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Análise integrativa dos mecanismos de patogênese em doenças lisossômicas

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CMA	autofagia mediada por chaperonas
DLs	doenças lisossômicas
DS	dermatan sulfato
GAGs	glicosaminoglicanos
Hh	hedgehog
HS	heparan sulfato
HSPGs	proteoglicanos de heparan sulfato
IDUA	alfa-L-iduronidase
IR	receptor de insulina
JNCL	lipofuscinose ceróide neuronal juvenil
KS	queratan sulfato
LIMPs	proteínas integrais lisossômicas de membrana
LMPs	membrana associadas ao lisossomo
M6P	manose 6-fosfato
MEC	matriz extracelular
MHC-II	complexo principal de histocompatibilidade de classe II
MMPs	metaloproteinases
MPS	mucopolissacaridoses
mTOR	proteína quinase alvo mecanicista da rapamicina
NPC	Niemann-Pick tipo C
NK	natural killer
ORL	organelas relacionadas ao lisossomo
RE	reticulo endoplasmático
ROS	espécies reativas de oxigênio
SNC	sistema nervoso central
TFE3	fator de transcrição E3
TFEB	fator de transcrição EB
TLR4	receptores toll like-4

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Resumo

Doenças lisossômicas (DLs) causam acúmulo intracelular de substratos e deficiência no tráfego de macromoléculas. O armazenamento do substrato pode impactar uma ou várias vias que contribuem para o dano celular. Vias morfogênicas e de crescimento como Hedgehog (Hh), mTOR e insulina estão envolvidas na fisiopatologia das DLs. A via Hh é afetada com expressão anormal e alterações nos níveis e distribuição de proteínas Hh. mTOR pode ter um atraso em sua reativação e desregular o término da autofagia e manutenção dos lisossomos. A resistência à insulina causada por mudanças nas jangadas lipídicas também foi descrita em diferentes DLs. Portanto, exploramos como estas vias podem estar relacionadas, mostrando que uma abordagem de medicina de redes pode ser uma ferramenta valiosa para o melhor entendimento da patogênese em DLs. Assim, utilizamos ferramentas de biologia de sistemas para investigar novos elementos associados com a dilatação da aorta em mucopolissacaridoses (MPS). Identificamos genes candidatos associados com processos biológicos, incluindo respostas inflamatórias, deposição de colágeno e metabolismo de lipídeos que podem contribuir para a patogênese da dilatação da aorta em MPS I e MPS VII. Por último, foram identificados novos genes candidatos e vias que convergem em mecanismos funcionais envolvidos nos defeitos de formação precoce do circuito neural, no qual podem indicar pistas sobre o comprometimento cognitivo em pacientes com MPSII. Tais mudanças moleculares durante o neurodesenvolvimento podem preceder as evidências morfológicas e clínicas, destacando a importância do diagnóstico precoce e do desenvolvimento de novas drogas.

Abstract

Lysosomal storage diseases (LSDs) cause intracellular accumulation of substrates and deficiency in trafficking of macromolecules. The substrate storage can impact one or several pathways which contribute to cell damage. Morphogenic and growth pathways such as hedgehog (Hh), mTOR and insulin are involved in the pathophysiology of LSDs. Hh pathway is affected with abnormal expression and changes in protein levels. mTOR may have a delay in reactivation and deregulate termination of autophagy and reformation of lysosomes. Insulin resistance caused by changes in lipids rafts also has been described in different LSDs. Therefore, we explored how specific signaling pathways can be related to specific LSDs, showing that a system medicine approach could be a valuable tool for the better understanding of LSD pathogenesis. Moreover, we used systems biology tools to investigate new elements that may be involved in aortic dilatation in Mucopolysaccharidoses (MPS) syndrome. We identified candidate genes associated with biological processes related to inflammatory responses, deposition of collagen, and lipid metabolism that may contribute to pathogenesis of aortic dilatation in the MPS I and MPS VII. Finally, we identified new candidate genes and pathways that converge into functional mechanisms involved in early neural circuit formation defects and could indicate clues about cognitive impairment in patients with MPSII. Such molecular changes during neurodevelopment may precede the morphological and clinical evidence, highlighting the importance of an early diagnosis and the development of new drugs.

1. Introdução

1.1. O lisossomo

Descrito pela primeira vez em 1950 por Christian de Duve, o lisossomo é conhecido como a principal organela celular capaz de degradar e reciclar resíduos celular (Duve2005; Perera and Zoncu 2016). Nos últimos anos, o lisossomo atingiu um novo status que destaca suas múltiplas funções celulares. A participação desta organela se estende a diversos papéis biológicos fundamentais à homeostase da célula, incluindo a transdução de sinais, morte celular, homeostase do colesterol, controle metabólico, autofagia, e exocitose, via na qual contribui em papéis fisiológicos específicos, como o reparo de membrana plasmática, resposta imune, e remodelação óssea e tecidual (Huizing et al. 2008; Parenti et al. 2015; Ballabio 2016; Perera and Zoncu 2016). Defeitos de degradação, exportação de catabólitos ou defeitos que conduzem à disfunção lisossômica no geral, tem sido implicado em várias doenças humanas, como câncer, obesidade, doenças neurodegenerativas, infecções, e em doenças lisossômicas (Ballabio 2016).

