, but the recommended dose and the efficiency of this treatment have not yet been determined¹².

Ataxia with coenzyme Q10 deficiency

Primary deficiency of coenzyme Q10 (CoQ10) is a genetically heterogeneous disorder, with a highly variable clinical spectrum, which includes multi-systemic manifestations as well as CNS compromise¹⁴⁻¹⁶. Five clinical subtypes have been recognized: (1) Encephalomyopathic, with mitochondrial myopathy, recurrent myoglobinuria and CNS symptoms and signs; (2) Early infantile multi-systemic, with severe visceral and brain manifestations; (3) Leigh syndrome; (4) Pure myopathic; (5) Ataxic^{14,15,17}.

The ataxic subtype is the most common presentation of CoQ10 deficiency^{14,15}. It is characterized by progressive ataxia, cerebellar atrophy and reduced muscle CoQ10^{14,15}. Early symptoms might include developmental delay, hypotonia and frequent falls. Global, progressive ataxia, and dysarthria start before the adolescence¹⁷. Epileptic seizures, proximal or distal muscle weakness, dysphagia, ophthalmoparesis, nystagmus, peripheral axonal neuropathy, pyramidal signs and scoliosis might also be present^{14,15,17}. Mental retardation or cognitive decline is also sometimes seen^{14,17}. The adult onset form of ataxia and CoQ10 deficiency is usually associated with hypergonadotrophic hypogonadism¹⁴.

CoQ10 (also known as ubiquinone) is a lipophylic com-

pound which is involved in electron transport from complex I and II to complex III of mitochondrial respiratory chain¹⁴⁻¹⁷. CoQ10 deficiency impairs proton transfer across the internal mitochondrial membrane and consequently to a reduction in ATP production^{14,16}.

The main source of CoQ10 is endogenous synthesis, which involves a still-uncharacterized complex pathway. Four genes are known to be involved in CoQ10 biosynthesis: *PDSS1* e *PDSS2* (subunits 1 and 2 of prenyldiphosphate synthase), *COQ2* (OH-benzoate polyprenyltransferase) and *ADCK3*, which acts as a chaperone^{14,16}.

Diagnosis is based on reduced amount of CoQ10 in muscle, as plasma CoQ10 levels are usually normal^{14,15,17}. Muscle histopathology is essentially normal and brain MRI discloses global cerebellar atrophy^{14,17}. Treatment with oral CoQ10 should be adjusted according to clinical results, with doses varying from 300 to 3000 mg/day^{14,17}. Treatment outcome is quite variable: in some patients disease stabilized while in others it progressed relentlessly. It is probable that treatment response is dependable of underlying biochemical defect as well as stage of disease^{14,15,17}.

Ataxia with mutation in polymerase gamma

Polymerase gamma (POLG) is a nuclear encoded gene, whose product is responsible for maintaining the integrity of mitochondrial DNA¹⁸. Mutations in POLG are associated with a variety of clinical phenotypes, which includes Alpers disease, parkinsonism, and external progressive ophthalmoplegia¹⁸. Two similar forms of autosomal recessive ataxias are associated with mutations in POLG: Mitochondrial Recessive Ataxic Syndrome (MIRAS) and Sensory Ataxia, Neuropathy, Dysarthria, and Ophthalmoplegia (SANDO)^{18,19}.

MIRAS is the most frequent recessive ataxia in Finland^{1,20}. Clinical manifestations, which start between 5 to 40 years of age, are characterized by cerebellar ataxia, nystagmus, dysarthria, ophthalmoplegia, tremor, cognitive decline, and myoclonus. Loss of vibratory and position perception is commonly seen^{19,20}. Epilepsy is a frequent manifestation in MIRAS, but not in SANDO, with both partial and generalized seizures, sometimes becoming refractory to antiepileptic drugs and evolving to status epilepticus¹⁸⁻²⁰. Brain MRI discloses cerebellar atrophy and T2 weighed hypersignal on thalamus, and dentate and inferior olivary nuclei^{19,20}. Nerve conduction studies also demonstrate axonal sensory neuropathy^{19,20}. Elevated protein might be detected in CSF^{19,20}. Muscle biopsy is not diagnostic, but Southern blotting analysis might detect multiple deletions in muscle mitochondrial DNA²⁰. Diagnosis is based on sequencing of the POLG gene, with two mutations (p.A467T and p.W748S) being responsible for most cases of this disorder in Caucasians^{19,20}. There is no clear genotype-phenotype in this condition.

