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JOSÉ ERITON GOMES DA CUNHA

DESENVOLVIMENTO DO BANCO DE DADOS EPIDEMIOLÓGICO E NOVA ABORDAGEM DE DIAGNÓSTICO PARA DOENÇAS PRIÔNICAS

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RESUMO

As Doenças Priônicas, também conhecidas como Encefalopatias Espongiformes Transmissíveis (EET), fazem parte de um grupo de doenças raras e invariavelmente fatais e incidem em diversos animais, incluindo o homem, e são ocasionadas pelo acúmulo de uma proteína insolúvel denominada *Príon*Scrapie (PrP^{sc}). Fazem parte deste grupo a Encefalopatia Espongiforme Bovina (EEB), *CronicWastingDisease* (CWD) nos cervídeos, *Scrapie* em ovinos e nos humanos Doença de *Creutzfeldt-Jakob*(DCJ), *Kuru*, *Gerstmann-Straussler-ScheinkerDisease* (GSS) e Insônia Familiar Fatal. Uma forte característica das EET é a alta heterogeneidade clínica e histopatológica, tendo como o códon 129 da proteína *príon* o maior fator para esta variabilidade. Por conta desta variabilidade e a falta de biomarcadores determinantes para as Doenças Priônicas, o estudo clínico dos casos se torna crucial para o diagnóstico diferencial. Nesta tese, criamos um banco de dados online, o epiCJD, para centralizar informações epidemiológicas a fim de orientar médicos e servir como apoio aos gestores de saúde. Utilizamos os registros dos casos até então coletados pelo Centro de Doenças Priônicas da Alemanha. Além disso, foram analisados Aβ42 e a proteína Tau através de eletroquimioluminiescência (MSD) como possíveis biomarcadores para diagnóstico diferencial. Após a análise dos dados, observamos que a distribuição geográfica dos registros não indicam *clusters* ou *hotspots*, obedecendo basicamente o tamanho populacional. A distribuição de acordo com o gênero não difere de outras populações descritas na literatura e os primeiros sintomas são compatíveis com os descritos previamente, bem como a idade média do inicio da doença. A distribuição genotípica do códon 129 está de acordo com a literatura. Quanto ao MSD, observamos que a quantificação foi ligeiramente superior nos níveis de TAU e significativamente superior Aβ42 em relação aos controles, DA e DCJ. Baixos níveis de Aβ42 e alto nível de TAU se mostraram características em DA e DCJ, comparado aos controles. Como perspectivas futuras, o banco de dados será alimentado por outros dados, além dos provenientes da Alemanha, a fim de observar se há ou não variações entre outras populações. A técnica MSD se mostrou promissora podendo, depois de melhoramentos, ser uma ferramenta de diagnóstico molecular.

Palavras chaves: BANCO DE DADOS. DOENÇA PRIÔNICA.*PRÍON*. TAU. Aβ42

ABSTRACT

Prion diseases, or Transmissible Spongiform Encephalopathies (TSEs), are part of a group of rare diseases and invariably fatal and focus on various animals, including humans, and are caused by the accumulation of insoluble protein called Prion Scrapie (PrP^{sc}). Bovine Spongiform Encephalopathy (BSE), CronicWastingDisease (CWD) in deer, scrapie in sheep and Creutzfeldt-Jakob disease (CJD), Kuru, Gerstmann-Straussler-ScheinkerDisease (GSS) and Fatal Familial Insomnia, in humans, are example of TSEs. A strong feature of the TSE is the high heterogeneity clinical and histopathological, with the codon 129 of the prion protein the biggest factor for this variability. Because of this variability and lack of biomarkers to determine the prion diseases, the clinical study of cases becomes crucial for the differential diagnosis. In this thesis, we have created an online database, epiCJD, to centralize epidemiological information in order to guide physicians and serve as support for public health managers. We use records from German Prion Disease Center. Furthermore, Ab42 were analyzed and Tau protein by electrochemiluminescence-based detection system (MSD) as biomarkers for the differential diagnosis. The distribution according to gender does not differ from other populations described in the literature and the first symptoms are consistent with those described previously, as well as the average age of onset of the disease. The genotype distribution of the 129 codon is in agreement with the literature. As for the MSD, we noted that the quantification was slightly higher levels of TAU and Ab42 significantly higher compared to controls, AD and CJD. Low levels of Ab42 and high TAU proved characteristics in AD and CJD, compared to controls. As future prospects, the database will be fed by other data, in addition to from Germany, in order to observe whether there are variations among other populations. MSD technique proved promising and may, after improvements, to be a molecular diagnostic tool.

Key Words: DATABASE. PRION DISEASE. PRION. TAU.Aβ42

Sumário

1 INTRODUÇÃO

1.1 Doenças Priônicas

As Doenças Priônicas (PrD) ou Encefalopatias Espongiformes Transmissíveis (EET) são desordens neurológicas raras que acometem tanto humanos quanto outros mamíferos e são, até o momento, invariavelmente fatais. Fazem parte deste grupo a Encefalopatia Espongiforme Bovina (EEB), *CronicWastingDisease* (CWD) nos cervídeos, Scrapie em ovinos, Doença de Creutzfeldt-Jakob (DCJ), Kuru, *Gerstmann-Straussler-ScheinkerDisease* (GSS), Doença priônicas sensível à protease (VPSP) e Insônia Familiar Fatal (IFF) nos humanos.

Através da crescente divulgação na mídia de casos em torno dessas doenças, percebemos um aumento no interesse sobre o entendimento das Doenças Priônicas, nem sempre é acompanhado de informações corretas, tomamos, por exemplo, a insistência em denominar em casos humanos a doença do "mal da vaca louca", ou mesmo de informações acessíveis, visto que familiares de pacientes muitas vezes se veem diante de longos textos técnicos e em inglês, possibilitando, assim, a permanência do mal entendimento sobre aquelas doenças.

Para um melhor entendimento das Doenças Priônicas, devemos primeiro entender o "agente etiológico" e o mecanismo de ação deste nessas doenças. (BROWN; MASTRIANNI, 2010)·(LIBERSKI, 2012)

1.2 "Agente etiológico" das Doenças Priônicas: a proteína *Príon*

Para um melhor esclarecimento tanto sobre o "agente etiológico" quanto aos mecanismos que deflagram as EET devemos nos ater a um breve relato histórico, uma vez que esses aspectos transcendem o que era visto como dogma até 1982, quando Stanley B. Prusiner publicou o seu trabalho *Novel ProteinaceousInfectiousParticles Cause Scrapie.*(PRUSINER, 1982)

Por séculos, fazendeiros relataram uma doença em caprinos e ovinos na qual notavam mudança de comportamento, irritabilidade e instabilidade motora, entre outros sinais neurológicos. Essa desordem ganhou diversos nomes dependendo do país, sendo mais conhecida como *scrapie*.

Em 1732 foi relatada uma epidemia de *scrapie* em ovelhas merino espanhola,desencadeando no Reino Unido uma série de medidas para se descobrir o agente etiológico desta enfermidade que causava tantos prejuízos econômicos, mas sem sucesso. (LIBERSKI, 2012)Nessa época eram inexistentes vários dos conceitos atuais de bioquímica e microbiologia usados comumente para definir a relação de causa e efeito em uma doença. Antoine Fourcroy ainda desenvolvia estudos básicos sobre gelatina e albumina enquanto GerhardusJohannes Mulder caracterizava bioquimicamente uma substância fundamental a todos os seres vivos, batizada por Jakob Berzelius como proteína (do grego proteus, que significa de grande importância). (ZABEL; REID, 2015)

A *scrapie*, entre outras características, possui um longo período de incubação, levando à criação do termo "*slow vírus*" para explicar o possível agente para a enfermidade dos ovinos.

Em 1944, W. S. Gordon tratou o tecido de animais infectados com formalina, que inativa ácidos nucléicos, e posteriormente injetou em animais sadios, os quais, mesmo assim, contraíram e morreram decorrentes da*scrapie*.(ZABEL; REID, 2015)

Por volta de duas décadas antes de Gordon, Alfons Maria Jakob e Hans Gerhard Creutzfeldt, independentemente, relataram uma nova enfermidade neurológica cunhada por Spielmier, e em 1921 passou a ser denominada de Doença de Creutzfeldt-Jakob. Curiosamente, o próprio Creutzfeldt declarou que seus casos nada tinham a ver com os de Jakob. (WILL, 2003)'(CREUTZFELDT; JAKOB, 1998)

Em 1957, o médico e antropólogo Carleton Gajdusek e VinZigas investigaram as causas de uma encefalopatia que atingia o grupo étnico Fore, de Papua Nova Guiné, denominada de Kuru. Gajdusek e Zigas mostraram que o Kuru estava relacionado ao ritual de canibalismo realizado por aquele povo. Dois anos depois, W. J Hadlow mostrou a relação entre Kuru e Scrapie e propôs infectar chimpanzés com extratos de cérebros humanos de pacientes que morreram de Kuru para comprovar seu caráter infeccioso. Gajdusek e

Alpersrealizaram o experimento recomendado por Hadlow com êxito e comprovaram que Kuru poderia ser transmitido através de inoculação intracerebral. (HADLOW, 1959)

Em 1967, novos estudos demonstravam que o agente da *scrapie* não era inativado por procedimentos que usualmente desestabilizava ácidos nucléicos, como raios ionizantes. Para Alpers, o agente era desprovido ou teria uma porção diminuta de DNA, como um viróide. (MANUELIDIS et al., 2007)

Griffith, Parttinson e Jones, estes dois últimos com trabalhos em conjunto, propuseram que esse "agente etiológico" da *scrapie*poderia ter natureza proteica, mas não conseguiram demostrar qual era essa proteína. Como já mencionado, foi Stanley B. Prusiner que identificou esta proteína dando-a o nome de *Príon*, surgindo assim a teoria conhecida como "*protein*only" e que seria agraciada com o prêmio Nobel de 1997. (POSER, 2002)

A noção de um agente infeccioso, com longo tempo de incubação, poder ser uma proteína, foi visto quase como uma heresia por muitos biólogos moleculares. Em bem da verdade, até os dias atuais alguns grupos contestam essa visão, atribuindo as EET a uma co-infecção com um vírus.

Para muitos pesquisadores essa *príon* seria sintetizada a partir de algum vírus ou viróide, levando a uma resposta do organismo e desencadeando as doenças. Entretanto, em 1985, Weissmann e colaboradores apontaram que a *príon* era sintetizada, em humanos, por um gene situado no braço curto do cromossomo 20, sendo denominado de PRNP. A proteína gerada por esse gene possui de 33 a 35 KDa, enquanto a encontrada nos estratos da *scrapie* tem 27-30 KDa. A partir de então, os estudos demostraram que a *príon* possui duas isoformas, uma denominada *príon* celular (PrP^{c}, PrP^{sen} ou apenas PrP) e o seu "estado *scrapie*" (PrP^{sc}, PrP^{res}, PrP^{d}).(WEISSMANN; FLECHSIG, 2003)

A *príon* celular, PrP^{c}é codificada, em humanos, pelo gene PRNP, localizado no braço curto do cromossomo 20. A PrP^{c} possui 3 α-hélices na região globular (correspondente aos resíduos 144-154; 173-194; 200-228) e uma folha ß-antiparalela (128-131 e 161-164). A PrP^{c} pode ser encontrada de forma não glicosilada, monoglicosilada ou diglicosilada e é solúvel. Estes níveis de glicosilação ocorrem nos aminoácidos de asparagina (181 e 197). Outra característica da PrP^{c} é a presença de ponte de disulfeto entre os aminoácidos de cisteína (179 e 214). A figura abaixo mostra uma representação gráfica da *príon*celular.(LINDEN; CORDEIRO; LIMA, 2012)

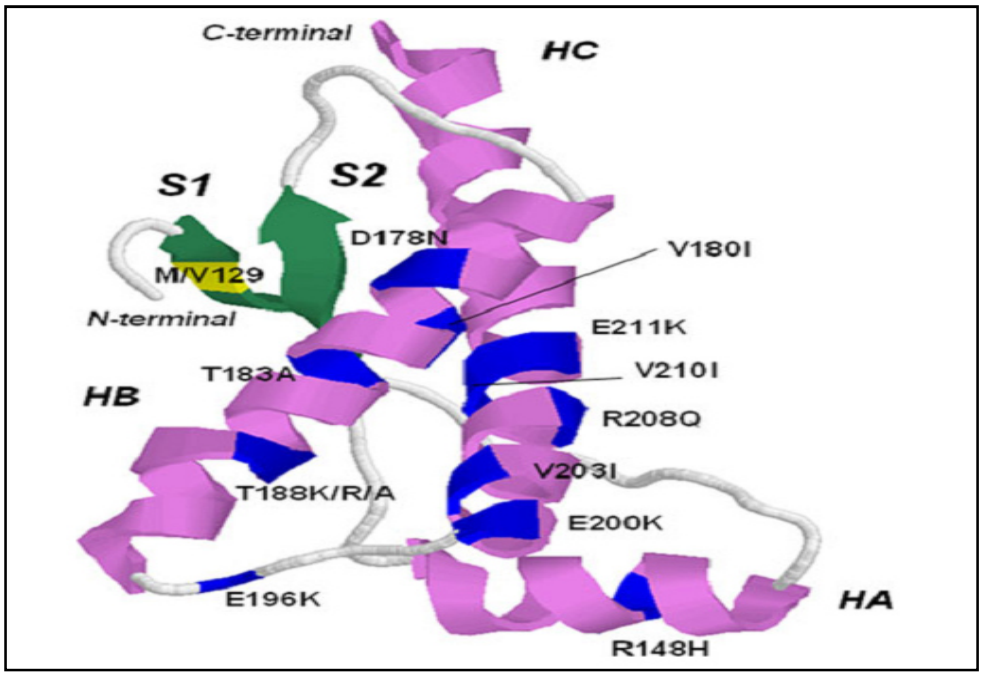


Figura 1 – Estrutura tridimencial da PrP^{c} mostrando as principais mutações associadas às Doenças Priônicas. HC- Porção hidrofóbica. $H^{Á}$ - H^{C} – formação em α – hélice. S1 e S2 – formação em folhas β-pregueadas. S-S – Ponte de disulfeto. (figura retirada de Capellari*etal*(CAPELLARI et al., 2011). Uso da imagem autorizado pela revista. LicenseNumber – 2833820137731.

Segundo Linden et *al*, a *príon* celular está associada direta ou indiretamente a diversos eventos fisiológicos, influenciando eventos moleculares, celulares e até mesmo sistêmicos. Entre esses eventos podemos destacar comportamento, regulação sono-vigília, memória e eventos pré e pós-sinápticos além de elos com o sistema imune. (LINDEN et al., 2008)

Diferente da PrP^{c}, a PrP^{sc} é insolúvel, parcialmente resistente a proteases, tende a formar fibrilas naturalmente e é rica em folhas β-pregueadas. A parcialidade na resistência a proteases e o grau de glicolização da proteína e do códon 129 da mesma, concede variações denominadas cepas (*strain*). Por ser de extrema importância, o códon 129 será melhor abordado em separado.