1.1.1. Biogênese lisossomal

Os lisossomos são constituídos por uma bicamada lipídica com um pH perto de 4,5. Os principais componentes presentes nesta organela, incluem hidrolases solúveis, proteínas de membrana associadas ao lisossomo (LMPs), proteínas integrais lisossômicas de membrana (LIMPs), organelas relacionadas ao lisossomo (ORL), e outros constituintes celulares (De Duve 1975; Saftig and Klumperman 2009; Schultz et al. 2011). Em torno de 60 diferentes de enzimas hidrolíticas estão presentes no lúmen lisossomal, sendo elas: lipases, proteases e glicosidases - todas estas envolvidas na degradação de metabólitos (Xu and Ren 2015). Estas proteínas são sintetizadas no retículo endoplasmático (RE) e encaminhadas para o complexo Golgi, onde ocorre a modificação da maioria das hidrolases ácidas com a incorporação de resíduos de manose 6-fosfato (M6P), permitindo seu reconhecimento pelos receptores M6P na rede trans-Golgi, no qual segue o transporte para o sistema endossomal/lisossômico (Saftig and Klumperman 2009; Schwake et al. 2013). Além disso, receptores de membrana LIMP-2 ou sortilina são responsáveis por transportar

outras enzimas solúveis e proteínas não enzimáticas para os lisossomos de maneira independente de M6P (Maxfield and Willard 2016; Braulke and Bonifacino 2009). Hidrolases e LMPs sintetizadas que não são encaminhadas para a rede trans-Golgi entram em uma via que as direciona para a membrana plasmática, onde podem ser guiadas aos lisossomos via endocitose (Maxfield and Willard 2016).

1.1.2. Vias de degradação celular

Os lisossomos realizam a degradação de uma ampla variedade de macromoléculas, como proteínas, glicosaminoglicanos, esfingolipídios, oligossacarídeos, glicogênio, ácidos nucleicos e lipídeos (Parenti et al. 2021). Os processos catabólicos são mantidos e regulados por endocitose (fagocitose, pinocitose) e autofagia (macroautofagia e autofagia mediada por chaperonas-CMA), no qual são responsáveis pela entrega de substratos ao lisossomo (Parenti et al. 2021). O material extracelular atinge o lisossomo por endocitose, internalizado por vesículas endocíticas na superfície celular formadas pela fissão da membrana plasmática. Estas vesículas passam por uma variedade de endossomos intermediários, distinguidos pelo conteúdo, composição molecular, morfologia e pH (Saftig and Klumperman 2009; Xu and Ren 2015). Portanto, estas vesículas atingem diferentes processos de maturação para se tornarem endossomos maduros, também denominados de corpos multivesiculares. Estas estruturas fundem-se com lisossomos compostos de hidrolases e tornam-se endolisossomos que medeiam a degradação celular (Xu and Ren 2015; Ballabio and Bonifacino 2020).