Infantile-onset spinocerebellar ataxia

Infantile-onset spinocerebellar ataxia (IOSCA) is currently identified only in Finland and is characterized by acute or subacute cerebellar signs triggered by unspecific infection around the age of 1 year^{21,22}. Their clinical features are similar to MIRAS. Hypotonia, athetosis of hands and face, and ataxia with absent reflexes are the early symptoms this disease. Later at pre-school age, ophthalmoplegia and neurosensory deafness might be seen. Tactile, proprioceptive, and vibratory impairment, without pain or temperature compromise are detected after the first decade. Teenagers are usually wheelchair bound with severe distal muscular atrophy, pes cavus, mild to moderate cognitive impairment and optic atrophy without significant visual impairment. Refractory epilepsy and status epilepticus might contribute to rapid neurological deterioration and death. Further recognized abnormalities are autonomic dysfunction and, in females, primary hypogonadism^{21,22}.

There is no biochemical marker for IOSCA. Nerve conduction studies and nerve biopsy demonstrate a severe, mostly sensory, axonal neuropathy. Sensory ganglia are more severely affected than motoneurons^{21,23}. Neuroimaging studies at early stages of disease demonstrate reduced size of cerebellar hemispheres which progress to a more widespread olivopontocerebellar atrophy²³. Muscle biopsy is non-diagnostic but mitochondrial DNA depletion might be seen in this tissue. Pathological studies disclose spinal cord atrophy (more intense on the posterior funiculi), cerebellum and brainstem; there is also marked loss of myelinated fibers on peripheral nerve²¹.

IOSCA is caused by mutation in *C10ORF2* gene, which codes for twinkle, a specific mitochondrial DNA helicase, and one of its smaller isoform, twinkly. Twinkle is important for maintenance and replication of mitochondrial DNA, and twinkly function is currently unknown. The same founder mutation (p.Y508C) was detected in most Finnish patients with classical IOSCA. Mutations in *C10ORF2* might also be associated to different phenotypes, such as Alpers disease (early onset encephalopathy with untreatable epilepsy, mtDNA depletion and liver failure) and autosomal dominant progressive external ophthalmoplegia²².

METABOLIC ATAXIAS

Metabolic ataxias are treatable disorders, and it is particularly important to make an early and accurate diagnosis in this group of ARA. Ataxia with vitamin E deficiency, abetalipoproteinemia, hypobetalipoproteinemia, Refsum disease and cerebrotendinous xanthomatosis will be discussed in this section.

Ataxia with vitamin E deficiency

Ataxia with vitamin E deficiency (AVED) is similar to

Friedreich ataxia (FA). Age of onset usually varied from 4 to 20 years, with outliers ranging from 2 to 52 years^{24,25}. Clinical manifestations are characterized by progressive trunk and limbs ataxia, dysarthria, disturbance of positional and vibratory lower limbs senses, Babinski sign and abolished deep tendon reflexes. Scoliosis and pes cavus are commonly seen²⁴⁻²⁶, yet retinopathy is less frequent^{25,26}. Dystonia (13%) and head titubation (28%) are more commonly seen in AVED than in FA^{24,26}. Cardiomyopathy and an acute cardiac event might be associated with premature death among AVED cases^{25,26}.

AVED is caused by mutations in the α -tocopherol transfer protein gene (*TTPA*), which codes a protein responsible for transferring α -tocopherol from chylomicrons to VLDL^{26,27}. *TTPA* disfunction causes very low level of circulating α -tocopherol and tissue deficiency of this vitamin²⁷. Several different pathogenic mutations have been reported so far. Two mutations, associated with a severe phenotype, are particularly frequent in Europe, North Africa and North America: c.744delA and c.486delT^{24,25}. On the other hand, the mutation p.H101G was only detected in Japanese families and is associated with later onset and pigmentary retinopathy²⁴. Age of onset, clinical manifestations and progression velocity are quite variable in AVED. It is usually stated that mutations causing profound *TTPA* protein depletion are responsible for a more severe phenotype and mutations leading to amino acid substitution are associated to a milder form of disease²⁴⁻²⁶.

Diagnosis in a symptomatic individual is established with the determination of α -tocopherol (vitamin E) serum level, which is always below 2.5 $\mu\text{g/ml}$ (reference values: 5-15 $\mu\text{g/ml}$)^{24,25}. Brain MRI is usually normal, but mild cerebellar atrophy might also be seen^{1,26}. A pattern of axonal sensitive neuropathy is often observed at nerve conduction studies²⁴.

AVED treatment consists of vitamin E oral administration at a dose of 600 to 2,400 mg/day. Serum levels might be used as guidance for oral dose adjustment²⁴⁻²⁶. Differential diagnosis of α -tocopherol primary deficiency includes intestinal fat mal-absorption and abetalipoproteinemia²⁶. As a rule, vitamin E serum levels should be checked in all patients with FA clinical phenotype without molecular confirmation for this condition.