É postulado que a diferença entre a PrP^{c} e a PrPsc se dá basicamente por mudanças na conformação tridimensional, excetuando-se a presença de mutações no PRNP. Além de proteases, a PrP^{sc} é termoestável e não sofre alterações com radiações, o que comprovaria a ausência de ácidos nucléicos, desacreditando a teoria do "*slow vírus*" ou qualquer organismo com material genético. (CAUGHEY, 2003)

Apesar de incomum, o acúmulo de proteínas com mau dobramento não é único nas EET. O termo "*misfoldingdisease*", ou proteínopatias, engloba uma série enfermidades que são ocasionadas, direta ou indiretamente, pelo acúmulo de proteínas. Podem ser genéticas ou esporádicas, mas se diferenciam das Doenças Priônicas por não serem transmissíveis. Entre estas estão a Doença de Alzheimer (DA), Parkinson (DP), Huntington entre outras. (REVIEW, 2013)

Embora classicamente as EET sejam diferenciadas das demais proteínopatias pelo seu caráter transmissível, recentemente alguns estudos vêm mostrando a possibilidade da DA, pelo menos esta, também poder ser transmissível. Em fato, alguns pesquisadores questionam se todas as proteínas, ou centenas delas, possuem um estado "scrapie" e assim, em determinadas condições, deflagrarem alguma desordem biológica. (MILLER, 2009)(JAUNMUKTANE et al., 2015)

1.3 Gene PRNP

O gene PRNP está localizado, em humanos, na região 20p12, no braço curto do cromossomo 20. É constituído por dois exons, tendo a região codificantesituada no ultimo. Cerca de 30 mutações estão associadas as formas genéticas das doenças priônicas além de alguns polimorfismos, destacando-se o códon 129 que será abordado separadamente. (JEONG; KIM, 2014)

O gene PRNP está presente em 26 famílias, abrangendo cerca de 80 espécies. Estudos filogenéticos sugerem que o PRNP, ou prnp para os demais animais, possui regiões conservadas sendo mantido durante a evolução por conta da importância fisiológica da príon. Além disso, é alguns pesquisadores acreditam que quanto maior a semelhança entre as espécies, maior será a facilidade de transmissão das enfermidades de um grupo filogenético para outro, sendo isto chamada de "barreira de espécies". (RONGYAN et al., 2008)(BASKAKOV; BREYDO, 2007)

1.4 Códon 129

Vários estudos relacionam o códon 129 da proteína *príon* com a susceptibilidade às Doenças Priônicas. O mesmo encontra-se em uma das folhas β-preguedas da proteína. Isso se deve à existência do polimorfismo M129V (rs1799990). A frequência alélica, na população saudável, varia entre as diversas populações humanas, como será visto na Tabela2 abaixo. No Brasil, a distribuição alélica e genotípica do códon 129 na população mostra uma maior frequência do alelo M, condizente com dados de demais populações mundiais. Cabe ressaltar que os trabalhos brasileiros foram feitos com populações do sudeste.(DYRBYE et al., 2008)·(MARTINS et al., 2007)·(SMID et al., 2007).

Tabela 1:Frequênciagenotípica para o códon 129 do PRNP emalgumaspopulaçõeshumanas.

População	Grupo Étnico	Cromossomos (N)	MM	MV	VV	M	V
CEPH		184				0.560	0.440
HapMap-CEU	Européia	120	0.433	0.433	0.133	0.650	0.350
HapMap-HCB	Asiática	90	0.933	0.067		0.967	0.033
HapMap-YRI	Áfricana	120	0.400	0.517	0.083	0.658	0.342
HapMap-CEU	Européia	226	0.442	0.425	0.133	0.655	0.345
HapMap-HCB	Asiática	86	0.930	0.070		0.965	0.035

Esse polimorfismo está associado a diversas características das Doenças Priônicas como tempo de incubação, diferenças clínicas e histopatológicas, susceptibilidade entre outros. Curiosamente, aMetionina e a Valina são classificadas como aminoácidos polares, com carga positiva e não apresentam alteração estrutural na forma nativa da PrP^c. (DYRBYE et al., 2008)

A influência do códon 129 pode ser percebida ao compararmos casos de gDCJ com pacientes de IFF. No primeiro caso, o paciente possui o haplótipo D178N-129V, enquanto que no segundo caso o haplótipo é D178N-129M. Como visto na tabela 1, os fenótipos dessas duas doenças possuem diferenças acentuadas. (CAPELLARI et al., 2011)

Outros dois elementos associados ao códon 129, que são de vital importância no entendimento das EET, são o grau de glicolisação da *príon* (não glicosilado a diglicolisado) e o tamanho dos fragmentos gerados após o tratamento da proteinase K. A combinação desses três fatores geram fenótipos diversos sendo evidenciado na eDCJ na qual já foram descritos 6 subtipos Essa variação fenotípica tem grande impacto no diagnóstico das EET. (KRASNIANSKI et al., 2006)·(KLEMM et al., 2012)

1.5 Mecanismo de conversão da PrP^{c} em PrP^{sc}

A descoberta e caracterização da *príon* e da *príon* scrapie, embora esta última ainda não possuía a estrutura tridimensional conhecida, apenas um modelo, é sem dúvida um divisor de águas no campo da biologia molecular. Entretanto, o mecanismo patogênico ainda é controverso, sendo discutidos basicamente dois mecanismos: nucleação e polimerização.

No primeiro caso, a forma correta da *príon* recém sintetizada pela maquinaria celular passaria por diversas etapas pós-traducional, tendo alguns intermediários, podendo estes interagirem entre si, formando oligômeros. Esta ideia explicaria o fato das EET surgirem, em humanos, na idade entre 55-60 anos. Na segunda hipótese, seria introduzido no organismo, através da alimentação, por exemplo de moléculas da PrP^{sc}. Esta interage com a PrP^{c} induzindo um processo autocatalitico, semelhante aos encontrados no processo enzima-substrato. A figura abaixo, retirada de Corsaro e colaboradores, sintetiza essas duas teorias. (AGUZZI; BAUMANN; BREMER, 2008)·(CORSARO et al., 2012)

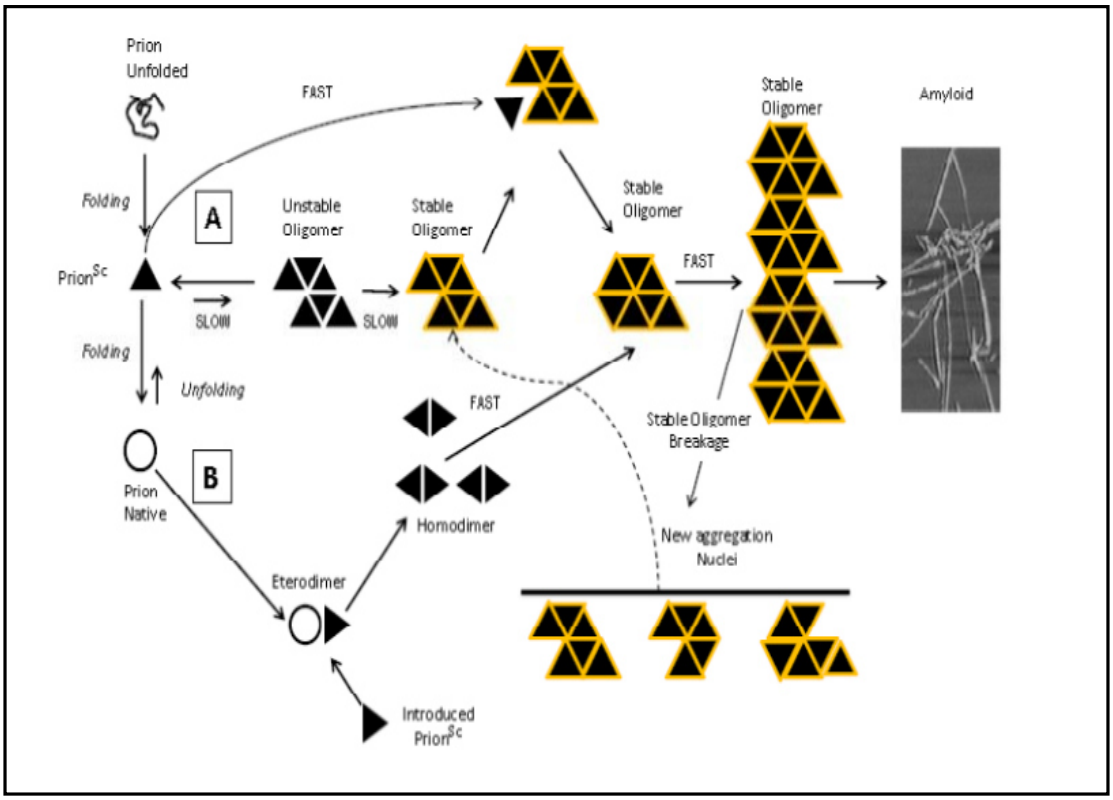


Figura 2- Representação esquemática da conversão da PrP^{c} em PrP^{sc}. Em (A) modelo de nucleação e em (B) modelo da polimerização por indução. Em (A) ocorre a formação de oligômeros e estes se agregam dando origem as fibras amiloides. Em (B), o processo ocorre por polimerização através de um molde de PrP^{sc}.

Independente da teoria a ser seguida, o fato é que esses agregados formam fibrilas e estas são tóxicas ao tecido nervoso, levando à morte celular. Curiosamente, ambas as teorias, bem como outras já preditas, são bioquimicamente desfavorecidas tendo, em humanos, o códon 129 um fator de risco para o desenvolvimento das EET.

1.6 Classificação das Doenças Priônicas

As EET podem ser classificadas de acordo com a etiologia em Esporádicas, Genéticas/Familiar ou Adquiridas. O diagnóstico tem como base dados clínicos, de neuroimagem e alguns testes genéticos e moleculares. Em resumo podemos definir a formas etiológicas desta forma:

•Esporádica - Corresponde a 85% dos casos. Não possui fonte aparente de infecção, como neurocirurgias, hormônioterapias ou transfusões de sangue bem como ausência de

mutações no gene PRNP. Exemplos: DCJ esporádica (eDCJ) e a forma esporádica da insônia fatal (eIF).

•**Genética:** Quando ocorrem mutações especificas no gene PRNP. Como exemplo, temos a Gerstmann-Straussler-ScheinkerDisease (GSS), a Insônia Familiar Fatal (IFF) e a forma genética/familiar da DCJ, gDCJ.

• **Adquirida:** Contaminação através carnes contaminadas com PrP^{sc}, no caso da nova variante da DCJ (vDCJ); Através de instrumentação cirúrgica não esterilizados de maneira adequada; hormônios hipofisários não sintéticos e transfusão de sangue, sendo chamada de DCJ iatrogênica (iDCJ). Ainda nesse grupo, encontra-se uma forma rara de doença priônicas, ainda mais nos dias de hoje, e restrita à região de Papua Nova Guiné, denominada de Kuru e está associada a rituais canibalísticos daquele povo. A tabela seguinte mostra um breve resumo das principais características das Doenças Priônicas. Essa tabela foi adaptada do trabalho de Glatzel e colaboradores. (GLATZEL et al., 2005)·(NOZAKI et al., 2010)

Até recentemente, as EET eram tidas como únicas doenças que podiam ter essas três formas etiológicas. Entretanto, como já mencionado, estudos recentes indicam a possibilidade da transmissão da DA através de procedimentos médicos levantando a possibilidade da DA também possuir aspecto infeccioso, bem como outras doenças neurodegenerativas. (MILLER, 2009)·(REVIEW, 2013)

Tabela 2: Resumo das características clínicas, genética e hispotalógica das Doenças Priônicas.

Doença Priônica	Idade do inicio da doença	Duração da Doença	Sintomas Clínicos	LCR	Ressonância Magnética	Dados genéticos		Achados Histopatológicos
						Códon 129	Mutações no PRNP	
eDCJ	60-70	6 meses (1-35)	Demência progressiva; mioclonias, ataxia cerebelar e sinais piramidais e extrapiramidais	+ em 90% dos casos	Atrofia cerebral e hipersinal nos gânglios basais e/ou cortical em 67% dos casos	70% homozigoto para M	Sem mutações	Perda neuronal, aspecto espongiforme do tecido nervoso, astrogliose
gDCJ	50-60	6 meses (2-41)	Similar a eDCJ	+ em 90% dos casos	Similar a eDCJ	V para o alelo multado	Mutações no PRNP (E200K e D178N)	Similar a eDCJ
iDCJ	*	6 meses (similar a eDCJ)	Similar a eDCJ	Positivo em cerca de 77% dos casos	Similar a eDCJ	60% homozigoto para M	Sem mutações	Similar a eDCJ
vDCJ	26	14 meses (6-24)	Sintomas psiquiátricos	Positivo em 50% dos casos	Hipersinais no tálamo posterior (sinal pulvinar em 78%)	MM (100% dos casos)	Sem mutações	Similar a eDCJ e "placas flóridas"
GSS	50-60	5 anos (3 meses a 13 anos)	Disfunções cerebelares	Geralment e negativo	Normal ou alteraçõesinespecíficas	M para o alelo multado	A mutação mais frequente é a P102L	Similar a eDCJ, envolvimento talâmico e placas multicêntricas
IFF	50	13-15 meses (6-42 meses)	Insônia, disfunções autônomas	Negativo	Normal ou alterações inespecíficas	Mpara o alelo multado	D178N	Envolvimento do Tálamos

2 BANCO DE DADOS E VIGILÂNCIA EPIDEMIOLÓGICA

2.1 Epidemiologia

Por serem doenças raras, de difícil diagnóstico e com claros impactos na saúde pública, diversos países criaram ferramentas de vigilância epidemiológica (VE) para uma melhor monitoração das EET, tais como Japão, França, Alemanha, Canadá, Bélgica, etc. Além dos centros individuais, países da União Europeia, criaram o EROCJD consórcio para facilitar troca de informações e medidas de controle, principalmente no caso da Encefalopatia Espongiforme Bovina.(NOZAKI et al., 2010)

No Brasil, uma maior atenção a estas doenças começou em 2005 com a inserção destas no grupo das doenças de notificação compulsória. Entretanto, o fluxograma estabelecido pelo Ministério da Saúde (MS) não é executado de forma adequada na grande parte do país, deixando pacientes e familiares sem o acompanhamento adequado. Ainda sim, o único ponto positivo desta medida foi a criação de uma ficha individual clara, objetiva e de fácil preenchimento por médicos e demais profissionais de saúde, o que foi debatido pelo nosso grupo. (MARTINS et al., 2007)(GOMES DA CUNHA; OLIVEIRA, 2008)

Uma vez que a VE se baseia na informação para tomadas de decisões, o uso de ferramentas na coleta, no armazenamento e na análise dos dados de forma adequada são fundamentais para o sucesso dos Sistemas de Vigilância Epidemiológicos (SVE). A criação de bancos de dados de livre acesso é uma ferramenta que pode ser desenvolvida para todos os tipos de condições e facilitar a consulta dos profissionais de saúde, principalmente em desordens complexas e raras. (WOLONGEVICZ; BROWN; MILLEN, 2010)(XIE; BAEK; GROSSMAN, 2011)

A Doença de Creutzfeldt-Jakob (DCJ) é a prionpatia mais freqüente, sendo descrita em diversos países, inclusive no Brasil. Possui incidência de um caso a cada milhão de habitantes, afeta homens e mulheres na mesma proporção e ocorre principalmente na faixa dos 55 aos 65 anos. É causada pelo acúmulo da príonscrapie (PrP^{sc}) nos neurônios. Pode ocorrer de forma Adquirida, Genéticas ou Esporádicas, sendo a última a forma mais prevalente, cerca de 85% dos casos[5].