Por outro lado, os componentes intracelulares e agregados de proteínas são entregues ao lisossomo por meio da autofagia (**Figura 1**). A autofagia é o mecanismo celular responsável por deter e transportar componentes citoplasmáticos e organelas para reciclagem e degradação lisossomal (Kaushik and Cuervo 2012; Parenti et al. 2015). Desta forma, a autofagia exerce um papel essencial na homeostase celular, regulando a depuração intracelular e a reciclagem de múltiplas moléculas e componentes celulares, influenciando no metabolismo energético da célula (Parenti et al. 2021). Esse mecanismo inicia com a formação de uma vesícula de dupla membrana que resulta em autofagossomos, estas estruturas encapsulam o conteúdo citoplasmático e se fundem direta ou indiretamente com endossomos maduros, em seguida, com os lisossomos para formar os autolisossomos. Estas estruturas são responsáveis pela degradação dos substratos autofágicos (Xu and Ren 2015; Perera and Zoncu 2016).
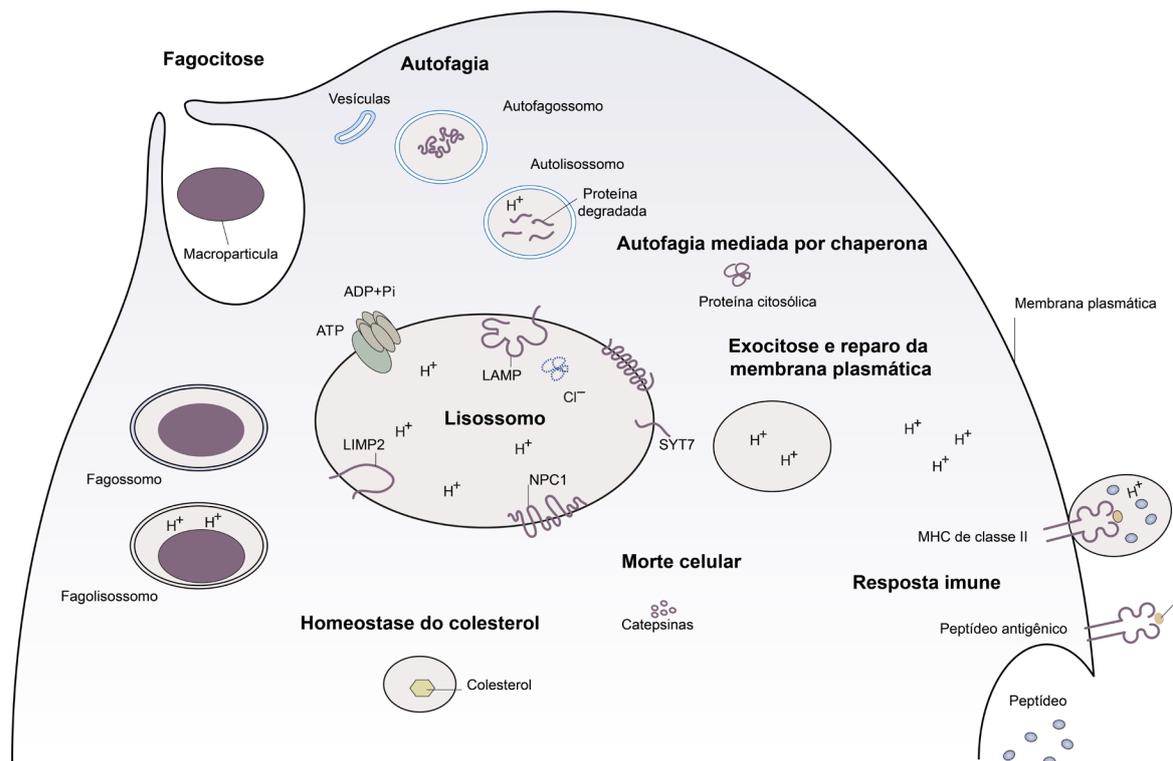
Figura 1. Principais funções dos lisossomos. O lisossomo é uma organela envolvida em vias de degradação através da autofagia, fagocitose, e autofagia mediada por chaperonas. O lisossomo também tem o papel na apresentação de antígenos durante a reposta imune, homeostase do colesterol, morte celular, e reparo da membrana plasmática. SYT7: sinaptotagmina 7. Adaptado de Saftig and Klumperman 2009.

CMA é um processo de transporte lisossomal regulado por proteínas lisossômicas de membrana. Este mecanismo permite associar substratos de proteínas citosólicas à membrana lisossomal, no qual são internalizados diretamente nos lisossomos para a degradação do material (Xu and Ren 2015). As proteínas destinadas à degradação por CMA apresentam motivos específicos em sua sequência, no qual são produzidos por modificações pós-traducionais. A identificação desses motivos pela chaperona HSC70 causa o recrutamento do substrato e o seu transporte para a superfície dos lisossomos (Maxfield and Willard 2016). Os produtos de degradação do lisossomo são exportados por proteínas específicas de membrana (Sagné and Gasnier 2008), ou via tráfego de membranas vesiculares (Saftig and Klumperman 2009). Estes metabólitos são usados na geração de novos componentes e na resposta energética às necessidades nutricionais da célula.

1.1.3. Exocitose e reparo de membrana

Em contraste às vias de tráfego lisossomal que recebem material intra e extracelular, a exocitose estimula a saída de componentes celulares por meio de eventos de fusão

dependentes de Ca2+ e sinaptotagmina 7. A exocitose desempenha papéis fundamentais na célula, como a defesa contra microrganismos, o crescimento de neuritos durante o desenvolvimento neuronal em humanos, e reparo da membrana plasmática após lesão mecânica (Saftig and Klumperman 2009; Perera and Zoncu 2016). Há dois diferentes mecanismos de exocitose. Na exocitose constitutiva, fatores de crescimento e citocinas podem ser liberados continuamente no espaço extracelular para a manutenção do organismo, independente do lisossomo. Por outro lado, o segundo mecanismo é marcado pela participação direta do lisossomo, consequente a um sinal extracelular que causa o transporte ativo do material até a membrana (Stenmark 2009; Maxfield and Willard 2016). Neste caso, o lisossomo pode se movimentar da região perinuclear para a membrana plasmática por meio de microtúbulos, estimulado pelo o aumento de Ca+ que leva a fusão do lisossomo com a membrana plasmática, onde pode secretar seu conteúdo no espaço extracelular (Xu and Ren 2015).

Em lesões celulares, o influxo de Ca2+ causado pelo dano na membrana plasmática desencadeia uma resposta rápida de reparo que é fundamental para a sobrevivência da célula (Maxfield and Willard 2016). A exocitose fornece membrana adicional na superfície celular e gera uma diminuição da tensão na membrana plasmática, fundamental para a união e encerramento da bicamada lipídica. Além disso, o conteúdo lisossomal pode fornecer hidrolases, como a esfingomielinase que fomenta o reparo da célula, convertendo a esfingomielina da membrana em ceramida (Steinhardt et al. 2000; Maxfield and Willard 2016). A produção de ceramida desencadeia a via endossomal que elimina os danos presentes na membrana plasmática (Tam et al. 2010).