Abetalipoproteinemia and hypobetalipoproteinemia

Abetalipoproteinemia (ABL), a multisystem disorder caused by a defect in lipoprotein metabolism, is characterized by acanthocytosis, atypical pigmentary retinopathy and spinocerebellar degeneration^{28,29}. In the first year of life, main manifestations are chronic diarrhea and failure to thrive. Neurological features, present after the first decade of life, include absent deep tendon reflexes, superficial and deep sensory abnormalities, weakness and

global ataxia²⁹. As disease progresses, atypical pigmentary retinopathy characterized by small, irregularly distributed white spots, and night and color blindness is detected²⁹. Clinical manifestations of ABL are secondary to deficient absorption of the lipid-soluble vitamins A, D, E, and K^{1,28}.

Apolipoprotein B (ApoB) is the main protein of both VLDL and LDL, and their assembly is dependent on microsomal triglyceride transfer protein (MTP)²⁸. Mutations in the gene coding for the large (88 kD) subunit of MTP is responsible for ABL and determine very low levels of LDL and VLDL cholesterol. Decreased levels of vitamins A, K, and E, anemia, very low sedimentation rate, increased prothrombin time and elevated creatine kinase are also observed. Deficient MTP can also lead to lipid infiltration of small bowel mucosa and hepatic steatosis^{28,29}. Nerve conduction velocity usually discloses sensory axonal peripheral neuropathy^{1,29}.

ABL treatment is done with supplementation of vitamin A (100 to 400 IU/Kg/day), vitamin E (2,400 to 14,400 IU/day), and vitamin K (5 mg/day)²⁹. It is also recommended a low fat diet combined with essential fatty acid supplementation^{28,29}. Coagulation tests are used to monitor vitamin K and serum levels of vitamin A and E to check supplementation adequacy for these vitamins^{28,29}.

Hypobetalipoproteinemia (HBL) is similar to ABL. It is caused by mutations in *APOB* gene, which encodes apolipoprotein B (ApoB)². *APOB* heterozygotes have lower levels of ApoB, VLDL- and LDL-cholesterol, while MTP heterozygotes have normal levels of these substances^{2,29,30}.

Refsum disease

Refsum disease (RD) is a peroxisomal disorder clinically characterized by pigmentary retinopathy, cerebellar ataxia, mixed motor-sensory neuropathy and elevated CSF protein^{31,32}. Its onset usually occurs before 20 years of age, with night blindness secondary to retinopathy, followed by progressive constriction of visual field and optic atrophy, cataracts, vitreous opacities and nystagmus^{32,33}. Other common clinical manifestations are anosmia, cochlear deafness, ichthyosis, bone dysplasia and cardiac abnormalities³¹⁻³³. Psychiatric disorders are uncommon^{31,33}. If not adequately treated, RD can cause premature cardiac death^{31,32}.

Elevation of serum phytanic acid (>200 $\mu\text{M/L}$; reference value <30 $\mu\text{M/L}$) is very suggestive, but not specific of RD^{31,33}. Phytanic acid is a branched long chain fatty acid present on dairy products and red meat³³. It is a by-product of chlorophyll catabolism and is not endogenously synthesized³¹⁻³³. Diagnostic confirmation can be made measuring the activity of phytanoyl-CoA hydroxylase in fibroblasts or by molecular analysis of the responsible gene³¹.

RD is a genetically heterogeneous disorder. Most cas-

es are caused by mutations in *PHYH* (encoding phytanoyl-CoA hydroxylase), a peroxisomal matrix enzyme which catalyses β -oxidation of branched chain fatty acids³¹⁻³³. Deficiency in *PEX7* (encoding peroxin-7), a protein involved in peroxisomal import of some enzymes, including phytanoyl-CoA hydroxylase, also results in this phenotype^{2,31}. *PEX7* mutations may also cause a severe peroxisomal biogenesis disorder known as rhizomelic chondrodysplasia punctata³².

Treatment is based on phytanic acid dietary restriction, combined, if necessary, with plasmapheresis. With reduction of phytanic acid serum levels, RD symptoms stabilize and there may be some improvement of ataxia and ichthyosis, albeit effects on pigmentary retinopathy are uncertain^{32,33}.

Cerebrotendinous xanthomatosis

Cerebrotendinous xanthomatosis (CTX) is a rare bile acid synthesis disorder³⁴. Its main clinical manifestations are juvenile cataracts, chronic diarrhea, and tendinous xanthomas³⁴⁻³⁶. In the neonatal period, a potentially lethal cholestatic syndrome has been reported³⁴. After the second decade of life, progressive neurological deterioration may occur, characterized by cognitive decline, psychiatric manifestations, cerebellar ataxia, progressive spastic paraplegia, dysphagia, and less frequently, seizures and peripheral neuropathy^{34,36,37}. Exceptionally, neurological manifestations are restricted to the spinal cord³⁵. There is a wide intra- and inter-familial clinical variability^{34,37}. Coronary heart disease without elevated cholesterol is an important cause of morbidity and mortality in adults^{34,35}.