Clinicamente a DCJ é caracterizada por demência progressiva menor que dois anos, mioclonias, ataxia e outros sintomas. O diagnóstico é feito associando esses com exames de ressonância magnética (RM), eletroecenfalograma (EEG) e pesquisa no líquor da proteína 14-3-3. [6]

Entretanto, na prática clínica reconhecer pacientes com DCJ requer muito cuidado e habilidade do médico visto que os sinais e achados de exames para a DCJ se confundem com outras doenças neurológicas, como a DA. [7]

Esta dificuldade é potencializada pela falta de campanhas de conscientização sobre o tema e maior aporte de informações para os profissionais de saúde. No Brasil, não há como traçar, no momento, uma comparação visto que temos falhas na notificação dos casos. [8]

Com a finalidade de melhorar a VE, em 2005, o Ministério da Saúde (MS) incorporou a DCJ à Lista de Doenças de Notificação Compulsória o que obriga profissionais de saúde notificar casos suspeitos às secretarias estaduais de saúde. [9]

Infelizmente, essa iniciativa por si só não melhorou a notificação no aspecto quantitativo, visto que ainda temos uma taxa de notificação muito abaixo da média mundial e do que é previsto para uma população de cerca de 190 milhões de habitantes. Na figura abaixo, observamos o número de casos de sDCJ por ano ajustado para cada um milhão de habitantes em alguns países.

A análise do gene PRNP é fundamental para os estudos de Doenças Priônicas visto que, além das formas genéticas da doença, temos a presença do polimorfismo do códon 129 que é associado a fatores de risco para a forma esporádica. Indivíduos homozigotos para metionina parecem ter uma maior eficiência de conversão de PrP^{c} em PrP^{SC} do que indivíduos heterozigotos. Em contrapartida, homozigotos para Valina estão fortemente associado à vDCJ.[10]

A DCJ é vista como uma preocupação de saúde pública por causa das formas Adquiridas da doença uma vez que o consumo de carne contaminada pode afetar milhares de pessoas ao ano. Além disso, recentemente vem se debatendo o risco de contágio através de sangue de doadores assintomáticos. Ao menos 5 casos até o momento foram rastreados, sendo mais associados a indivíduos homozigotos para metionina. (IRONSIDE, 2012)

Pouco conhecimento sobre a DCJ, reduzido número de profissionais habilitados para uma análise clínica e laboratorial detalhada e poucos trabalhos nessa área no Brasil e a falta de coordenação entre secretarias municipais e estaduais com o Ministério da Saúde (MS) contribuem para que a notificação seja ineficaz.

Adicionalmente, a política estabelecida pelo MS para as Doenças Priônicas estão voltadas para a vDCJ que é ainda mais rara, acomete poucos casos por ano no mundo inteiro e não foi descrita, até o momento no Brasil. Visto a forma de contágio da vDCJ, as Doenças Priônicas estão na Unidade Técnica de Hídricas e Alimentares – UHA/CGDT/ DEVEP o que não é adequado para uma desordem neurológica e que tem quase 90% dos casos de origem Esporádica.

No Brasil, uma maior atenção a estas doenças começou em 2005 com a inserção destas no grupo das doenças de notificação compulsória. Entretanto, o fluxograma estabelecido pelo MS não é executado de forma adequada em grande parte do país, deixando pacientes e familiares sem o acompanhamento adequado e basicamente o único ponto positivo desta medida é a criação de uma ficha individual clara, objetiva e de fácil preenchimento por médicos e demais profissionais de saúde, o que foi debatido pelo nosso grupo de pesquisa. (MARTINS et al., 2007)·(GOMES DA CUNHA; OLIVEIRA, 2008)

2.2 Diagnóstico das Doenças Priônicas

Um dos principais desafios nas EET é, sem sombra de dúvidas, o diagnóstico. Até o presente momento, os diagnósticos têm uma forte base clínica já que alguns exames (Ressonância Magnética (RM) e exame da proteína 14-3-3) auxiliam apenas na exclusão de outras doenças. O exame *standard* para a confirmação destas enfermidades se dá apenas *post-mortem*. Além disso, a pesquisa no liquido cefalorraquidiano (LCR) para a 14-3-3 é um procedimento invasivo, necessitando de internamento e trazendo mais desconforto a pacientes e familiares já debilitados. (ZAFAR, 2016)

O Centro de Estudos de Memória e Envelhecimento da Universidade da Califórnia, São Francisco (UCSF), elaborou, baseado em textos da Organização Mundial de Saúde (OMS), critérios de diagnóstico para as formas da DCJ (Anexo I). Este grupo leva em consideração estudos com D*iffusion-WeightedImaging* (DWI) no qual tiveram

precisão de 97%. (KIM; GESCHWIND, 2015) Devido à heterogeneidade clínica dos casos de EET, principalmente da DCJ, o grupo da UCSF, liderado por Michael D. Geschwind criou o anagrama VITAMINS. Essa ferramenta, apesar de mais simples, orienta o médico ou demais profissionais de saúde a desconfiar ser está diante de um caso suspeito de DCJ ou não. Segue abaixo:

Vascular

Infecção

Tóxico - Metabólica

Autoimune

Metástases/neoplásica

Iatrogênica

Neurodegenerativa

Sistêmica

Entre os marcadores biológicos, a 14-3-3, proteína Tau e p-Tau, são utilizadas em caso de suspeita de Doenças Priônicas. Em recente estudo, pesquisadores da Alemanha analisaram 231 casos de eDCJ e 2035 controles para a 14-3-3, obtendo como resultado uma sensibilidade de 88% e sensibilidade de 96%. Entretanto, os resultados quando são comparados a outras Doenças Priônicas e quando se separa indivíduos baseados no códon 129, esses valores passam a ter diversas alterações, como por exemplo, apenas 50% dos casos de vDCJ são positivos. (SCHMITZ et al., 2015)

Recentemente, diversos grupos de pesquisa começaram a utilizar a técnica denominada de RT-Quic(*Real-time quaking-inducedconversion*). Basicamente, essa técnica estimula a formação de agregados de PrP^{sc} de forma exponencial. Através de uma série de diluição da amostra, combinado com cofator e a adição de pequena quantidade de PrP^{sc} com a PrP^{c}, o sistema determina o quanto tinha de PrP^{sc} antes. Essa técnica vem levantando bons resultados, diferenciando pacientes conforme o códon 129 e mesmo entre eDCj, gDCJ e FFI, sendo um futuro promissor para o diagnóstico das Doenças Priônicas. (ORRÚ et al., 2015),(CRAMM et al., 2014)

2.3 -Objetivos

- Desenvolver um banco de dados online com dados epidemiológicos, de exames e moleculares dos casos de Doenças Priônicas já relatados e permitir o acesso à informação desses dados de forma centralizada.

- Disseminar a informação sobre as Doenças Priônicas em website para que as pessoas tenham acesso a esse tipo de informação em português.

- Analisar a Aβ42 e a proteína Tau através de eletroquimioluminiescência (MSD) como possíveis biomarcadores para diagnóstico diferencial das doenças priônicas.

2.4 Metodologia

2.4.1 - Banco de dados

O epiCJD foi criado baseado em estrutura MySQL por sua flexibilidade e compatibilidade com diversos módulos de interface e por ser um software livre e está hospedado no servidor do grupo ideas (http://www.ideias.ufpe.br/portal/), sendo acessado no endereço http://www.ideias.ufpe.br:8180/prions/public/login.jsf. A figura abaixo mostra a tela inicial do epiCJD. Nesse primeiro momento, o epiCJD está em fase de implementação sendo requerido *log in* e senha para acessar o seu conteúdo.

Após efetuar o *log in*, o epiCJD oferece a opção de alimentar o banco de dados através da ficha de notificação de Doenças Priônicas do MS, através de relatos de casos publicados em periódicos internacionais e através da importação via *upload* de arquivo excel. Para o presente trabalho, foi utilizado como projeto piloto a importação via excel.

Figura 3: Tela de acesso do banco de dados epiCJD.

Após a alimentação do epiCJD, o usuário pode escolher dados em um intervalo de tempo, gênero, país de origem ou optar por baixar toda base de dados. Em um futuro próximo, o usuário poderá selecionar qualquer tipo de informação que estiver no epiCJD.

2.4.2 - População piloto

Para efeito de validação e testes do banco de dados, alimentamos o epiCJD com dados provenientes do *NationalesReferenzzentrumfür die SurveillanceTransmissiblerSpongiformerEnzephalopathien*, da Alemanha. Foram selecionados 4210 casos reportados entre 1993 a 2014. Neste banco, por questões legais, foram suprimidos dados como a idade dos casos.

3 RESULTADOS E DISCUSSÕES

Dos 4210 casos notificados como suspeitos, apenas 2866 foram associados a alguma Doença Priônica ao fim da investigação médica. Os demais foram descartados por serem inconclusivos ou terem diagnóstico para outras doenças neurológicas, como a DA. A distribuição dos casos, com a maioria sendo associada à eDCJ não difere do descrito na literatura. A **Tabela 1** abaixo mostra a distribuição destes casos.

Tabela 4: Distribuição dos casos de acordo com a Doença Priônica. Dados provenientes do *NationalesReferenzzentrumfür die SurveillanceTransmissiblerSpongiformerEnzephalopathien*, Alemanha no período de 1993 a 2014.

Doença Priônica	**Número de Casos**
Doença de Creutzfeldt-Jakob Definitiva (DCJ)	
DCJ idiopática	1213
DCJ genética	105
DCJ iatrogênica	11
DCJ Possível ou Provável	1453
Insônia família Fatal (IFF)	65
Gerstmann-Straussler-Scheinker Disease (GSS)	19
Total	2866

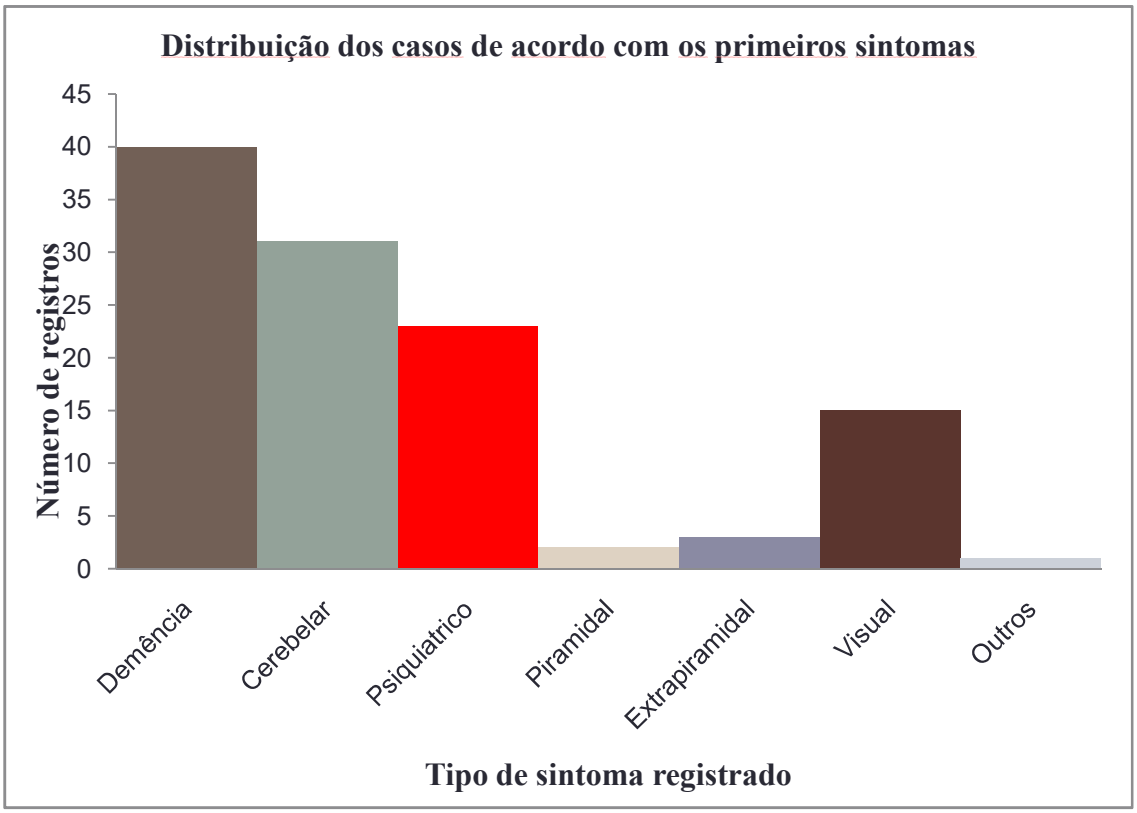
Apenas 3 dos 2866 casos não possuíam informações sobre o gênero dos pacientes. Cerca de 45% dos casos (1280 registros) foram do sexo masculino enquanto 55,3% foram do sexo feminino (1583 registros). A proporção masculino/feminino foi de 0,80, não sendo diferente da literatura mundial. Apesar de estudos indicarem uma maior relação de mulheres no Japão, após ajustes na idade das autópsias. (NOZAKI et al., 2010)

Infelizmente, os dados da Alemanha não continham informações sobre a idade dos casos notificados, inviabilizando o cálculo da idade média destes. Entretanto, a faixa etária dos primeiros sintomas estava disponível para 2215 registros, tendo média de 65,3 anos. Como as Doenças Priônicas, possuem média de menos de um ano de sobrevida dos pacientes, podemos estimar que os registros possuíam idade compatível com a literatura mundial. (IMRAN; MAHMOOD, 2011)

Quanto aos dados clínicos, apenas 192 registros possuíam a descrição dos primeiros sintomas, sendo Demência presente em 74 casos (38,5%), seguido por sintomas cerebelares. Entretanto, o agrupamento em sintomas cerebelares, piramidais, extrapiramidais é interessante para questões didáticas. Tratando-se de doenças raras, com pouca informação disseminada, o maior detalhamento se faz necessário para assegurar que médicos recém formados não deixe passar sinais e sintomas devido a confusões que

possa, surgir ao se reportar, por exemplo, distúrbios motores, o que pode ser uma ataxia ou uma hemiparesia, por exemplo.

Gráfico 1: Distribuição dos primeiros sintomas reportados em pacientes com Doenças Priônicas.



O epiCJD possui campo para os primeiros sintomas, diferente do que se apresenta, infelizmente, no banco de dados alemão. Uma grande vantagem de bancos de dados digitais frente a guias impressos, como o CID-10 e DSMIV, é o fator dinâmico. Podemos a qualquer momento incorporar novas informações de forma mais ágil, com alcance mais abrangente e barato que qualquer um dos guias citados.