1.1.4. Resposta imune e morte celular

O sistema endossomal-lisossômico é responsável por diversos processos envolvidos em infecções e respostas imunes no organismo (**Figura 1**), incluindo a digestão de bactérias fagocitadas, liberação de antígenos, e o processamento do complexo principal de histocompatibilidade de classe II (MHC-II) e sua apresentação às células T CD4 (Münz 2012; Marques and Saftig 2019). Durante processos inflamatórios, o sistema endossomal encaminha proteínas para a degradação no lisossomo, gerando um pool de peptídeos apresentados via moléculas MHC-II para a estimulação de células T CD4 (Münz 2012). Previamente à apresentação de antígeno, endossomos maduros liberam proteases para

processar moléculas MHC II e permitir o recebimento de peptídeos desconhecidos. Antígenos chegam à célula por fagocitose e são encaminhados aos endossomo/lisossomos para serem parcialmente degradados e internalizados na estrutura de MHC-II. A formação de microtúbulos a partir de compartimentos lisossomais permite a fusão direta destas vesículas com a membrana plasmática, e a liberação de exossomos contendo MHC II. A apresentação destes peptídeos na superfície celular é vital para o reconhecimento pelos leucócitos, como as células T e consequente ativação da resposta imune (Chow et al. 2002; Bousso 2008).

Lisossomos também podem atuar em outros contextos durante a resposta imunológica. Células T e natural killer (NK) do sistema imune possuem lisossomos secretores, denominados de grânulos líticos contendo MHC II, perforin, ou agente citolíticos, como a granzima A. Estes grânulos são secretados para produzir a sinapse imunológica, no qual consiste na interação entre um linfócito e uma célula apresentadora de antígeno, causando a permeabilização e morte da célula-alvo (Saftig and Klumperman 2009).

Além disso, lisossomos cumprem um papel na morte celular que pode ocorrer durante a remodelação do tecido, resposta imune, envelhecimento, e doenças neurodegenerativas. A presença de dano direto ou estresse extracelular, os lisossomos respondem com a permeabilização de sua membrana (Serrano-Puebla and Boya 2016). Este mecanismo leva ao bloqueio de funções degradativas por endossomos e a liberação de hidrolases no citosol. Hidrolases, como catepsinas, podem ativar a via de apoptose dependente ou independente de caspase, ou mesmo levar a necrose. Neste sentido, hidrolases podem produzir mediadores que sinalizam morte celular programada ou causar a hidrólise generalizada dos componentes citoplasmáticos, resultando em necrose celular (Repnik et al. 2014; Maxfield and Willard 2016).

1.1.5. Controle metabólico da célula e regulação transcricional do lisossomo

Os lisossomos possuem a capacidade de detectar o estado nutricional da célula por meio de um mecanismo sensível à disponibilidade energética no ambiente celular. Este mecanismo é composto pela proteína quinase alvo mecanicista da rapamicina (mTOR) que controla processos anabólicos e catabólicos em resposta aos sinais extracelulares e intracelulares (Zoncu et al. 2011; Pu et al. 2015). Desta forma, o sistema mTOR-lisossomos é capaz de monitorar o estado nutricional intracelular para adaptar seu metabolismo às

oscilações das condições energéticas do momento. A via mTOR é formada pelo Complexo 1 (mTORC1) e 2 (mTORC2) (Kim et al. 2002; Pearce et al. 2007; Saxton and Sabatini 2017). mTORC1 regula o crescimento e o metabolismo celular, promovendo a síntese de proteínas, nucleotídeos, lipídios e o metabolismo da glicose. mTORC2 está envolvido no controle da proliferação e sobrevivência celular, regulando o citoesqueleto, o transporte de íons e a migração celular (Jacinto et al. 2004; Porstmann et al. 2008; Saxton and Sabatini 2017). Portanto, o complexo mTORC1 exerce atividades anabólicas celulares, abolindo as vias de renovação de proteínas, como autofagia, sistemas de ubiquitina-proteassoma e biogênese lisossomal (Kim et al. 2011; Zhao et al. 2015).

O lisossomo pode se adaptar a diferentes condições, demonstrando que a biogênese e a função lisossômica estão sujeitas à regulação transcricional global (Settembre et al. 2013). O funcionamento do lisossomo requer a transcrição coordenada de uma rede de genes que regulam a biogênese lisossomal. Estes genes apresentam um motivo em comum, nomeado como elemento de regulação e expressão lisossomal coordenada, no qual são alvos dos fatores de transcrição EB (TFEB) e E3 (TFE3) (Martina et al. 2014; Ballabio and Bonifacino 2020). Ambos TFEB e TFE3 promovem a expressão de um conjunto genes envolvidos na regulação da autofagia, biogênese lisossomal, atividade de hidrolases lisossomais, eliminação de resíduos celulares e metabolismo energético da célula (Sardiello et al. 2009; Martina et al. 2014; Ballabio and Bonifacino 2020).