CTX is caused by mutations in *CYP27A1* gene, which codes for sterol 27-hydroxylase, a protein expressed mostly in liver^{34,36}. This enzyme is essential for bile acids synthesis, including chenodeoxycholic acid³⁶. In the absence of sterol 27-hydroxylase, the substrate of this enzyme is converted to cholestanol by the action of 7 α -hydroxylase^{34,36}. Elevated serum cholestanol is the biochemical hallmark of CTX^{35,37}. Increased urinary excretion of bile alcohol glucuronides might also be present³⁷. This disorder can be treated by oral administration of chenodeoxycholic acid, which inhibits 7 α -hydroxylase and consequently cholestanol synthesis³⁴⁻³⁷. Liver transplant is another therapeutic alternative³⁴. Cholestanol determination in asymptomatic siblings of CTX patients is recommended to improve clinical outcome^{34,37}.

Brain MRI most distinctive abnormalities are detected at T2-weighted and FLAIR sequences, which demonstrate bilateral, heterogeneous and hyperintense sign in dentate nuclei and adjacent cerebellar white matter (Fig 2)^{35,36}. Other less characteristic abnormalities which might be detected are brain stem, cerebellar and cerebral atrophy and diffuse hyperintense cerebral white matter lesions^{35,36}.

Fig 2. Cerebellar atrophy and hyperintense sign in dentate nuclei and adjacent cerebellar white matter on T2-weight RMI images (arrow), for a patient with cerebrotendinous xanthomatosis.

MR spectroscopy (MRS) of CTX patients discloses reduction of N-acetylaspartate and increase in lactate³⁶.

ATAXIAS WITH DNA REPAIR DEFECTS

This group has as a common pathogeny a defect in double or single strand DNA repair; besides ataxia, extrinsic ocular movements are frequently affected. Ataxia-telangiectasia, ataxia telangiectasia-like, apraxia and oculomotor apraxia types 1, 2 and 3, and spinocerebellar ataxia with axonal neuropathy type 1 belong to this group.

Ataxia-telangiectasia

Ataxia-telangiectasia (AT) has an estimated frequency in the USA of 1/40,000 individuals³⁸ and it is predicted that 0.5% of UK population carries one mutation in *ATM* gene, which is responsible for AT³⁹. Progressive ataxia with onset before 3 years of age is the main clinical characteristic^{38,40}. Telangiectasias (Fig 3), another hallmark of disease, are seen in at least 90% of affected individuals and their age onset ranges from 2 to 8 years; they are more easily seen in conjunctivas, ears, face and neck^{38,40,41}. A large range of ophthalmological abnormalities might also be detected, including: optokinetic nystagmus (present in 81% of cases), gaze induced nystagmus (seen in 29%), hypometric or delayed saccades (76%), delayed eye tracking (63%), strabismus (38%), and oculomotor apraxia (30%)⁴¹. Dysarthria, dysphagia, facial hypomimia, generalized hypotonia, peripheral neuropathy and movement disorder, as tremor or choreoathetosis, are seen after five years of age^{38,40,41}. Cognitive level is usually normal, even though

Fig 3. Ocular telangiectasia for a patient with ataxia telangiectasia patient.

severe dysarthria and incoordination might leave an impression of mental retardation. Independent gait is lost by the end of first decade^{38,40}. Immunodeficiency (mainly humoral) leading to chronic sinopulmonary infection and increased susceptibility to cancer are other important features in AT. Risk of lymphoproliferative disorders are dramatically increased in AT^{38,40}. Treating cancer in AT patients is particularly challenging, because of their increased radiosensitivity and adverse side effects to chemotherapy⁴⁰. Female carriers of one mutated copy of the gene have 3–4 fold increased risk of breast cancer when compared to general population³⁹.

ATM gene encodes for a ATM serine/threonine kinase, a large protein with 3,056 amino acids which is part of the phosphatidylinositol-3-kinase (PI3-K) complex, responsible for DNA repair during the cell cycle, avoiding incorporation of deleterious mutations^{38,39}. ATM gene is very large, containing 66 exons, and its sequencing with current technology is still cumbersome, but feasible^{38,39}. Most patients are compound heterozygotes for ATM mutations and a large variety of sequence variants have been recognized, thus making interpretation of results difficult. Pathogenic mutations are usually nonsense mutations (85%), and missense mutations are responsible for 10% of detected pathogenic changes^{38,39,40}.