O códon 129 é um dos fatores mais importantes para o entendimento das Doenças Priônicas. Este dado estava presente em 73 casos. Destes, 52,1% eram homozigotos para Metionina, 21,9 heterozigotos e 26% homozigoto para Valina. De acordo com alguns estudos, homozigose para o alelo M confere susceptibilidade para o desenvolvimento das Doenças Priônicas. Em diversas populações, a presença de homozigose para metionina varia de 37 a 49%, mostrando que os dados da população Alemã é condizente com a literatura. (DYRBYE et al., 2008)

4 CONCLUSÕES

A implementação do banco de dados epiCJD tem o potencial de ser uma grande ferramenta para catalogar e centralizar dados das Doenças Priônicas reportados no mundo. Como projeto piloto, foram analisados casos reportados na Alemanha, mas com a possibilidade de ser inseridos dados de qualquer população.

A distribuição de acordo com o gênero não difere de outras populações de Doenças Priônicas, tendo uma leve incidência mais elevada nas mulheres.

Infelizmente, dados sobre a idade não foram disponibilizados. Entretanto, extrapolando os dados sobre a idade em que o paciente apresentou os primeiros sintomas, podemos estimar que a idade média dos casos é condizente com o visto mundialmente.

A análise dos primeiros sintomas é compatível com o encontrado em diversos estudos. Porém, o detalhamento ao invés do agrupamento dos sintomas poderia ser mais útil na educação continuada dos jovens médicos.

Adicionalmente, a distribuição genotípica do códon 129 está de acordo com a literatura apesar do N ser de apenas 73 casos.

Em paralelo ao banco de dados, que será integrado futuramente, o website dcjBRASIL , bem como grupos em redes sociais, possibilitam o contato e apoio aos familiares brasileiros, informando sobre a doença bem como os procedimentos que são realizados. Graças a essas ações daqueles grupos, de centros de pesquisa e de nossas atuações através do contato contínuo com diversos pesquisadores tanto nacionais quanto estrangeiros, o Brasil agora faz parte do grupo da CJDISA e abre uma perspectiva de maior apoio aos familiares e pacientes destas desordens.

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Capítulo 1

Epidemiology in Prion Disease: a database on the Web (epiCJD)

Epidemiology in Prion Disease: a database on the Web (epiCJD)

Abstract

Introduction: Prion diseases (PrD), or Transmissible Spongiform Encephalopathies (TSEs), are part of a group of rare diseases and invariably fatal and focus on various animals, including humans, and are caused by the accumulation of insoluble protein called Prion Scrapie (PrP^{sc}). Bovine Spongiform Encephalopathy (BSE), Cronic Wasting Disease (CWD) in deer, scrapie in sheep and Creutzfeldt-Jakob disease (CJD), Kuru, Gerstmann-Sträussler-ScheinkerDisease (GSS) and Fatal Familial Insomnia, in humans, are example of TSEs. Clinically, PrDshavea huge spectrum of signals and symptoms and can overlap with some other diseases making the diagnosis a challenger. **Aims:**Implementation of an online database to centralize data about epidemiological, molecular and clinical information associated to PrD. **Methods**: epiCJD consist in a web based database designed to integrate clinical, molecular and epidemiological data of PrD reported worldwide. epiCJD can be feed collecting data from index articles, from compulsory notification databases and importing excel files from others databases. **Results:** From 1993 to 2014, 4210 cases are reported as suspected, butjust2866 were associated with PrD at the end of the medical investigation. As expected, sCJD is the most prevalent PrD with 1213 records. About first symptoms, dementia is the most frequent followed by cerebellar, psychiatric and visual. None unusual symptoms are mentioned in any case. In addition to epidemiological data, our database has information about molecular information such as polymorphism of condon 129 which isone of most important factors for the understanding of Prion Diseases. This figure was present in 73 cases. Of these, 52.1% were homozygous for Methionine, 21.9 heterozygotes and 26% homozygous for Valine. According to some studies, homozygosis for the M allele confers susceptibility to the development of Prion Diseases. **Conclusion:**Diagnosis of prion diseases are difficult because those illnesses are rare and have a broad of symptoms. Besides an advance in pursuit of a biomarker, clinical symptoms and signs still an important point for initial investigation and differential diagnosis. As a pilot project, cases reported in Germany were analyzed, but with the possibility of inserting data from any population. In addition, any user can access epiCJD and download all data or just for parameters, such as symptoms, age, country, etc. The implementation of the epiCJD database has the potential to be a great tool for cataloging and centralizing data from the world's reported Prion Diseases

Key words: Prion disease, epidemiology, database, first symptoms, codon 129.

1 – Introduction

Prion Diseases (PrDs) or Transmissible Spongiform Encephalopathy (TSE) is a group of rare and fatal diseases affecting several animals including humans. According to *prion only hypothesis*, they are caused by accumulation of a misfolded protein called Prion Scrapie (PrP^{sc}). [1].

PrDs can be classified in three different forms: sporadic (sporadic Creutzfeldt-Jakob disease [sCJD]; Fatal Insomnia [sFI]), genetic (genetic CJD [gCJD]; Fatal Familiar Insomnia [FFI]; Gerstmann-Sträussler-Scheinker [GSS] and Acquired (Kuru; variant CJD [vCJD]; and iatrogenic CJD [iCJD]). This remarkable condition gives to PrDs a huge spectrum of signals and symptoms, making them a great challenger to scientific community and for public health. [2]

Disease form and molecular profile, such as polymorphism at codon 129 on PRNP gene and glycosylation ratio of prion protein, alters drastically PrDs phenotype pattern including first symptoms, age of onset of disease, time survive, neuropathological changes and others. [3]

The identification of signals and symptoms associated to Prion Diseases is critical for differential diagnosis at the anamnesis phase. Nevertheless, as we discussed before, symptoms and signals in PrD can overlap with some other diseases and lead the health professional to a labyrinth of possible diagnostics [4].

International diseases classifications, such as DSM, are a useful way to catalogue and generate protocols to help diagnosis and thus, guide to a better conduction of case. However, the process of updating these classifications is expensive and too long, and sometimes may bring difficulties to retrieve actual information about diseases, mainly in genetic disorders. [5]

Online database can be more dynamics than traditional manuals, present global and fresh information about PrD. We can populate and spending less time and money than usual in this process which are very powerful tool for illnesses with great heterogeneity, allowing to us tabulate and share a huge amount of information easier than the traditional ones. In order to improve the health surveillance of PrD we have modeled an online database with clinical, molecular and epidemiological data related to those illnesses.

2. Methods

2.1 – Database building

EpiCJD was created based on MySQL structure for its flexibility and compatibility with several interface modules and for being free software and is hosted on the ideas group server, accessed at http://www.ideias.ufpe.br:8180/prions/public/login.jsf.

2.2- Database feeding

epiCJD consist in a web based database designed to integrate clinical, molecular and epidemiological data of PrD reported worldwide. To feed epiCJD, we used three different sources: cases reported at periodical international journals, data from prion's researchers groups and an input designed for Brazilian's public health purpose.

Retrieving data from indexed articles: we performed an active search on Pubmed database, using the term "prion disease". We used the fallow filters: case report to avoid redundancies in reviews and metanalysis articles; free full text available, regarding copyrights issues; Species Human, to include just human PrD; and publication dates, from 1th January of 1920 to 30th May of 2013.

Import excel file: we received an xls file with 4210 cases reported as suspected of prion diseases between 1993-2014 from the national register of prion diseases Germany (NationalesReferenzzentrumfür die Surveillance TransmissiblerSpongiformerEnzephalopathien) and was incorporated in our database using excel import input.

Retrieving data from compulsory notification databases: this input was created for Brazilian public health purpose. The form is an electronic version of notification report sheet used in Brazilian public health to report any case suspect does be a Prion disease.

For the purposes of validation and database testing, we simulated a download of records from our database accessing excel import area and we did a brief report of part of those data. At this moment, this field is available only for registered users.

3. Results and discussion

From 1993 to 2014, 4210 cases are reported as suspected. However,just2866 were associated with PrD at the end of the medical investigation. The others were discarded to be inconclusiveor for other neurological disorders, such as Alzheimer Disease. sCJD is the most prevalent PrD, 1213 records. In the period studied, 1993-2014, Germany's populations correspond to around eighty million habitants and the number of cases of

sCJD is in accordance to literature. Table 1 below shows the distribution of all PrD cases.[6]

Table 1 - Dristribution of Prion Disease in period of 1993 to 2014.

Prion Disease	Number of Cases
Definitive Creutzfeldt-Jakob Disease (CJD)	
Sporadic CJD	1213
Genetic CJD	105
Iatrogenic CJD	11
Possible or Probable CJD	1453
Fatal Familial Insomnia	65
Gerstmann-Sträussler-Scheinker Disease (GSS)	19
Total	2866

Only 3 of the 2866 cases did not have information about gender of the patients. About 45% of the cases (1280 records) were male while 55% were female (1583 records). The male/female ratio was 0.80. Unfortunately, information about age of the reported cases is suppressed.

First symptoms are present in 101 cases. Dementia is the most frequent followed by cerebellar, psychiatric and visual. None unusual symptoms are mentioned in any case. Recently, Krasnianski and colleges published a paper about first symptoms in Germany population using data similar what we used to populated epiCJD. [7]

Codon 129 is one of the most important factors for the understanding of Prion Diseases. This figure was present in 73 cases. Of these, 52.1% were homozygous for Methionine, 21.9 heterozygotes and 26% homozygous for Valine. According to some studies, homozygosis for the M allele confers susceptibility to the development of Prion Diseases. In several populations, the presence of homozygous for methionine ranges from 37 to 49%, showing that data from the German population is consistent with the literature.[8]

4 - Conclusions

Diagnosis of prion diseases are difficult because those illnesses are rare and have a broad of symptoms. Besides an advance in pursuit of a biomarker, clinical symptoms and signs still an important point for initial investigation and differential diagnosis.

As a pilot project, cases reported in Germany were analyzed, but with the possibility of inserting data from any population. Besides, any user can access epiCJD and download all data or just for parameters, such as symptoms, age, country, etc.

The implementation of the epiCJD database has the potential to be a great tool for cataloging and centralizing data from the world's reported Prion Diseases

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CAPÍTULO 2

How Heterogeneous Can the Clinical Presentation for Creutzfeldt-Jacob Disease Be?

How Heterogeneous Can the Clinical Presentation for Creutzfeldt-Jacob Disease Be?

To the Editor: Creutzfeldt-Jakob disease (CJD) is a heterogeneous clinical condition, usually presenting in a sporadic form (sCJD) and affecting about 0.5–1.5 cases per million inhabitants, showing particularities on pathological and clinical features, according to western blot analysis of Prion scrapie protein (PrP^{sc}).[1]

A clinical case published in this journal recently reported the unusual association of CJD and catatonia.[2] Others reports are also showing that psychiatric symptoms, such as depression, are more common than previously assumed.[3]

There is a current interest in reporting a more detailed clinical profile of patients with prion disorders, and this is crucial to increasing the rate of early detection.

The most recent cases reported in our institution reinforce this concept; here, we summarize four cases confirmed in Recife, a major city (about 1.5 million people) in northeastern Brazil.

Histopathological and imunohistochemistry assays confirmed CJD; Cases #3 and #4 were MM at 129 codon in the PRNP gene, and no mutations were observed. Table 1 summarizes the major symptoms and profiles of each case.

Patient #1 was admitted to the hospital service because of delusions and behavioral changes, but diplopia followed by cognitive impairment, characterized by progressive memory decline, were retrospectively mentioned by relatives several weeks before. In the '70s, this patient had had neurosurgery for a pituitary adenoma in the United States. Neuro-ophthalmologic evaluation showed a mild palsy in the sixth nerve.

Case #2 was hospitalized in 2009, but medical records report depressive symptoms 2 years before, with sudden psychomotor agitation, aggression, and hallucinations, and the patient's being hospitalized in the psychiatric clinic. Confusion and aggression increased progressively, associated with intense hypertonia in all four limbs and neck, with later feeding impairment.

Curiously, the first and second cases died on the same day, and this brought about major concern of local sanitary authorities and general media.

Relatives of Patient #3 reported disorientation, memory loss, and behavior changes characterized by disinhibition. There is a history of neuropsychiatric illness in his family. He presented with myoclonia, apraxia, and acute weight loss.

The last and most recently reported case was a 52-year-old man. The first complaints were changes in sleeping pattern, dizziness with imbalance, ataxia, and myoclonia. This was another patient with a familial history of mood disorder, but not associated with prion diseases. This patient also presented difficulty in communication and comprehension of simple commands.

Brazil has a recent history of epidemiological surveillance for prion disorders, and, since 2005, we have dealt with the challenge to make compulsory notification a reality, still far from the optimal expected number of annual reports for a country with almost 200 million inhabitants.

Martins et al.[4] confirmed the high spectrum phenotype of this disease in Brazil and other countries are possibly due also to ethnic influences.

Brazil's examples highlight the importance of additional attention for possible CJD cases starting with behavioral changes. Neurologists should be attentive to more atypical symptoms, and CJD must be a differential diagnosis in rapidly-progressive dementia when neurodegenerative, infectious, and others syndromes was suspected.[5]

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TABLE 1. Summary of Main Characteristics: Four Definite Cases of Creutzfeld-Jakob Disease

Case	Age, years	Gender	Illness Duration, months	Major Characteristics	Magnetic Resonance Imaging	14–3–3 Protein
#1	62	M	7	Visual impairment; delirium; cerebellar syndrome; axial and appendicular ataxia	Increase of ventricular volume	+
#2	62	F	4	Behavioral disturbance; hallucinations; extrapyramidal syndrome	Bilateral hypersignal in parietal and occipital cortex	Not performed
#3	65	M	8	Seizures; trismus; behavioral changes; pyramidal signs; myoclonus	Hypersignal in T2 and Flair; diffuse atrophy; basal ganglia affected	—
#4	52	M	6	Motor impairment; myoclonus; dysphagia; pyramidal signs	Hypersignal in T2 and Flair; diffuse atrophy; basal ganglia affected; frontal and parietal damage	+

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CAPÍTULO 3

Quantification of CSF biomarkers using an electrochemiluminescence-based detection system in the differential diagnosis of AD and sCJD

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ORIGINAL COMMUNICATION

Quantification of CSF biomarkers using an electrochemiluminescence-based detection system in the differential diagnosis of AD and sCJD

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Abstract The identification of reliable diagnostic tools for the differential diagnosis between sporadic Creutzfeldt–Jakob Disease (sCJD) and Alzheimer's disease (AD) remains impeded by the existing clinical, neuropathological and molecular overlap between both diseases. The development of new tools for the quantitative measurement of biomarkers is gaining experimental momentum due to recent advances in high-throughput screening analysis and with the optimization of assays for their quantification in biological fluids, including cerebrospinal fluid (CSF). Electrochemiluminescence (ECL)-based immunoassays have demonstrated to achieve clinical quality performance in a variety of sample types due to its high sensitivity and dynamic range. Here, we quantified the CSF levels of Tau-protein, β-amyloid 1-42 (Aβ42) and α-synuclein, as important biomarkers in CSF used in the differential diagnosis of neurodegenerative disorders in 12 AD, 12 sCJD and 12 control cases by singleplex ECL-based technology. Its performance has been compared to classical enzyme-linked immunosorbent assays (ELISA) to confront their clinical accuracy. ECL-based technology validates previous data obtained with ELISA and presents a higher performance in the discrimination of three analysed groups as determined by increased area under the curve (AUC) values for the three biomarkers. Importantly, α-synuclein levels detected by ECL allow an excellent discrimination between sCJD cases and AD and control cases, unveiling a new clinical approach for the differential diagnosis of sCJD.