Na presença de nutrientes e fatores de crescimento, mTORC1 bloqueia vias catabólicas por meio da fosforilação e inibição da translocação nuclear do TFEB, (Settembre et al. 2011; Saxton and Sabatini 2017; Marques and Saftig 2019). No entanto, o recrutamento de mTOR na superfície lisossomal é exigida para regular sua ativação, destacando o lisossomo como um hub no controle da homeostase celular (Sancak and Sabatini 2009; Liu et al. 2017; Ballabio and Bonifacino 2020). Contudo, sob privação de nutrientes ou na ausência de fatores de crescimento, TFEB provoca o reposicionamento lisossomal perinuclear, reprimindo a atividade de mTORC1 e estimulando a atividade lisossomal (Schultz et al. 2011; Saxton and Sabatini 2017). Portanto, a atividade anabólica e catabólica são mutuamente antagônicas na célula, em que o aumento da sinalização de mTOR poderia suprimir as funções lisossomais, e vice versa (Zoncu et al. 2011; Ballabio and Bonifacino 2020).

1.2. Doenças lisossômicas

As doenças lisossômicas (DLs) são erros inatos do metabolismo que pertencem a um subgrupo de mais de 70 distúrbios hereditários raros. DLs resultam da disfunção do lisossomo causada por mutações em genes que codificam proteínas envolvidas no funcionamento e manutenção do sistema lisossômico, incluindo proteases, lipases, glicosidases, sulfatases, proteínas de membrana, transporte, modificadoras ou ativadoras enzimáticas (Platt et al. 2018). DLs são desordens monogênicas que afetam 1 em 5000 nascido vivos, em que a maioria é herdada de modo autossômico recessivo, com exceção de três condições ligadas ao X (doença de Fabry, mucopolissacaridose tipo II; doença de Danon) (Platt 2018; Platt et al. 2018).

O defeito da atividade de proteínas lisossomais e não-lisossomais resulta em mau funcionamento do lisossomo, e consequentemente, no gradual acúmulo intralisossomal de metabólitos ou substratos não degradados, o que caracteriza a presença do depósito celular (**Figura 2**) (Futerman and Van Meer 2004). Lisossomos recebem seus substratos através de vias que levam a degradação de moléculas e componentes celulares, como a endocitose e autofagia. Portanto, o acúmulo de macromoléculas específicas ou compostos monoméricos dentro de organelas do sistema endossomal-autofágico-lisossômico é uma característica em comum em DLs (Platt et al. 2012). Os efeitos dessas disfunções são indicados por alterações em diversos processos biológicos associados à organela (Bezprozvanny 2009; Vitner et al. 2010).

As DLs apresentam um curso clínico progressivo com alguns sintomas comuns, incluindo a visceromegalia, anormalidades esqueléticas, problemas cardíacos, defeitos auditivos, dificuldades motoras, e comprometimento cognitivo. Indivíduos com DLs podem apresentar sintomas no início da vida, no entanto, muitos são clinicamente normais ao nascimento (Poswar et al. 2019). A idade de início e a gravidade dos sintomas pode estar relacionada com a atividade residual da proteína deficiente, do substrato armazenado, da linhagem celular afetada pela formação do depósito, e outros fatores pouco esclarecidos, como a regulação epigenética, genes modificadores, doenças infecciosas, e fatores ambientais (Platt et al. 2012; Platt et al. 2018). As formas mais graves infantis apresentam comprometimento cerebral, no qual pacientes podem morrer nos primeiros anos de vida. Em formas adultas, os sintomas se desenvolvem mais devagar e a deficiência geralmente surge principalmente de sintomas periféricos como a hepatoesplenomegalia, injúrias no rim,

coração, e formação óssea anormal (Platt et al. 2018). Em torno de 70% dos indivíduos com DLs apresentam comprometimento do sistema nervoso central (SNC), indicado por sinais de neurodegeneração e inflamação em múltiplas regiões do cérebro (Futerman and Van Meer 2004; Platt et al. 2018).
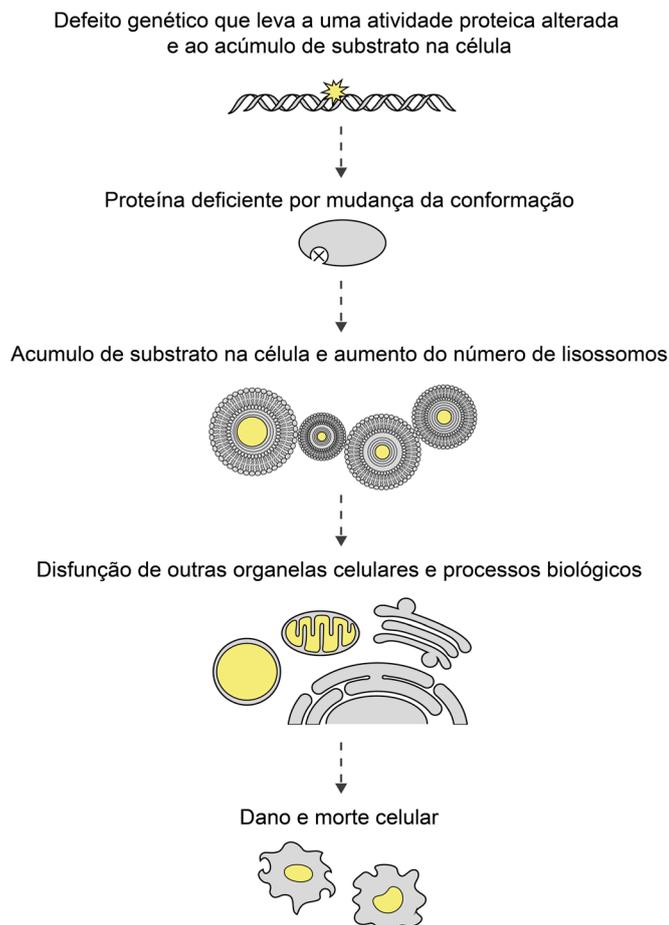
Figura 2. Cascata de patogênese em DLs. A presença de um defeito genético gera mudançasda conformação da proteína comprometendo sua função. O resultado da disfunção das proteínas é o acúmulo de substrato no lisossomo. Efeitos das alterações lisossomais impactam o funcionamento de outras organelas e processos biológicos, desencadeando inflamação, dano e morte celular. Adaptado de Platt et al. 2018.