Some laboratory tests might help AT investigation: serum alpha-fetoprotein is elevated in more than 95% of cases and low levels of IgA, IgE and reduced T lymphocyte count, with normal or elevated B lymphocytes are usually detected. Karyotype might show translocation between chromosomes 7 and 14³⁸⁻⁴¹ and radiosensitivity test might be used to demonstrate chromosomal breakage predisposition.

Brain MRI discloses, except in early stages of disease, cerebellar atrophy, which is initially more evident on the hemispheres and superior vermis and later becomes dif-

fuse³⁸. CT-scan and plain radiography should be avoided because of increased X-ray sensitivity.

Ataxia-telangiectasia like

Ataxia-telangiectasia like (ATL) is a very rare clinical condition which is characterized by slowly progressive ataxia with onset between 1 and 7 years of age, associated to oculomotor apraxia and dysarthria⁴². No ocular or facial telangiectasias are detected and cognition is preserved^{39,42,43}. Reflexes might be initially brisk and became reduced⁴². At advanced stages of disease, tongue and facial dyskinesia, choreoathetosis, and dystonia, suggesting basal ganglia compromise, might be seen^{42,43}. ATL is progressive up to the adolescence, when it stabilizes⁴³. There is no increased risk for infections or neoplasias, as is seen in AT, but occasionally microcephaly is present^{39,42}.

Brain MRI detects cerebellar atrophy, and laboratory tests are non-informative⁴³. Radiosensitivity test is usually present but in a lesser degree than in AT^{35,42-44}.

ATL is caused by mutation in MRE11 gene, located in chromosome 11q21, near the ATM gene. Its product is part of the MRN complex, which recognizes DNA double strand breakage. Both missense and null mutations have been reported. Severity varies according to the type of molecular defect. Most of reported cases were original from Saudi Arabia^{39,42-44}.

Ataxia with oculomotor apraxia type 1

Ataxia with oculomotor apraxia type 1 (AOA1) is a condition characterized by involuntary movements (chorea and dystonia) and/or progressive global ataxia, with dysarthria associated with hands and head tremor. Onset can vary between 1 to 20 years of age and developmental delay might be seen before clinical symptoms became apparent. As disease progresses, movement disorder are attenuated and peripheral neuropathy signs, as distal atrophy,

pes cavus, superficial and deep sensory impairment, hypo/ areflexia, become apparent. The most distinctive clinical signs in AOA1 are related to external eye movements: gaze-evoked nystagmus (found in all patients), oculomotor apraxia (seen in 86%), saccadic pursuit, hypometric saccades, fixation instability, and excessive blinking⁴⁵⁻⁴⁷. In advanced stages, oculomotor apraxia might be masked by progressive external ophthalmoparesis, which starts with upward gaze paralysis⁴⁵. Optic atrophy and retinal exudative lesions have been occasionally reported (Barbot, 2001; Le Ber, 2003). Variable cognitive impairment might be seen and mental retardation is not uncommon^{46,47}.

Laboratory findings include hypoalbuminemia and hypercholesterolemia^{46,47}. Elevated creatine kinase is occasionally detected⁴⁷. Nerve conduction velocity studies disclose sensory-motor axonal neuropathy. MRI reveals marked cerebellar atrophy, mild brainstem atrophy and, in advanced cases, cortical atrophy⁴⁵⁻⁴⁷. Loss of myelinated fibers with maintenance of amyelinic ones is seen at sural nerve^{45,47}.

AOA1 is caused by mutation in *APTX* gene, which encodes aprataxin, a nuclear protein involved in single-strand DNA repair, acting in the same pathway of the ATM protein^{39,46}. Several mutations have been reported so far, most of them in exons 5, 6, and 7 of *APTX* gene⁴⁷. This condition was originally reported in Japan (where it is the most common cause of ARA) but is found worldwide. In Portugal, AOA1 is the second most common cause of ARA⁴⁶.

Ataxia with oculomotor apraxia type 2

Ataxia with oculomotor apraxia type 2 (AOA2) is characterized by global progressive ataxia with onset usually between 8 and 25 years of age^{48,49}, dysarthria, axonal motor sensory neuropathy, and oculomotor apraxia, which is seen in less than 50% of cases⁴⁸⁻⁵⁰. Saccadic pursuit is seen in all patients, gaze evoked nystagmus in 89%, and bilateral limited abduction of the eyes with strabismus in 61% of the patients⁴⁸. Dystonia, head and postural tremor, chorea, dysphagia, pes cavus, and scoliosis are occasionally seen. Cognitive function is usually preserved, but executive dysfunction is sometimes observed⁴⁸⁻⁵⁰. Premature ovarian failure was also reported in some patients⁴⁸. Progression is slow, and most patients are wheelchair bound 10 years after its onset^{48,49}.