Keywords Cerebrospinal fluid · Biomarkers · Neurodegeneration · Alzheimer's disease · Creutzfeldt–Jakob disease · Electrochemiluminescence-based detection system · ELISA

Introduction

Alzheimer's disease (AD) and Creutzfeldt–Jakob disease (CJD) are neurodegenerative diseases characterized by the presence of pathogenic protein aggregates leading to an alteration of brain functions and to profound dementia. AD usually displays a slow progression rate, although rapid progressive AD forms (known as rapid progressive AD) have been also described [28]. A broad range of pharmacological treatments can temporarily alleviate the symptoms of AD patients slowing down disease progression. On the contrary, CJD is a fatal progressive disease with an invariable rapid progression rate for which there is no current treatment [1, 7].

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Therefore, biomarkers to discriminate between CJD and AD are required to generate an appropriate therapeutic intervention before pathological changes spread throughout the brain.

CSF analyses have demonstrated that the ratio between phosphorylated and total Tau levels [25], the presence or absence of the 14-3-3 protein [39, 41] and the ability of PrPsc to induce the seeding of a template PrPc [3, 8, 9] can discriminate between CJD and AD cases. However, different disease duration, progression rates, disease subtypes, blood contamination, CSF storage conditions as well as the presence of co-pathology significantly alter the sensitivity and specificity of these biomarkers. In addition, an overlap exists between CJD and AD in the levels of main biomarkers used for clinical diagnosis such as Tau and Aβ42 [21, 36, 37].

Different approaches are explored to overcome the inherent limitations of a differential diagnosis. On one side, the screening of new molecules with better clinical performance to those currently in use and, on the other side, the development of new techniques and methodologies able to detect the presence of well-known biomarkers with higher sensitivity and larger dynamic ranges are two of the main research focus in the field of biomarkers development.

In this context, the use of an electrochemiluminescence (ECL)-based detection system developed by Meso Scale Discovery (MSD, Gaithersburg, MD, USA) has been shown to present high sensitivity, reproducibility, recovery rates and low background in the detection of several biomarkers in biological fluids [4, 5, 24]. However, the analytical and clinical performance of ECL technology in CSF samples has not been explored in detail for the differential diagnosis of neurodegenerative dementias.

In the present study, we have analysed in sCJD, AD and control samples the levels of the CSF biomarkers Tau, Aβ42 and α-synuclein, previously reported to be deregulated in several neurodegenerative diseases using ECL technology. Details about the comparative performance between MSD's ECL and conventional ELISA are reported.

Methods

Demographics

This diagnostic study is based on data from an ongoing surveillance study of the German National Reference Centre for Transmissible Spongiform Encephalopathies [11].

Lumbar puncture was performed for diagnostic purposes with analysis of CSF standard parameters (e.g. cell count, proteins and immunoglobulins). CSF was centrifuged and stored at −80 °C until analysis. All patients with sCJD were classified as definite cases by neuropathological examinations or as probable CJD cases according to diagnostic consensus criteria [40, 41]. All sCJD cases were tested positive for 14-3-3 CSF protein. AD diagnosis was based on the ICD-10 definition (F.00 G.30). Controls are patients with neurological disorders that were diagnosed according to clinical syndrome, neuroimaging and standard neurological clinical and paraclinical findings. The presence of neurodegenerative disease in the control cohort was excluded in the follow-up clinical diagnosis. In addition, CSF biomarkers proposed to predict development of dementia (p-Tau, Tau, Aβ42/Aβ40) were negative in controls cases. A total of 12 CSF samples from age- and sex-matched cases per each group were analysed. Mean and Standard deviation from ECL measurements, as well as mean of patients' age are reported (Suppl. Table 1). For comparisons between detection methods for Tau, Aβ42 and α-synuclein, AUC values derived from ROC curves from ELISA and ECL measurements were calculated. AUC values for ELISA measurements reported in the present study are in line with those previously reported for the German National Reference Centre for Transmissible Spongiform Encephalopathies cohort [14, 15, 31].

ECL-based analysis

Quantification of Tau and Aβ42 was performed using Human Total Tau Kit V-PLEX™ (Meso Scale Discovery®) and Human Aβ42 kit V-PLEX™ (Meso Scale Discovery®), respectively, following manufacturer's instructions. Quantification of α-synuclein was performed as described before [17, 18]. Tau and α-synuclein values were analysed logarithmically to the base of 10 when necessary for clear visualization of differences between studied groups.

ELISA analysis

Total tau levels were also measured using a commercially available ELISA according to the manufacturer's instructions (Innogenetics) [23]. Aβ42 was detected with a commercially available ELISA kit [INNOTEST® ß-AMYLOID(1–42) Innogenetics] following manufacturer's instructions. α-synuclein was analysed as described before [19, 35]. In addition, S100B protein was analysed as reported before [26, 27].

Statistical analysis

The ANOVA test followed by post-test Tukey's Multiple Comparison Test was used to compare the values from

different groups; $*p < 0.05$; $**p < 0.01$ $***p < 0.001$. The box plot was used for the graphs. ROC curves and statistical analyses were performed using Graph Pad Prism 5 software. Correlations (Pearson r) and statistical significance (p value) between data obtained from ELISA and ECL methodologies in the same set of samples were calculated.

Ethics

The present study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines and has been approved by the local ethics committee in Göttingen (No. 9/6/0). Informed consent was given by all study participants or their legal next of kin. All samples were anonymized.

Results

CSF levels of neurodegenerative diseases biomarkers in AD and sCJD

First, we examined whether Tau, Aβ42 and α-synuclein levels were altered in the CSF of AD and sCJD cases using the ECL-based MSD system (Fig. 1). ROC analysis was performed and AUC was calculated for each group in a comparative analysis between ECL and ELISA technologies (Table 1).

There was a statistically significant difference between Tau levels in both clinical groups when compared to control samples in agreement with previous reports [36]. While Tau levels are able to discriminate sCJD from control (AUC = 0.9931) and AD samples (AUC = 0.9583) with an excellent accuracy ($p < 0.001$), some overlap is observed between control and AD cases (AUC = 0.8125 and $p < 0.01$) (Fig. 1a; Table 1).

Low levels of Aβ42 were detected in AD ($p < 0.001$) and sCJD ($p < 0.05$) samples when compared to control cases (Fig. 1b) in agreement with previous data [33, 37]. Although no differences were observed between both clinical groups, Aβ42 levels can discriminate with higher accuracy controls from AD cases (AUC = 0.9097) than from sCJD samples (AUC = 0.7813) (Table 1).

Increased α-synuclein levels were detected in sCJD (AUC = 0.9306) when compared to control cases. No changes in α-synuclein levels could be detected between AD cases and control donors (AUC = 0.5030). Intriguingly, α-synuclein levels allow an excellent discrimination rate between AD and sCJD samples ($p < 0.001$, AUC = 0.9861) due to decreased levels, although not statistically significant, of α-synuclein in AD cases when compared to controls (Suppl. Table 1).

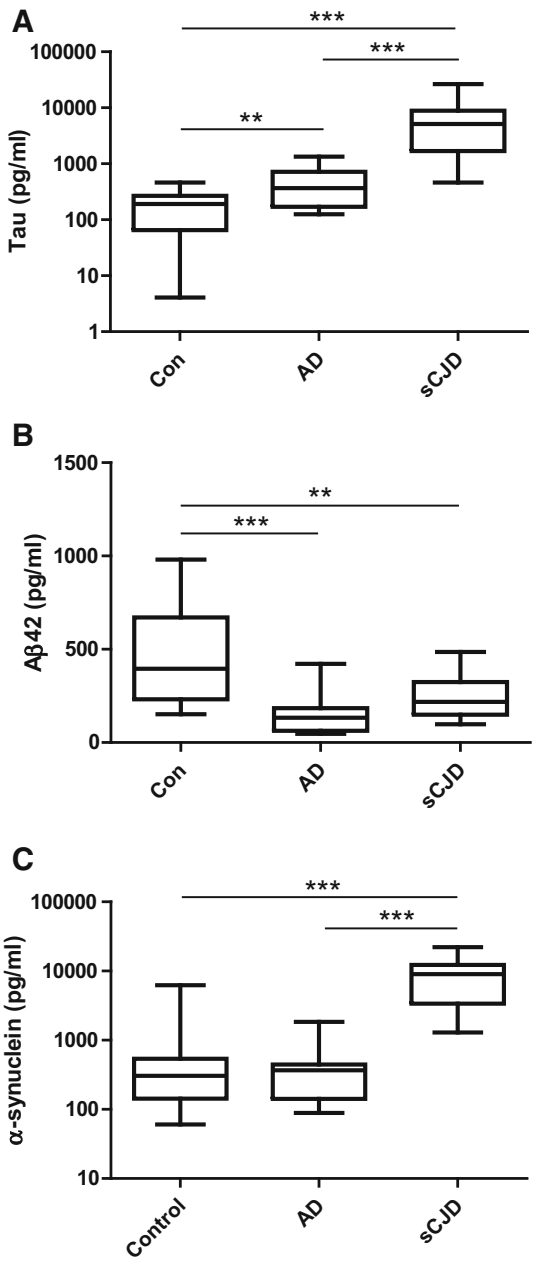

Fig. 1 ECL-based analysis of Tau, Aβ42 and α-synuclein in control, AD and sCJD cases. *Box plots* showing (**a**) Tau levels (**b**) Aβ42 levels and (**c**) α-synuclein levels in the CSF of Control, AD and sCJD patients. Tau and α-synuclein levels are displayed on a logarithmic scale (*y*-axis). Whiskers represent min to max for each group. ANOVA test followed by post-test Tukey's Multiple Comparison Test was used to compare the values from different groups. *P* values for the comparisons of the three groups are indicated in the figure: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

By comparing both detection systems, we found a good correlation between ECL and ELISA methods for Tau ($r = 0.81$) and Aβ42 ($r = 0.78$), while a weaker but positive correlation was observed for α-synuclein ($r = 0.61$) between both methods (Table 1). Increased AUC values

Table 1 Comparison of the clinical value of Tau, Aβ42 and α-synuclein biomarkers between ECL and ELISA methodologies. Regression values (*r*) correlating ECL and ELISA methodologies in the combined analysis of Control, AD and sCJD cases and AUC values for ELISA and ECL methodologies for each pair of groups

	r value	AUC					
		Con vs AD		Con vs sCJD		sCJD vs AD	
	ELISA vs MSD	ELISA	MSD	ELISA	MSD	ELISA	MSD
Tau	0.81	**0.8002**	**0.8125**	**0.9858**	**0.9931**	**0.9557**	**0.9653**
Aβ42	0.79	**0.8155**	**0.9097**	0.7058	0.7951	0.5489	0.7632
α-synuclein	0.61	0.6843	0.5139	**0.8269**	**0.9306**	0.7666	**0.9861**

AUC values greater than 0.8 are indicated in bold

are observed when using ECL methodology in comparison to ELISA kits. Increased AUC values are modest for Tau, but highly significant for Aβ42 and α-synuclein (Table 1).

Correlation between α-synuclein and Tau levels

Among the biomarkers analysed herein by ECL, Tau and α-synuclein levels present optimal discrimination rates between AD and sCJD patients. Thus, we explored a potential correlation between α-synuclein and Tau levels in sCJD and AD cases. A strong and significant positive relationship between the levels of both biomarkers was observed in sCJD patients using α-synuclein concentrations as measured by ECL and Tau concentrations as measured in both systems [ECL ($r = 0.85$) and ELISA ($r = 0.73$)] (Fig. 2a). A weaker but positive and significant correlation between α-synuclein and Tau ECL ($r = 0.85$) or ELISA ($r = 0.60$) data was also detected in AD cases where the levels of both proteins are significantly lower than those detected in sCJD (Fig. 2b). In addition, α-synuclein levels in sCJD significantly correlate with the levels of S100B ($r = 0.54$) (Suppl. Figure 1). S100B is an astroglial marker widely reported as a CSF biomarker for prion diseases, whose levels are highly upregulated in the CSF of CJD cases when compared to controls and AD [13]. α-synuclein levels were unrelated to the age of control and diseased patients (data not shown).

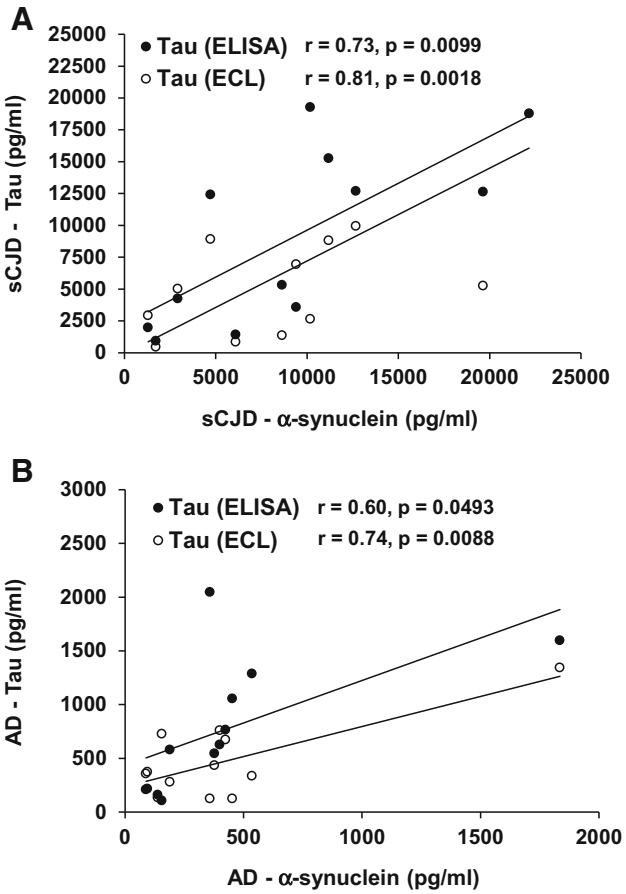

Fig. 2 Correlations between Tau and α-synuclein levels in AD and sCJD cases. Graphs showing the correlation between Tau and α-synuclein levels in the CSF of **a** sCJD and **b** AD cases. *R* values are indicated for each analysis. For Tau measurements, data obtained from ECL analysis (*empty circles*) and ELISA analysis (*full circles*) are plotted

Discussion

In the field of neurodegenerative diseases, the need for improved diagnostic accuracy urges for the identification of more reliable biomarkers as well as for the development of new optimized assays and methodologies for their quantification.