A caracterização baseada na proteína ou enzima defeituosa é a abordagem mais útil para a classificação das DLs (Futerman and Van Meer 2004). No entanto, estas desordens podem ser categorizadas pelo tipo de substrato acumulado, ou pelos mecanismos que causam o depósito de metabólitos na célula (**Tabela 1**). Nestes casos, podem envolver a deficiência enzimática, defeito no transporte, ou modificação pós tradução.

Tabela-1. Doenças lisossômicas

Doença	Proteína defeituosa	Material de depósito
Mucopolissacaridoses (MPS)		
MPS I (Hurler, Scheie, Hurler / Scheie)	α-Iduronidase	Dermatan sulfato e heparan sulfato, GM2, GM3, SCMAS
MPS II (Hunter)	Iduronato-2-sulfatase	Dermatan sulfato e heparan sulfato, GM2, GM3, SCMAS
MPS IIIA (Sanfilippo)	Heparan N-sulfatase	Heparan sulfato, GM2, GM3, GD2, SCMAS, ubiquitina
MPS IIIB (Sanfilippo)	N-Acetil-α-glucosaminidase	Heparan sulfato, GM2, GM3, GD2, colesterol não esterificado, SCMAS
MPS IIIC (Sanfilippo)	Acetil-CoA: α-glucosamida N-acetiltransferase	Heparan sulfato, GM2, GM3, GD2
MPS IIID (Sanfilippo)	N-acetilglucosamina-6-sulfatase	Heparan sulfato, GM2, GM3, GD2
MPS IV A (Morquio-A)	N-acetilgalactosamina-6-sulfato-sulfatase	Queratan sulfato, condroitina-6-sulfato
MPS IV B (Morquio-B)	β-Galactosidase	Queratan sulfato, oligossacarídeos
MPS VI (Maroteaux-Lamy)	N-acetilgalactosamina-4-sulfatase (arilsulfatase B)	Dermatan sulfato, GM2, GM3, colesterol não esterificado
MPS VII (Sly)	β-Glucuronidase	Heparan sulfato, dermatan sulfato, condroitina-4- e -6-sulfatos, GM2, GM3, ubiquitina
Deficiência múltipla de sulfatase (Austin)	Enzima geradora de formilglicina	Heparan sulfato, dermatan sulfato, condroitina-4- e -6-sulfatos, sulfolipídeos
Sfingolipidoses		
Fabry	α-Galactosidase A	Globotriaosilceramida, galabiosilceramida, globotriaosilsfingosina
Farber lipogranulomatose	Ceramidase	Ceramide
Gaucher	β-Glicosidase	Glicosilceramida, GM1, GM2, GM3, GD3, glucosilsfingosina
Doença de Krabbe	Galactocerebrosídeo β-galactosidase	Galactosilceramida, psicosina lactosilceramida
Leucodistrofia metacromática	Arilsulfatase A	Sulfatida, 3-O-sulfolactosilceramida, lisossulfatida, gangliotetraosilceramida-bis-sulfato, GM2
Niemann–Pick A e B	Sphingomyelinase	Esfingomielina, colesterol, GM2, GM3, glucosilceramida, lactosilceramida, globotriaosilceramida
Gangliosidose GM1	β-Galactosidase	GM1, GA1, GM2, GM3, glucosilceramida, oligossacarídeos, queratan sulfato
Gangliosidose GM2 (Tay – Sachs)	β-Hexosaminidase A	GM2, GA2
Gangliosidose GM2 (Sandhoff)	β-Hexosaminidase A and B	GM2, globosídeo, oligossacarídeos
Oligossacaridoses e glicoproteinoses		
Aspartilglucosaminúria	Aspartilglucosaminidase	Aspartilglucosamina
Cistinose	Cistinosina	Cistina
Fucosidose	α-Fucosidase	Fucose contendo oligossacarídeos e antígeno H-glicolipídeo
Sialidose	Sialidase	Sialiloligossacarídeos e sialilglicopeptídeos
Glicogenose		
Doença de Pompe	α-glucosidase	Glicogênio