Serum alpha-fetoprotein is mildly to moderately elevated in all patients with AOA2^{39,48-50}. Increased serum creatine kinase, cholesterol, and immunoglobulin IgG and IgA, and reduced serum albumin are inconstantly seen^{48,50}. Brain MRI discloses diffuse cerebellar atrophy, more intense in the vermis, occasionally associated with pontine atrophy⁵⁰. Nerve conduction studies detect sensory-motor axonal neuropathy and nerve biopsy demonstrates that large myelinated fibers are more severely affected than thin ones^{48,50}.

AOA2 is caused by mutation in *SETX* gene (encoding senataxin), a protein with DNA and RNA helicase activity and which is involved in RNA processing and DNA repair^{39,49,50}. Amyotrophic lateral sclerosis type 4 (ALS4), is caused by dominant mutations in senataxin⁵⁰.

Ataxia with oculomotor apraxia type 3

Ataxia with oculomotor apraxia type 3 (AOA3) is a recently described ARA with a phenotype similar to ataxia-telangiectasia, but with onset after 8 years of age. Reported clinical features are ataxic gait, dysarthria, oculomotor apraxia and cerebral atrophy. No telangiectasia, biochemical abnormalities, or nerve conduction impairment was detected. Other forms of ataxia with oculomotor apraxia were excluded. Studies performed in fibroblasts demonstrated a defect in repairing DNA, making these cells sensitive to agents that cause single strand breaks in DNA. Nevertheless, locus for AOA3 remains elusive⁵¹.

Spinocerebellar ataxia with axonal neuropathy type 1

Spinocerebellar ataxia with axonal neuropathy type 1 (SCAN1) is a rare disorder recognized in 2002 in a large consanguineous family of Saudi Arabia⁵². Age of onset is around 14 years, characterized by moderate ataxia, dysarthria, muscular weakness, distal atrophy, pes cavus and reduction of vibratory and postural sense. Epilepsy may occur, but there is no cognitive decline or oculomotor abnormality. Nerve conduction studies disclose motor-sensory axonal neuropathy and biochemical tests are non diagnostic, but low serum albumin and elevated cholesterol are occasionally seen⁵². Mild cerebellar and cerebral atrophy might be present on MRI studies. SCAN1 is caused by mutation in *TDPI* gene, which codes for tyrosyl DNA phosphodiesterase 1 (TDPI), a protein involved in single strand DNA repair^{39,52,53}. SCAN1 is an additional example of nervous system vulnerability to impaired DNA repair, as occurs in AOA1, AOA2 and AT^{52,53}.

DEGENERATIVE ATAXIAS

Degenerative ataxias have as a common feature the compromise of a protein that acts as a chaperone, helping protein folding. Two conditions belong to this group: autosomal recessive ataxia of Charlevoix-Saguenay and Marinesco-Sjögren syndrome.

Autosomal recessive spastic ataxia of Charlevoix Saguenay

Spastic ataxia of Charlevoix-Saguenay (SACS) was first reported in Charlevoix and Saguenay region of northeast Quebec province, Canada^{1,54}. In this area, its incidence was estimated in 1/1,932 newborns, and 1 in every 22 of its inhabitants are supposed to be carrier of the mutation responsible for SACS^{2,55}. This condition has now been re-

Table 3. Clinical characterization and diagnostic exams of the autosomal recessive ataxias.