Development of quantitative assays for CSF biomarkers on the MSD platform has been discussed in several publications [5, 24] but detailed descriptions about their clinical performance for the differential diagnosis of neurodegenerative diseases are very limited.

The aim of the present work was to study whether the MSD platform presents a better performance in discriminating control, AD and sCJD cases than the well-established colorimetric ELISA platforms.

ECL-based quantification proves to be slightly superior (Tau) or significantly superior (Aβ42 and α-synuclein) in the discrimination of controls, AD and sCJD groups on

these groups of biomarkers, whose levels are deregulated in both diseases and currently used for their clinical diagnosis.

Low Aβ42 and high Tau levels are shown to be a characteristic feature in AD and sCJD cases, when compared to control samples and thus, an overlap between the detection of these biomarkers is commonly observed [32, 33]. The increased AUC values between the analysed groups for Tau and Aβ42 using ECL indicate that implementation of the ECL-based technologies system in the clinical work-up for both biomarkers could improve the diagnostic discrimination between AD and sCJD.

A new promising clinical outcome is derived from analysis of α-synuclein. The presence of an increased α-synuclein level in sCJD patients has already been reported during the last years. However, a large discrepancy on its clinical accuracy, most likely due to the different measurement tests used impeded the implementation in the clinical practice [16, 19, 20].

ECL shows superior performance to the established ELISA methods for the differential detection of α-synuclein in sCJD cases when compared to control and AD samples. This is in line with a previous report using a second generation of ELISA methodology, where only sCJD cases presented upregulated levels of α-synuclein in a cohort of several neurodegenerative diseases [20]. Interestingly, α-synuclein, contrary to Tau and Aβ42, is specifically regulated in sCJD. Although increased levels of α-synuclein have been reported in AD cases when compared to controls, these increases range from low to moderate and present no relevant clinical significance [34, 38].

Therefore, the introduction of α-synuclein in the clinical work-up of dementia diseases could improve the diagnostic accuracy and possibly monitor disease progression. Indeed, α-synuclein levels correlate with those observed for Tau, especially in sCJD. Since Tau has been extensively reported to be a marker of neuronal/axonal injury [6] and sCJD presents increased neuronal damage when compared to AD, it is tempting to speculate that α-synuclein could reflect the extent of synaptic damage in a similar manner as Tau reflects the extent of neuronal damage. In this line, we recently described that human α-synuclein in the CSF is mainly derived from neurons of the brain and spinal cord [22].

Since S100B levels in the CSF have been reported to reflect the disease-specific pathological mechanisms between sCJD and AD [13], the strong correlation between S100B levels and α-synuclein supports the idea that the differential α-synuclein levels in the CSF of sCJD and AD patients reflect the specific aetiology between both diseases.

On the other hand, CSF-Tau levels increased in parallel with CJD progression [29], while Tau levels remained stable along disease duration in patients suffering from AD and other non-prion dementias [2]. Thus, it will be interesting to study in larger cohorts of AD and sCJD samples if α-synuclein is able to track disease progression. In this regard, although α-synuclein is not clinically useful as a biomarker for α-synuclein-related disorders [10] and no association between α-synuclein levels in the CSF and PD severity has been observed [12], α-synuclein has been proposed to predict PD cognitive decline in one study [30]. New studies should also consider the presence of underlying modifying factors such as the presence of preclinical AD cases and co-morbidities (such as vascular events) which could influence the levels of the three CSF biomarkers analysed in the present study.

Major advantages of the ECL-based technology described here are: (1) Reduction in laboratory time: The ECL-based assay is performed in approximately 4 h. Conventional ELISAs sometimes take two days or longer due to overnight incubations, (2) Low volumes required for sample input, (3) Excellent reproducibility of quantification results: Compared to convention ELISA assays where up to 100 µl or more of precious samples are needed, the MSD platform requires only 25–50 µl diluted CSF, in our case diluted 1 in 8. Excellent reproducibility of the ECL-based system has also been reported (Kruse et al., manuscript in preparation).

Further studies on larger independent CSF cohorts of defined AD and sCJD patients, as well as in samples from patients suffering from other dementia types, are recommended to establish cut-off definitions and to validate the definitive performance of ECL measurements for Tau, Aβ42 and α-synuclein in the clinical routine for the differential diagnostic of neurodegenerative dementias.

However, the present data present a step forward toward their differential diagnosis. On one hand, from a methodological point of view, ECL-based MSD technology presents a high discrimination potential between control and diseased groups for Tau, Aβ42 and α-synuclein. On the other hand, we describe the gained value of α-synuclein measurements in the CSF as new sCJD biomarker which overcomes the overlap observed in classical dementia biomarkers (Tau, p-Tau and Aβ42) between AD and sCJD.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

Ethical standard The study has been approved by the appropriate ethics committee and have therefore been performed in accordance

with the ethical standards laid down in the 1964 Declaration of Helsinki. As stated in the text, all persons gave their informed consent prior to their inclusion in the study and samples were anonymised.

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Anexos

nature genetics

Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice

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Calcifications in the basal ganglia are a common incidental finding and are sometimes inherited as an autosomal dominant trait (idiopathic basal ganglia calcification (IBGC)). Recently, mutations in the *PDGFRB* gene coding for the platelet-derived growth factor receptor β (PDGF-Rβ) were linked to IBGC. Here we identify six families of different ancestry with nonsense and missense mutations in the gene encoding PDGF-B, the main ligand for PDGF-Rβ. We also show that mice carrying hypomorphic *Pdgfb* alleles develop brain calcifications that show age-related expansion. The occurrence of these calcium depositions depends on the loss of endothelial PDGF-B and correlates with the degree of pericyte and blood-brain barrier deficiency. Thus, our data present a clear link between *Pdgfb* mutations and brain calcifications in mice, as well as between *PDGFB* mutations and IBGC in humans.

Brain calcification is a common incidental neuroimaging finding with a prevalence ranging from ~1% in young subjects to >20% in the elderly[1,2]. Rarely, brain calcification is inherited as an autosomal dominant trait with clinical and genetic heterogeneity (IBGC). In patients with IBGC, calcifications occur in the basal ganglia and in certain other brain areas, including the cerebellum, thalamus and brainstem. These individuals display motor, cognitive and psychiatric symptoms but may also remain asymptomatic, despite prominent calcifications[3]. Thus far, mutations in *SLC20A2* leading to inactivation of the encoded inorganic phosphate transporter PIT2 have been linked to ~50% of IBGC cases[4–8]. A recent report also linked IBGC to mutations in *PDGFRB*, the gene encoding PDGF-Rβ (ref. 6). Two missense mutations were identified; however, the functional consequences of these alterations are currently unknown[6].

Here we report that mutations in *PDGFB*, the gene encoding PDGF-B, the main ligand for PDGF-Rβ, are a major cause of IBGC. PDGF-B is a growth factor for mesenchymal cells and has a particularly important role in the recruitment of pericytes during angiogenesis (reviewed in ref. 9). Pericytes fail to be recruited to developing blood microvessels in mice null for *Pdgfb* or *Pdgfrb*, thereby leading to vascular dysfunction and perinatal death[10–13]. Partially inactivating mutations in *Pdgfb* or *Pdgfrb* cause pericyte hypoplasia and milder renal and cardiovascular impairment compatible with adult survival[14–17]. Notably, mice with these mutations show impaired maturation of the blood-brain barrier (BBB)[18,19] resulting from a combination of activated endothelial transcytosis and abnormal astrocyte end-foot polarization[19]. So far, this phenotype has not been connected with any specific central nervous system (CNS) pathology, which is unexpected, given the assumed importance of the BBB for brain homeostasis. Here we link inactivating mutations in *PDGFB* to IBGC in humans and correlate pericyte deficiency and BBB impairment to an IBGC-like condition in mice.

We recently described a Serbian family (family S; **Fig. 1a**) with symmetrical IBGC in six family members from three generations[20]. After genome sequencing of two affected and one unaffected individual (**Fig. 1a**), we found 317,473 heterozygous genomic variants shared by the affected individuals but absent from the unaffected one (**Supplementary Table 1**). Of these variants, 998 were predicted to be protein-changing variants, including 5 presumably truncating variants, 2 of which were novel (**Supplementary Table 1**). The newly identified truncating variants were confirmed by Sanger sequencing; however, only the mutation c.433C>T (encoding p.Gln145*) in exon 4 of the *PDGFB* gene cosegregated with calcification in the remaining family members (**Fig. 1**). In an independent study,

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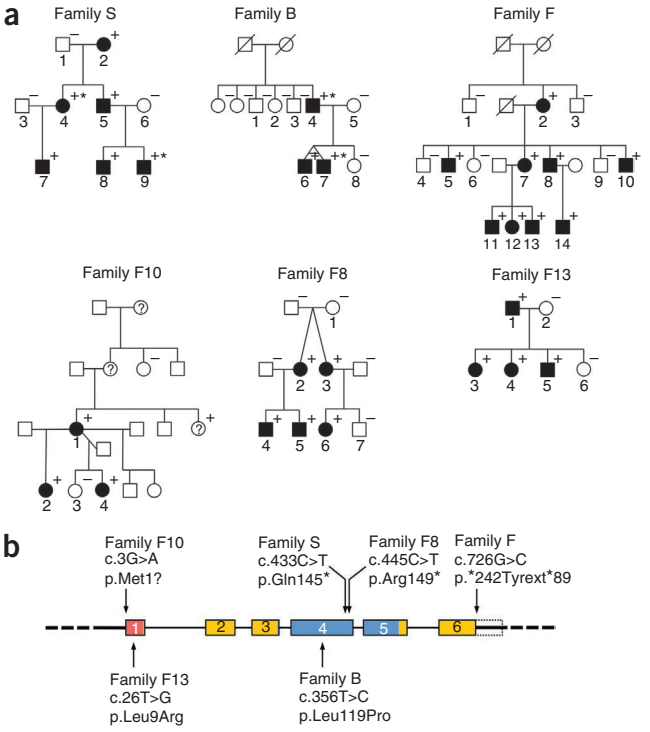
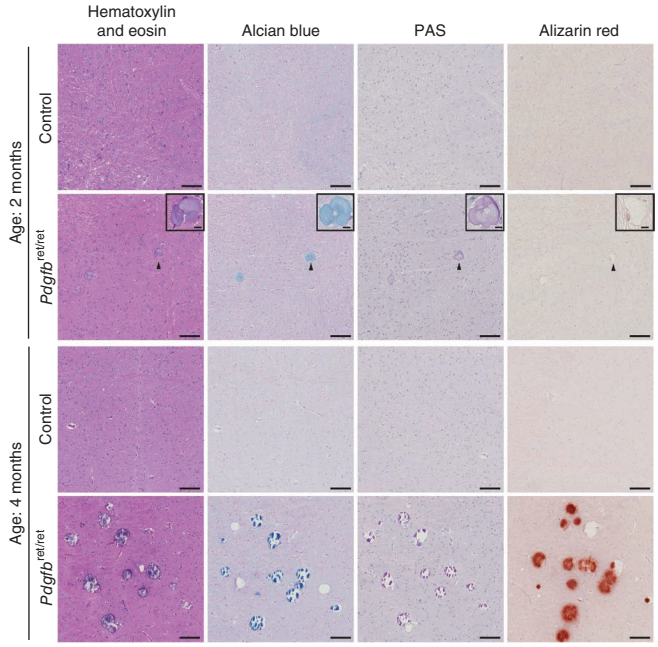


Figure 1 *PDGFB* mutations in IBGC. (**a**) Pedigrees of six families with IBGC with *PDGFB* mutations. Numbers below the symbols indicate individuals in whom CT scans were performed. Filled symbols, individuals affected with brain calcification, including both symptomatic and asymptomatic individuals; symbols with slashes, deceased individuals; symbols with question marks, individuals probably affected as inferred by history; +, mutation carriers; –, non-carriers; *, individuals in whom whole genomes or exomes were sequenced. (**b**) Schematic of the *PDGFB* gene with the positions of the IBGC-associated mutations indicated by arrows. The region coding for the mature form of the protein is highlighted in blue, and the regions coding for the signal peptide and propeptides are shown in red and yellow, respectively. The putative extended part of the nascent protein predicted to be translated from the c.726G>C allele is represented by a dotted box. Mutations predicted to severely impair the structure of the protein are shown above, and missense changes are shown below.

we performed exome sequencing in two individuals from a Brazilian family (family B) in whom a pair of identical twins and their father had IBGC. We found that 1,400 protein-changing variants were shared by the affected individuals (**Fig. 1**) and were not reported in dbSNP135 (**Supplementary Table 1**), including a c.356C>T substitution (encoding p.Leu119Pro) also located in *PDGFB* exon 4. To confirm *PDGFB* as a new causative gene for IBGC, we performed Sanger sequencing of its entire coding region in 30 additional families and 22 sporadic cases who were negative for mutations in *SLC20A2* and *PDGFRB* (ref. 5 and A.W., M.J.S., M.G.-M., A.D. and R.L.S. *et al.*, unpublished data). In these cases, we detected four additional *PDGFB* mutations (c.3G>A, p.Met1?; c.26T>G, p.Leu9Arg; c.445C>T, p.Arg149*; c.726G>C, p.*242Tyrext*89) that segregated with the presence of IBGC (**Fig. 1a**). The 6 identified *PDGFB* mutations were absent from more than 12,000 chromosomes from the National Heart, Lung and Blood Institute (NHLBI) Exome Variant Server, as well as from a large number of ancestry-matched controls and 173 in-house exomes (**Supplementary Table 2**). Of a total of 31 mutation carriers in the 6 families (17 male; mean age of onset of 23.9 ± 15.7 years), 30 with available computed tomography (CT) scans had neuroimaging findings characteristic of IBGC (**Supplementary Fig. 1**), and 12 had motor signs including dystonia/dyskinesias ($n = 8$), parkinsonism ($n = 3$) and chorea ($n = 3$). Cognitive impairment or psychiatric disease was present in 9 mutation carriers, and migraine or headache was present in 13 mutation carriers (**Supplementary Table 3**).