Portanto, de acordo com a localização da proteína, diferentes mecanismos de depósito lisossômico podem ser descritos em DLs (Platt et al. 2012). Por exemplo, na

doença de Gaucher, mucopolissacaridoses (MPS), ou na doença de Pompe, a deficiência abrange as enzimas lisossômicas propriamente ditas (Dasouki et al. 2014; Graziano and Cardile 2015; Vairo et al. 2015). Os defeitos na fosforilação de enzimas lisossômicas estão associados à mucolipidose tipo II e mucolipidosetipo IIIA (Lin and Pitukcheewanont 2012). Além disso, proteínas alteradas solúveis não enzimáticas e proteínas de membrana lisossômica estão deficientes em Niemann-Pick tipo C2 e cistinose, respectivamente (Elmonem et al. 2016; Evans and Hendriksz 2017).

Infelizmente, o diagnóstico pode levar anos para ser realizado na maioria dos indivíduos com DLs. Os testes aplicados hoje nos laboratórios, incluem a medição dos níveis da atividade de enzimas lisossomais, identificação de metabólitos não degradados em fluidos biológicos, e a detecção de mutações no DNA (Parenti et al. 2021). O sequenciamento genômico com o uso de painéis de genes ou abordagens que abrangem o sequenciamento de todo o genoma são extremamente úteis para que se alcance o diagnóstico com maior eficiência, evitando a execução de múltiplas análises bioquímicas ou reiteradas admissões hospitalares pelo paciente (Parenti et al. 2021). O transplante de medula óssea ou transplante de células-tronco hematopoiéticas foi o primeiro tratamento específico utilizado em DLs antes que houvesse a disponibilidade de novas terapias. Essa terapia fornece ao paciente células com níveis normais de enzimas lisossomais. Ao longo do tempo, o uso do cordão umbilical ao invés da medula óssea também vem sendo usado com a intenção de diminuir os efeitos colaterais através de uma abordagem menos invasiva (Platt 2018). Atualmente, outras opções terapêuticas são empregadas, como uso de reposição enzimática por infusão intravenosa, a redução de substratos que impede parte da biossíntese de macromoléculas de armazenamento na célula, ou ainda o uso de moléculas chaperonas que estabilizam a enzima deficiente, gerando maior meia vida para a proteína (Platt 2018).

No entanto, estas estratégias terapêuticas são ainda ineficientes ou indisponíveis para a maioria das DLs (Ballabio 2016; Giugliani et al. 2016). Além disso, a transposição da barreira hematoencefálica pela enzima exógena para o tecido nervoso continua sendo um desafio no tratamento destes pacientes. A infusão de enzimas recombinantes em regiões intracerebroventricular ou intratecal são possíveis soluções para o acometimento neuronal, estratégia recentemente aprovada para a lipofuscinose ceróide neuronal tipo 2 (Platt 2018).

1.2.1. Mucopolissacaridoses (MPS)

MPS compõem um grupo de 11 tipos diferentes de DLs (**Tabela 1**) caracterizadas pela deficiência de enzimas responsáveis pela degradação de glicosaminoglicanos (GAGs). O prejuízo da atividade enzimática nos lisossomos leva ao acúmulo de GAGs na célula, afetando múltiplos tecidos (Giugliani 2018; Kubaski et al. 2020). MPS é uma condição multissistêmica com um espectro clínico que pode variar entre a forma grave e atenuada da doença, dependendo do tipo de GAGs acumulado e da variante patogênica presente nos indivíduos (Yogalingam et al. 2004; Pollard et al. 2013; McBride and Flanigan 2021). Manifestações clínicas típicas incluem deformidades esqueléticas, hérnias, degeneração articular, dismorfias faciais, déficit auditivo, obstrução das vias aéreas superiores, hepatoesplenomegalia, disfunção cardíaca, e comprometimento do SNC (Stapleton et al. 2018; McBride and Flanigan 2021). O envolvimento cardíaco é uma característica comum em MPS. Com a exceção de MPS IX, anomalias cardíacas foram reportadas em todos os tipos de MPS, levando ao risco de morte súbita, insuficiência cardíaca, e oclusão coronariana. Portanto, as disfunções cardíacas em MPS são consideradas um importante fator de morbidade e mortalidade nos indivíduos acometidos, mesmo com a disponibilidade da terapia de reposição enzimática (Braunlin et al. 2011; Poswar et al. 2019). Achados cardiovasculares, como a hipertrofia cardíaca, redução da função cardíaca, aumento da câmara ventricular esquerda, e dilatação da aorta são frequentes em MPS. A incidência de dilatação da aorta é estimada em até 40% dos pacientes com MPS (Braunlin et al. 2011; Bolourchi et al. 2016; Poswar et al. 2019).