	CA	JS	CHVR	FA	ADCQ	APGM	IOSCA	AVED	ABL	RD	CTX	AT	AT like	AOA1	AOA2	SCANI	SACS	MSS
Age at onset [†]	<1	<1	<1	>5	<20	>5	<2	2-52	<20	<20	1-36	<5	1-7	<20	8-22	13-15	1-10	<20
Psychomotor delay	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	+	+
Hypotonia	+	+	+	-	+	-	+	-	-	-	-	+	-	+	-	-	-	+
Head titubation	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-
Ocular alterations	N	N, OA, OS RP, VD	S	N, GI	VD, N, OS, O	N, O, OS	VD, O	RP, VD	N, RP	RP, N, VD	C	N, OA, OS, S	N, OA, S	N, OA, O, GI, OS	N, OA, OS, S	-	N, OS	N, S, C
Sensory neuropathy	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Distal weakness	-	-	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
Deep tendon reflexes	NL	NL	NL or ↑	↓	↓, NL or ↑	↓	↓	↓	↓	↓	↓ or ↑	↓	↓ or ↑	↓	↓	↓	↑	↓
Spasticity	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	+
Babinski sign	-	-	+	+	+	-	-	+	+	-	+	-	-	-	+	-	+	-
Pes cavus	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-
Extrapyramidal signs	-	-	-	-	-	T, M	Ch	T, D	-	-	M, D	T, D, Ch	D, Ch	T, D, Ch	T, M, D, Ch	-	-	-
Psychiatric problems	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Cognitive impairment	-	+	+	-	+	+	+	-	-	-	+	-	-	+	-	-	+	+
Epilepsy	-	-	+	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+
Hearing loss	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	+
Cardiomyopathy	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-
Diabetes	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Radiosensitivity	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Skeletal deformities	-	-	-	+	+	-	-	+	-	+	-	-	-	+	+	-	-	+
Renal failure	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver alterations	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
Nerve conduction studies	NL	NL	NL	AS	NL	ASM	AS	AS	AS	DSM	ASM	ASM	ASM	ASM	ASM	ASM	ASM	DSM
MRI brain	Ca	Ca	Ca	NL	Ca	Ca, WM	Ca	NL	NL	NL	Ca, WM	Ca	Ca	Ca	Ca	Ca	Ca	Ca, WM
MRI spinal cord atrophy	-	-	-	+	-	-	+	-	lipo↓	phytan. acid ↑	choles	Ig↓	-	alb↓	α-eto ↑,	alb↓	+	CPK↑
Laboratorial exams	-	-	-	-	coeQ↓ muscle	-	-	vitE↓	vitE↓	acid ↑	tanol ↑	α-feto ↑	-	choles	choles	terol ↑	-	-
Treatment	-	-	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-

-: absent or uncommon; +: may be present; ↑: increased; ↓: reduced or absent; †: most frequently onset age; ABL: abetalipoproteinemia; ACQD: ataxia with coenzyme Q10 deficiency; AOA1: ataxia with oculomotor apraxia type1; AOA2: ataxia with oculomotor apraxia type2; APMG: ataxia with mutation in polymerase gamma; AS: axonal sensory neuropathy; ASM: axonal sensorimotor neuropathy; AT: ataxia telangiectasia; ATlike: ataxia telangiectasia like disorder; AVED: ataxia with vitamin E deficiency; C: cataracts; Ca: cerebellar atrophy; Ch: chorea or choreoathetosis; CHVR: cerebellar hypoplasia associated to VLDL receptor; CTX: cerebrotendinous xanthomatosis; D: dystonia; DSM: demyelinating sensorimotor neuropathy; FA: Friedreich ataxia; GI: gaze fixation instability; IOSCA: infantile onset spino cerebellar ataxia; JS: Joubert syndrome; M: myoclonus; MSS: Marinesco Sjögren syndrome; NL: normal; O: ophthalmoplegia; OA: oculomotor apraxia; OS: ocular saccadic impairment; RD: Refsum disease; RP: retinitis pigmentosa; S: strabismus; SACS: spastic ataxia of Charlevoix Saguenay; SCANI: spino cerebellar ataxia with axonal neuropathy type1; T: tremor; VD: visual deficiency; WM: white matter changes.

*Spastic legs. ABL: abetalipoproteinemia; ACQD: ataxia with coenzyme Q10 deficiency; AD: autosomal dominant; AVE: ataxia with vitamin E deficiency; AOA1: Ataxia with oculomotor apraxia type 1; AOA2: Ataxia with oculomotor apraxia type 2; APMG: ataxia with mutation in polymerase gamma; ARA: autosomal recessive ataxia; AS: axonal sensory neuropathy; ASM: axonal sensorimotor neuropathy; AT: ataxia-telangiectasia; ATlike: ataxia telangiectasia like disorder; CA: Cayman ataxia; CHVR: Cerebellar hypoplasia associated to VLDL receptor; CTX: cerebrotendinous xanthomatosis; DSM: demyelinating sensorimotor neuropathy; FA: Friedreich ataxia; IOSCA: Infantile onset spinocerebellar ataxia; JS: Joubert syndrome; MSS: Marinesco Sjögren syndrome; N: nystagmus; OA: oculomotor apraxia; RD: Refsum disease; SA: saccades abnormalities; SACS: Spastic ataxia of Charlevoix Saguenay; SCAN1: Spinocerebellar ataxia with axonal neuropathy type 1

Fig 4. Algorithm for diagnosis of the mains autosomal recessive ataxias.

ported worldwide, but the largest series remains from Canada^{1,54-56}.