All six *PDGFB* mutations are predicted to lead to loss of protein function. To test whether loss-of-function mutations affecting PDGF-B might be a direct cause of brain calcification, we analyzed mice with hypomorphic *Pdgfb* alleles. In 4-month-old homozygous PDGF-B retention-motif knockouts ($Pdgfb^{ret/ret}$)[14] but not in littermate controls, alizarin red staining detected clusters of calcified nodules in the midbrain and thalamus (**Fig. 2** and data not shown). The nodules were spherical or ovoid laminated acellular structures of ~20–100 µm in diameter, also visible by staining with hematoxylin and eosin, alcian blue and periodic acid shiff (PAS) (**Fig. 2**). At 2 months, smaller laminated nodules were detected in the midbrain and thalamus of $Pdgfb^{ret/ret}$ mice, but they were few in number and not positive for staining with alizarin red (**Fig. 2** and data not shown). These data suggest that *Pdgfb* hypomorphism causes the formation of matrix deposits that expand in size and number and eventually become mineralized. Because IBGC is progressive with age in humans, we also assessed 1-year-old $Pdgfb^{ret/ret}$ mice and controls. At this time, $Pdgfb^{ret/ret}$ mice showed abundant calcifications at multiple locations in the basal forebrain, thalamus, midbrain and pons, as detected by histological staining (**Fig. 3a**), micro-CT scan (**Fig. 3b**), magnetic resonance imaging (MRI) (**Fig. 3c**) and stereomicroscopy (**Supplementary Fig. 2**). Micro-CT scans showed bilateral punctate hyperdense lesions (**Fig. 3b**) with a mean density of 1,460 ± 260 Hounsfield units, indicating structures with bone density. Immunohistochemistry showed that these lesions were surrounded by reactive astrocytes and strongly CD45-positive

Figure 2 Calcified inclusions in the brains of $Pdgfb^{ret/ret}$ mice. Sections of the midbrain of 2- and 4-month-old $Pdgfb^{ret/ret}$ mice stained with hematoxylin and eosin show spheroid inclusions positive for staining with alcian blue and PAS, which are absent in littermate controls. In 4-month-old animals, these inclusions develop positivity for staining with alizarin red. Images are representative of analyses made on three mice per genotype. Scale bars, 100 µm (10 µm in insets). Arrowheads indicate the lesion shown in the inset at ×10 higher magnification.

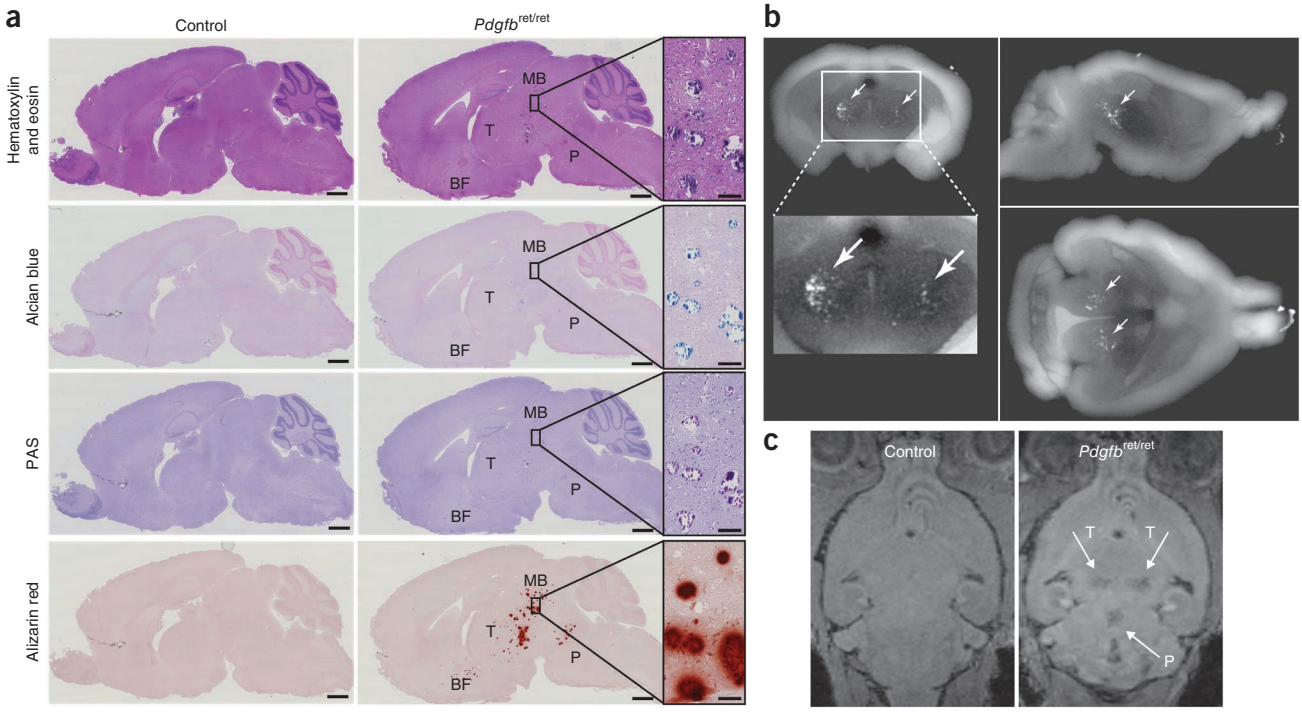


Figure 3 Progressive brain calcification in 1-year-old *Pdgfb*$^{ret/ret}$ mice. (**a**) Sagittal brain sections of a 1-year-old *Pdgfb*$^{ret/ret}$ mouse show calcified nodules in four distinct anatomical regions: basal forebrain (BF), thalamus (T), midbrain (MB) and pons (P) that are absent in the littermate control. A single calcospheroid can be seen in the medulla. Scale bars, 1 mm (100 µm in insets). Images are representative of analyses made on four mice per genotype. (**b**) Micro-CT scan of the brain of a 1-year-old *Pdgfb*$^{ret/ret}$ mouse. Coronal (left), sagittal (upper right) and axial (lower right) maximum-intensity projections are shown. The contrast of soft-tissue structures was increased by previous incubation in iodinated contrast agent. Note the bilaterally visible punctiform bone-dense structures within the midbrain-thalamus region (arrows). (**c**) Axial views of T1-weighted manganese-enhanced MRI scans of a 1-year-old *Pdgfb*$^{ret/ret}$ mouse and a littermate control. Images are representative of analyses performed on three mice per genotype. Postmortem analysis of each individual verified that hypointense areas corresponded with calcifications visualized by the histostains shown in **a**.

microglia (**Supplementary Fig. 3**). We next assessed the mineral content of the mouse lesions by scanning electron microscopy combined with energy-dispersive X-ray spectroscopy analysis (**Fig. 4**). This technique showed that calcium and phosphorus were the main constituent elements of the lesions, suggesting that the majority of their content was calcium phosphate. Taken together, these data suggest that the histological appearance, anatomical location and chemical composition of calcified lesions are highly similar in *Pdgfb* hypomorphic mice and humans with IBGC.

In the brain, PDGF-B is expressed in endothelial cells and neurons[9]. To clarify whether any of these sources are specifically relevant for IBGC pathology, we analyzed *Pdgfb*-null mice[10] rescued to adulthood by transgenic re-expression of PDGF-B in the endothelium. These mice lack neuronal PDGF-B expression but express endothelial PDGF-B at levels that depend on the copy number of the rescue allele (*R26P*)[19]. We found that *Pdgfb*$^{-/-}$ mice with two copies of the rescue allele (*Pdgfb*$^{-/-}$; *R26P*$^{+/+}$) failed to develop calcifications altogether (**Fig. 5**). However, *Pdgfb*$^{-/-}$ mice with one copy of the rescue allele (*Pdgfb*$^{-/-}$; *R26P*$^{+/0}$), hence expressing 50% less endothelial PDGF-B[19], developed substantial brain calcification at 1 year of age (**Fig. 5a**). In comparison with *Pdgfb*$^{ret/ret}$ mice, *Pdgfb*$^{-/-}$; *R26P*$^{+/0}$ mice developed smaller, fewer and more variable lesions but with similar anatomical location and histological appearance (**Fig. 5b**). These data exclude a role for neuronal PDGF-B and correlate levels of endothelial PDGF-B with brain calcifications in mice.

Our finding of *PDGFB* mutations in six independent families of different ancestry (6/32; 18.8%) establishes *PDGFB* as a key new gene in IBGC. So far, no *PDGFB* mutations have been found in sporadic IBGC cases. On the basis of available data for the three IBGC-relevant genes identified, *PDGFB* is the second most commonly mutated gene after *SLC20A2* (refs. 4–8). It is likely that additional IBGC-causing *PDGFB* mutations will be found, possibly also including other types

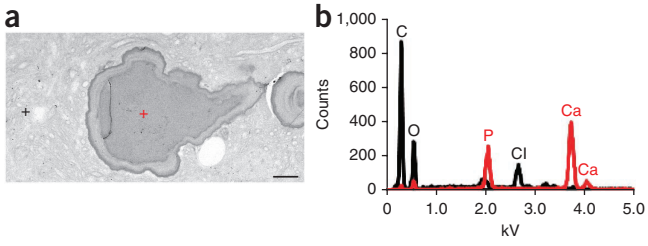


Figure 4 Elemental analysis of calcified nodules in mouse brain. The laminated nodules of a 1-year-old *Pdgfb*$^{ret/ret}$ mouse were examined with scanning electron microscopy combined with energy-dispersive X-ray spectroscopy. Two blocks of fixed tissue and several nodules on the same block were analyzed. (**a**) Scanning electron microscopy image of a lesion with laminated nodules. A nodule is located at the center of the image. Black and red plus signs indicate areas where spectral analysis was performed; the red plus sign is located in the middle of the nodule, and the black plus sign is located in the neighboring intact parenchyma. Scale bar, 10 µm. (**b**) Energy-dispersive X-ray spectroscopy spectra from the nodule (red line) and neighboring parenchyma (black line) shows that the nodule contains phosphorous and calcium, indicative of calcium phosphate being a primary constituent. Small peaks seen in the spectra obtained from brain parenchyma at 1.91 kV and 3.16 kV correspond with osmium and uranium, respectively, and are derived from the fixatives (black line).

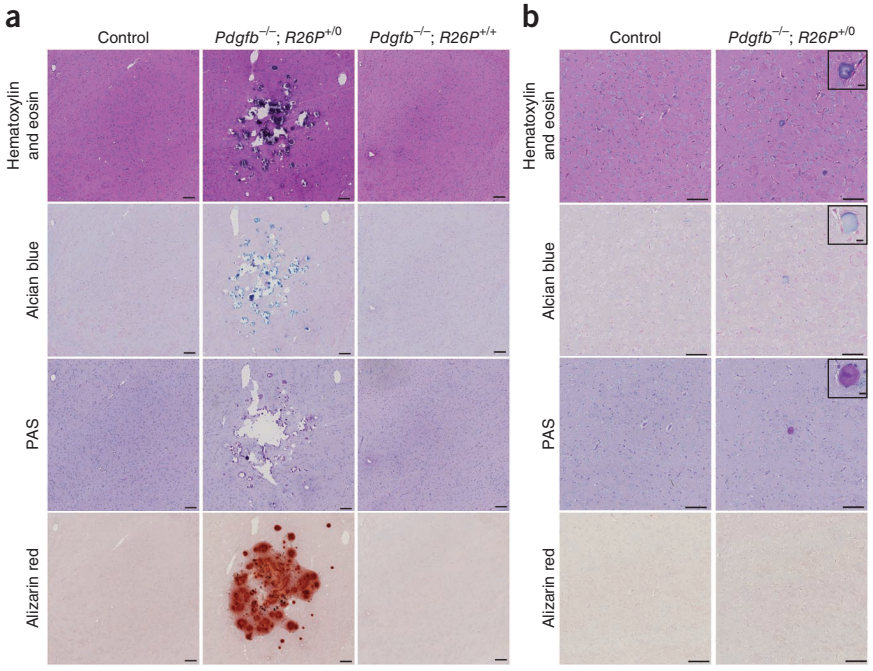


Figure 5 Brain pathology of *Pdgfb*$^{-/-}$; *R26P*$^{+/0}$ mice. (**a**) Laminated nodules in the thalamus of 1-year-old *Pdgfb*$^{-/-}$; *R26P*$^{+/0}$ mice visualized by staining with hematoxylin and eosin, alcian blue and PAS have developed positivity for staining with alizarin red. Calcified nodules are absent in littermates (control and *Pdgfb*$^{-/-}$; *R26P*$^{+/+}$ mice). Images are representative of analyses performed on four mice per genotype. (**b**) A laminated nodule in the thalamus of a 5-month-old *Pdgfb*$^{-/-}$; *R26P*$^{+/0}$ mouse visualized by staining with hematoxylin and eosin, alcian blue and PAS is negative for staining with alizarin red. These structures are absent in the littermate control. Images are representative of analyses performed on five mice per genotype. Note the close proximity of the laminated nodule to the microvessel (inset, hematoxylin and eosin staining). Scale bars, 100 μm (10 μm in insets).

of mutations, such as gene deletions or rearrangements. Of note, somatic rearrangement in *PDGFB* is a major cause of dermatofibrosarcoma protuberans, a slowly growing skin tumor (reviewed in ref. 9). These rearrangements increase PDGF-B synthesis and secretion, leading to autocrine growth stimulation and the neoplastic transformation of fibroblasts. Furthermore, somatic gain-of-function mutations in *PDGFRB* and *PDGFRA* are causative in certain leukemias and in a proportion of gastrointestinal sarcomas (reviewed in ref. 9). However, IBGC appears to be the first disease found to be caused by hereditary loss-of-function mutations in PDGF or PDGF receptor genes. Of the six *PDGFB* mutations described here, two are nonsense mutations (encoding p.Gln145* and p.Arg149* alterations) predicted to delete critical parts of the PDGF-B protein, including essential cysteine residues and receptor-binding loops[21]. One mutation replaces the start methionine (encoding p.Met1?), leading either to a complete lack of PDGF-B (p.0) or to a new translation initiation site up- or downstream of the original one, possibly with an alternative reading frame. Either way, the endogenous signal sequence for secretion is deleted. One missense mutation (encoding p.Leu9Arg) inserts a charged residue into the signal peptide, which is predicted to disrupt it. Of the remaining two missense mutations, one (encoding p.Leu119Pro) inserts a proline in a predicted receptor-binding loop, and the other (encoding p.*242>Tyrext*89) converts the translation stop site into a codon for tyrosine, leading to an elongation of the reading frame by 89 codons. The functional consequences of the latter mutation are unclear, but previous work on the PDGF-A protein showed that C-terminal protein extension inhibited its biological function[22]. In addition to the mentioned evidence for loss of function inferred through available structure-function data for PDGF proteins, we have provided direct evidence for a causative role for PDGF-B loss of function in brain calcification in mice. PDGF-B produced from the *Pdgfb*^{ret} allele (PDGF-B–ret) is C-terminally truncated and corresponds to the hypothetical human protein resulting from the p.Thr212* alteration[14]. Although PDGF-B–ret binds and activates PDGF receptors normally, it has reduced affinity for heparate sulfate proteoglycans[23], which probably alters the local concentrations and bioavailability of PDGF-B in tissues (reviewed in ref. 9). In *Pdgfb*$^{-/-}$; *R26P*$^{+/0}$ and *Pdgfb*$^{-/-}$; *R26P*$^{+/+}$ mice, *Pdgfb* expression from the *R26P* allele is confined to endothelial cells but occurs at levels that are likely lower than for wild-type *Pdgfb*, as fewer pericytes are recruited[19]. These mice are therefore PDGF-B expression hypomorphs of different degree.

Taken together, the analysis of three different hypomorphic mouse mutants correlates the degree of functional PDGF-B hypomorphism with the abundance and progression of brain calcification. These analyses further show that brain calcification is progressive with age and that the early lesions, observed already at 2 months of age in *Pdgfb*$^{ret/ret}$ mice, are non-calcified. Notably, the mouse data also provide a correlation between brain calcification and the extent of pericyte loss and BBB impairment reported earlier[19].