Clinically, SACS is characterized by delay in acquiring independent walk, frequent falls and gait instability⁵⁵. Disease progression is slow and ataxic gait; dysarthria and spastic paraplegia are the major manifestations in the first two decades. Later, lower limb peripheral neuropathy can also be detected. As disease evolves, pyramidal signs can be masked by progression of peripheral neuropathy, with the exception of the Babinski sign, which is usually present even in later stages of disease. Distal atrophy, pes cavus, and hammer toes are commonly seen as disease advances⁵⁴⁻⁵⁶. In some patients, funduscopy discloses hypermyelination of fibers radiating from optic disk and embedding the retinal vessels, a very peculiar finding. Horizontal nystagmus, saccadic alteration of smooth ocular pursuit and micturition urgency might be present^{54,55}. Mild mental retardation and cognitive decline were occasionally reported⁵⁴. Patients usually become wheelchair bound after the 3rd or 4th decades of life and life expectancy is reduced as they become bedridden. During pregnancy, disease progression is apparently accelerated in affected women⁵⁵.

Nerve conduction velocity studies usually disclose an axonal neuropathy with mild demyelination, sensory fibers are more severely affected than motor fibers. The most consistent neuroimaging finding is cerebellar vermis atrophy, mostly from its superior portion⁵⁴⁻⁵⁶. Cervical and thoracic spinal cord thinning are occasionally reported⁵⁵.

At an early stage, SACS can be misdiagnosed as cerebral palsy⁵⁵. Diagnosis is based on clinical manifestations and confirmed by mutation analysis of SACS gene located on 13q11^{54,56}. Putative role of its product, saccin, is to help protein folding, acting as a chaperone⁵⁶. How saccin deficiency causes neurodegeneration it is not known, but it has been reported to interact with Ataxin-1, the cause of Autosomal Dominant Spinocerebellar Ataxia type 1⁵⁶.

Marinesco-Sjögren syndrome

Marinesco-Sjögren syndrome (MSS) is a rare, multisystem disorder, characterized by congenital or early-onset cataracts, developmental delay, cerebellar ataxia and mild to severe mental retardation. Microcephaly, nystagmus, short stature, scoliosis, hypergonadotrophic hypogonadism and myopathy are common additional features^{57,58}. Peripheral neuropathy, deafness, optic atrophy, strabismus, spasticity and seizures might be present⁵⁷. Disease progression is slow and long survival can be expected².

Brain MRI usually discloses cerebellar atrophy or hypoplasia. Additional uncommon findings are cortical atrophy and leukoencephalopathy. Serum creatine kinase is usually elevated and muscle biopsy shows chronic myopathy with rimmed subsarcolemmal vacuoles^{57,58}.

MSS is caused by mutations in *SIL1* gene (encoding a nucleotide exchange factor for heat-shock protein 70 family member HSPA5). Heat-shock protein 70 family members are the highly conserved molecular chaperones that assist in stabilization and folding of newly synthesized polypeptides. Decrease of *SIL1* gene product leads to a reduction of protein synthesis in endoplasmic reticulum^{58,59}.

CONCLUSION

Differential diagnosis of ARA is a difficult task, as there is a clear overlap of clinical manifestations among several previously discussed conditions. Table 3 presents the main characteristics of each these disorders and Fig 4 is a proposed algorithm to help investigation of this group of diseases. Nevertheless, we should be aware that ataxia might be a symptom in many other progressive disorders, affecting primarily white matter (e.g., metachromatic leukodystrophy, leukoencephalopathy with vanishing white matter, Paelizeus-Merzbacher disease, X-linked adrenoleukodystrophy), neurons (e.g. neuronal lipofuscinosis ceroid, juvenile Tay-Sachs disease), or leading to a more widespread brain malformation or systemic manifestations, as it happens in pontocerebellar hypoplasia and congenital disorders of glycosylation (CDG). Non progressive cerebellar symptoms are also prominent in ataxic cerebral palsy, an important differential diagnosis for early-onset ARA. It is also important to remind that all spinocerebellar ataxias (SCAs) inherited as a dominant trait are out of the scope of this review.

Currently, neuroimaging studies, especially brain MRI, are particularly important in ARA work-up and to help detection of cerebellar malformations and atrophy. Normal MRI is expected in some disorders, as FA and AVED. Nerve conduction velocity studies and, in some cases, electromyography are useful for evaluation of some patients, even in the absence of clinical signs of peripheral neuropathy or myopathy. Elevation of serum alpha-fetoprotein is characteristic of AT and AOA2. More specific biochemical tests, as determination of vitamin E and phytanic acid should not be neglected, once they can help the diagnosis of some treatable forms of ARA. Determination of serum cholestanol and muscle CoQ10 are performed in a few specialized centers around the world but both CTX and ataxia with CoQ deficiency are potentially treatable.

Molecular analysis access is limited, but it is feasible for diseases as FA and, in lesser degree, AOA1 and AOA2. Many patients with putative ARA remain undiagnosed, and is expected that new forms of ARA will be recognized in the near future. The number of recessive ataxias is already high, and we will probably keep counting new arrivals for the forthcoming years.

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