A detailed autopsy of a case of familial IBGC provides further support for a connection with BBB dysfunction. In this case, calcifications were spatially associated with perivascular fibrinogen levels, prompting the interpretation that plasma proteins passing through a disrupted BBB may form a nidus for mineral deposition[24]. A causal role for BBB dysfunction in the pathogenesis of IBGC is also supported by the occurrence of blood vessel–associated brain calcifications in mice deficient for occludin, a component of epithelial and endothelial tight junctions[25], as well as by the occurrence of band-like calcification with simplified gyration and polymicrogyria in humans, a syndrome caused by loss-of-function mutations in the occludin gene (*OCLN*)[26]. Similar patterns of brain calcification as those reported here in *Pdgfb* hypomorphic mice have been described in 9- to 14-month-old vitamin D receptor (*Vdr*) knockout mice[27], as well as in mice transduced with interferon-γ–expressing viral vectors[28]. Although BBB impairment was not reported in those studies, it is conceivable that the neuroinflammatory responses elicited by interferon-γ perturbs BBB function. Brain calcification is further associated with a number of chronic as well as acute brain disorders, where BBB disruption has been described, including hypoxia, Alzheimer's disease, vascular dementia, Down's syndrome, Lewy body disease, epilepsy and brain tumors[29–32].

Future challenges will include elucidation of how different genetic etiologies for IBGC, such as loss of PDGF-B–PDGF-Rβ signaling on the one hand and loss of PIT2-mediated phosphate transport on the other, converge into a common pathogenesis mechanism. Currently, it is difficult to envisage a mechanistic link. No significant disturbance was observed in serum electrolyte concentrations, including those of Ca^{2+} and PO_4^{3-}, in hypomorphic mouse *Pdgfb* mutants

(**Supplementary Table 4**), which is also in agreement with the situation in human IBGC. Moreover, work on cultured smooth muscle cells has implicated PDGF-B–PDGF-Rβ signaling as an inducer of phosphate transport and calcification through the activity of the inorganic phosphate transporter PIT1 (ref. 6), a function that contrasts paradoxically with the protective role of PDGF-B against brain calcification, as elucidated in the present study.

In conclusion, we identify loss-of-function mutations in the gene encoding PDGF-B as a cause of IBGC in humans and we report a similar condition in mice deficient for PDGF-B. Our identification of the first disease-causing loss-of-function mutations in *PDGFB* leads to new directions where the biological and pathological functions may fall outside of the classical contexts of PDGF activities in the regulation of cell proliferation and migration. Further studies should now be directed toward confirming pericyte and BBB impairment in human IBGC, as well as clarifying how PDGF-B deficiency, pericyte loss and BBB impairment are mechanistically linked to brain calcification in mice. Such insights may provide new therapeutic opportunities for IBGC and other conditions that lead to brain calcification.

URLs. NHLBI Exome Variant Server, http://evs.gs.washington.edu/EVS/; 1000 Genomes Project, http://www.1000genomes.org/; dbSNP, http://www.ncbi.nlm.nih.gov/snp.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequences are available at NCBI for the PDGF-B gene (NC_000022.10), transcript (NM_002608.2) and protein (NP_002599.1).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.R.M.O., C.B., C.K. and V.S.K. initiated the project, which was subsequently developed and led jointly by A. Keller, A.W., M.J.S., C.B., C.K. and J.R.M.O. A. Keller, A.W., M.J.S., K.L., K.Z., I. Navas, C.B., C.K. and J.R.M.O. conceived the experiments. A. Keller, E.J.R., M.H., R.R., I.A., M.A.M., E.R., M.C.W., A.B. and A. Kaech performed the mouse experiments, which were financially supported by C.B. and A.A. A.W., M.J.S., M.G.-M., A.D., R.L.S., R.R.L., A.O.-U., G.N., J.E.G.d.C., K.L., V.D., A.C., I.P., J.M.M., M.Z., K.Z., J.K., E.S., J.M.P., I. Navas, M. Preuss, C.D., M.J., M. Paucar, P.S., K.S., H.R.K.K., I. Novaković, I.L.B., G.D., D.H., V.S.K., D.C., D.H.G., G.C., C.K. and J.R.M.O. recruited and examined patients, collected and analyzed human DNA and/or interpreted genetic data. C.B., A. Keller, A.W., M.J.S., C.K. and J.R.M.O. wrote the manuscript with critical input from A.D., K.L. and K.Z.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Genetic analysis. A description of how the human research subjects and their groups were recruited, chosen and investigated, an outline of clinical phenotyping criteria and methods for brain imaging are provided in the **Supplementary Note**. Briefly, patients with IBGC were identified by a process of medical record review, interviews and neurological examination followed by neuroimaging by computed tomography for establishment of a correct diagnosis. All patients and healthy family members gave written informed consent to participate in this study, which was approved by the ethics boards of each of the participating clinical centers. The ethics boards included the Ethics committee for Human subjects, Federal University of Pernambuco, Recife, Brazil; Comité de Ética de la Fundación Pública Galega de Medicina, Xenómica-Servicio Galego de Saude-Xunta de Galicia, Santiago de Compostela, Spain; Ethikkommission der Universität zu Luübeck, Luübeck, Germany; Etiĉ ki komitet Medicinskog Fakulteta Univerziteta u Beogradu, Belgrade, Serbia; Office of the Human Research Protection Program (OHRPP), Los Angeles, California, USA; Comité de Protection des Personnes, Ile de France II, Paris, France; Regionala etikprövningsnämnden i Stockholm; and Ethics Committee of the Avicenna Research Institute, Tehran, Iran. Genome sequencing was performed in two definitely affected (subject 4 (III-5) and subject 9 (IV-3)) and in one unaffected (paternal cousin; not shown on the pedigree) members of family S at Complete Genomics. Reads were aligned to the reference genome (hg19 Build 37). Variation files were analyzed using cga tools (Complete Genomics), software package R and ANNOVAR. Analysis was conducted as follows. First, using the calldiff function from cga tools, difference sets were built, both with respect to subject 4 (III-5) and the paternal cousin and with respect to subject 9 (IV-3) and the paternal cousin. Only high-quality variants were retained in this step. Second, an intersection set of both difference sets was built, and only variants that corresponded to the assumed dominant genetic model were included. Finally, remaining variants were annotated using the annotation software ANNOVAR (auto annovar Rev.502)[33,34]. The next filter steps included (i) a requirement that the variant be located in an exon or a splice site, (ii) exclusion of synonymous variants and (iii) a requirement that the variant was absent in public databases (dbSNP132, Exome Variant Server and 1000 Genomes Project). There were two candidate variants that were verified by Sanger sequencing and tested for segregation in all available family members of family S. In a family with IBGC from Brazil (family B), DNA samples from three affected family members (a father and two identical twin sons) and seven other family members were available. Mutations in *SLC20A2* and *PDGFRB* had previously been excluded in one affected family member. Exome sequencing was carried out in the father and one of the affected children at the sequencing facility of the Fundación Pública Galega de Medicina Xenomica. Enrichment was performed using the SureSelect Human All Exon v1 kit (Agilent Technologies), which targets 37.8 Mb of genomic sequence, following the manufacturer's protocol. Each exome library was sequenced using one-quarter of a SOLiD 4 slide (Life Technologies) as paired-end reads (50 + 35 bp). Reads were aligned to the reference genome (hg19 Build 37) using SOLiD BioScope software version 1.3.0 and GATK version 2.1. Coverage and quality measures for each sample are summarized in **Supplementary Table 1**. Variants were identified using SOLiD BioScope version 1.3.0, and annotation of the variants was performed with ANNOVAR (version 24 June 2012). The R statistical package was used to compare the variants in both exomes and to calculate statistics for the variants. Theoretical protein-changing variants present in coding regions or splice sites of both affected individuals and absent in dbSNP135 were considered candidates (**Supplementary Table 1**). Although not novel, variants present in other databases (1000 Genomes Project and Exome Variant Server) with minor allele frequency (MAF) < 0.005 were not ruled out for further analysis. To screen additional *SLC20A2* mutation–negative families with IBGC and sporadic cases for mutations in *PDGFB*, we designed primers that covered each of the six exons and at least 50 bp of the adjacent intronic regions. We performed Sanger sequencing of the index subject of each family and of sporadic cases (primer sequences are listed in **Supplementary Table 5**; the annealing temperature was 60 °C for all amplicons). We added 10% DMSO to the PCR for amplification of *PDGFB* exons 1 and 6. Detected new, possibly disease-causing variants were tested for segregation in all available family members by Sanger sequencing. Finally, controls were tested for the respective variants by Sanger sequencing, next-generation sequencing or restriction analysis (**Supplementary Table 2**). Additionally, the complete coding region of *PDGFRB* was sequenced in all 32 families and in 22 sporadic cases. Primers used for amplifying *PDGFRB* exons were as previously published[6]. Sanger sequencing was carried out in an ABI3730xl, the obtained traces were analyzed with the Staden package, and all identified variants were searched in dbSNP and the Exome Variant Server.

Mice. The following transgenic mouse lines were used in this study: PDGF-B retention-motif knockout mice (*B6-Pdgfb<tm3Cbet*) in the C57BL/6 genetic background[14]; PDGF-B knockout (*Pdgfb*$^{-/-}$)[10] (*Pdgfb<tm1Cbet*); Tie2Cre[35] (*Tg*(*Tek-cre*)1Ywa); and Rosa26-hPdgfb (*R26P*)[19] (*Gt*(*ROSA*)*26Sor<tm1*(*Pdgfb*)*Cbet*>). The generation of *R26P*$^{+/0}$ mice (*Tg*(*Tek-cre*)1Ywa; *Pdgfb<tm1Cbet*>; *Gt*(*ROSA*)*26Sor<tm1*(*Pdgfb*)*Cbet*>) in the hybrid C57BL/6/129sv genetic background has been described elsewhere[19]. Both males and females were used for experiments, and no sex-related phenotypic differences were observed. Animal experiments were approved by Stockholm's North Ethical Committee for Animal Research (Sweden) and by the Cantonal Veterinary Office (Switzerland).

Manganese-enhanced MRI. Before scanning, mice received three intraperitoneal injections of $MnCl_2$ (40 mg/kg, 20 mM in water and bicine, pH 7.4) at 12-h intervals[36]. Manganese-enhanced MRI was performed 24 h after the last injection with $MnCl_2$. Mice were imaged in a 4.7-Tesla small-animal MRI system (Bruker Pharmascan) under isoflurane anesthesia. Mice were placed in a bed equipped with a mouse whole-body radio-frequency transmitter coil and a mouse head surface-coil receiver (Bruker Pharmascan). During the scan, body temperature was maintained with a warming blanket. T1-weighted brain images were obtained using a three-dimensional gradient-echo sequence (TR, 15 ms; TE, 2.5 ms; FA, 20 degrees; average, 10; matrix, 265/265/126; field of view, 2 × 2.56 × 2 cm^3; acquisition time, 1 h; voxel size, 78 × 100 × 156 μm^3).

Scanning electron microscopy and energy-dispersive X-ray spectroscopy. Mice were anesthetized and transcardially perfused with 3% formaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The brain was post-fixed in 3% formaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h, and post-fixation was then carried out with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2). Subsequently, samples were block stained with 2% uranyl acetate in water for 1 h, dehydrated in an ethanol series and embedded in epon (Catalys). Epon blocks were trimmed with a diamond knife, mounted on a standard aluminum stub for scanning electron microscopy with conductive carbon and coated with a carbon layer by electron beam evaporation to render the surface conductive. Block faces were analyzed at an acceleration voltage of 15 kV in a Zeiss Supra 50 VP scanning electron microscope (Carl Zeiss) using the in-lens secondary electron detector and an EDAX Genesis energy-dispersive X-ray detector (Ametek).

Serum analysis of electrolytes and albumin. Retro-orbital blood sampling was performed under isoflurane anesthesia, and subsequent serum isolation was carried out using BD Microtainer Serum Separator Gel tubes (Becton Dickinson). Serum concentrations of albumin, phosphate, magnesium and calcium were measured by chromogenic tests on the COBAS8000 autoanalyzer (Roche Diagnostics).

Micro-CT scanning. Micro-CT scanning was performed on whole-brain samples fixed in 4% paraformaldehyde. To increase soft-tissue contrast, brain specimens were incubated in iopromid (Ultravist 300, Bayer Schering Pharma) at 4 °C for 20 h. Micro-CT imaging was carried out in a high-resolution micro-CT (Skyscan 1176, Bruker micro-CT) with the following scan parameters: tube voltage, 50 kV; tube current, 500 mA; exposure time, 275 ms; rotation step, 0.5°; covered angle, 360°; voxel size, 18 × 18 × 18 μm^3; field of view, 20 × 35 × 35 mm^3; filter, 0.5-mm aluminum. Images were reconstructed with a modified Feldkamp algorithm, using the following parameters: beam hardening correction, 35%; ring artifact reduction, 9; Gaussian smoothing kernel with full width at half maximum of 4 voxels. Images were calibrated to Hounsfield units via measurement of a phantom. To assess the density of apparent lesions, region-of-interest (ROI) analysis was performed.

Histochemistry and immunohistochemistry. Mice were transcardially perfused under anesthesia with PBS followed by 4% paraformaldehyde in PBS, pH 7.2. Brains were removed and embedded in paraffin. Tissue sections (2 µm) were stained for hematoxylin and eosin, PAS and alcian blue using standard protocols. For staining with alizarin red, deparaffinized and hydrated sections were incubated for 1 h in 0.5% alizarin red in borate buffer (pH 9.0) and for 5 min in 0.5% alizarin red in PBS (pH 7.0) at room temperature. GFAP immunostaining was performed on 2-µm paraffin sections using a rabbit antibody to GFAP from DakoCytomation (20334) at 1:200 working dilution followed by HRP-conjugated goat secondary antibody to rabbit from Jackson ImmunoResearch. Stained paraffin sections were scanned using a NanoZoomer (Hamamatsu Photonics) equipped with a 20× objective (UPlanSapo, 0.75 NA, Olympus). Images were analyzed using Digital Image Hub software (SlidePath). Brain coronal vibratome sections (30 µm) were incubated in blocking/permeabilization solution (1% BSA, 0.5% Triton X-100 in PBS) overnight at 4 °C and were then incubated in primary antibody solution and subsequently in secondary antibody solution, both overnight at 4 °C. Sections were mounted in Prolong Gold Antifade reagent with DAPI (Invitrogen). The following primary antibodies were used: rat antibody to CD45 from BD Pharmingen (553076) at 1:100 working dilution and goat antibody to podocalyxin from R&D Systems (AF 1556) at 1:200 working dilution. Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch. Images were captured using a CLSM Leica SP5 (Leica Microsystems) equipped with a 20× objective (multi-immersion, 0.7 NA). Images were analyzed using the image-processing software Imaris (Bitplane) and with Adobe Photoshop and Adobe Illustrator. Stereomicroscope images were taken with an SZX12 microscope equipped with SZX-FBV (Ex400-440/Em475) filter and DP71 camera (Olympus).

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