O envolvimento neurológico progressivo com a presença de comprometimento cognitivo, dificuldades comportamentais e regressão em marcos de desenvolvimento, também é frequente em formas mais graves de MPS (Whiteman and Kimura 2017). Lesões multifocais ou difusas da substância branca são comumente vistas em pacientes com MPS com comprometimento cognitivo. Espaços perivasculares dilatados são observados na substância branca periventricular e subcortical. Além disso, pode ocorrer atrofia cerebral com diminuição do volume do corpo caloso, aumento dos sulcos corticais, e aumento da produção de líquido cefalorraquidiano (Bigger et al. 2018; Stapleton et al. 2018). Lesões de substância branca e atrofia cerebral são associadas ao armazenamento de GAGs em neurônios e células gliais, o que pode levar a anormalidades de mielinização e morte neuronal (Schwartz et al. 2007; Bigger et al. 2018; Viana et al. 2020). Manifestações

neurológicas com deterioração neuronal e problemas comportamentais são identificadas em diversos tipos de MPS. Além disso, o prejuízo do SNC tende a ser irreversível, mesmo com o tratamento por reposição enzimática usado em humanos (Schwartz et al. 2007; Scarpa et al. 2017; Bigger et al. 2018; Viana et al. 2020). Portanto, as MPS são descritas como um grupo heterogêneo de doenças com variada apresentação clínica, no qual o comprometimento cardíaco e neurológico pode estar presente em pacientes com as formas mais graves de MPS I, MPS II e MPS VII. A MPS tipo I é uma desordem autossômica recessiva causada pela deficiência de alfa-L-iduronidase (IDUA) levando ao acúmulo de GAGs heparan sulfato (HS) e dermatan sulfato (DS) (Giugliani 2018). MPS I classifica-se em 3 formas clínicas que impactam na conduta terapêutica, mas praticamente indistinguíveis do ponto de vista bioquímico. A categorização clínica é estabelecida entre a forma grave (Hurler), que representa em torno de 60% dos pacientes afetados, e as formas atenuadas, denominadas como Hurler-Scheie e Scheie que abrangem 23% e 13% dos indivíduos, respectivamente (Beck et al. 2014). A MPS tipo I tem uma prevalência estimada de 1: 100.000 e a idade média de início dos sintomas é de 0,9 anos em pacientes Hurler; 3,4 anos em Hurler-Scheie; e 8,7 anos para a forma Scheie (Moore et al. 2008; Beck et al. 2014). Os principais achados clínicos são dismorfias faciais, hepatoesplenomegalia, anormalidades esqueléticas, limitação articular, baixa estatura e, na forma grave, pode ocorrer o declínio cognitivo (Poswar et al. 2019).

MPS tipo II, também conhecida como síndrome de Hunter, é uma doença recessiva rara ligada ao X. A síndrome de Hunter é causada por variantes patogênicas no gene iduronato-2-sulfatase (IDS) que levam a redução de sua atividade da enzimática (Beck 2011). A diminuição da atividade da proteína IDS afeta a sua função de degradação de GAGs, resultando no acúmulo DS e HS dentro de células e tecidos (Beck 2011). MPS II tem uma prevalência estimada em 1-9 em 1.000.000 nascidos vivos, e a idade de início, severidade da doença e progressão é muito variável entre indivíduos do sexo masculino. Manifestações como a obstrução das vias aéreas, deformidades esqueléticas, e cardiomiopatia estão presentes (Baehner et al. 2005; Wraith et al. 2008). MPS II é classificada em duas formas clínicas, de acordo com a presença ou ausência de comprometimento cognitivo: não neuropática e neuropática com presença de manifestações neurológicas, geralmente presentes na primeira e segunda década de vida (Whiteman and Kimura 2017).

MPS tipo VII, também conhecida como doença de Sly, apresenta prevalência <1: 1.000.000 nascidos vivos. MPS VII é causada por variantes patogênicas no gene GUSB, gerando deficiência da enzima lisossomal β-glucuronidase (Muenzer 2004). O depósito de GAGs heparan sulfato, condroitina sulfato e dermatan sulfato nas células estão associados com macrocefalia, hidrocefalia, dismorfias faciais, hepatoesplenomegalia, anormalidades nas válvulas cardíacas, hérnias, e deficiência intelectual progressiva (Platt et al. 2018). MPS são causadas por variantes patogênicas que compõem enorme heterogeneidade molecular, incluindo mutações nonsense, splicing alternativo, indels, e rearranjos complexos que parcialmente explicam a variabilidade clínica encontrada em indivíduos afetados (Muenzer 2004; Beck et al. 2014; Mohamed et al. 2020). As formas graves podem ser causadas por uma mudança mais significativa na estrutura das proteínas com perda da atividade da enzima, enquanto variantes patogênicas em formas atenuadas tendem a afetar em menor grau a estrutura da proteína com a manutenção da atividade enzimática residual (Kato et al. 2005; Beck et al. 2014; Mohamed et al. 2020). No entanto, durante a prática clínica, a dosagem enzimática não é confiável para diferenciar a forma grave da atenuada.

Além disso, é difícil prever o fenótipo em MPS por análise de variantes patogênicas. Por exemplo, grandes alterações estruturais são frequentemente descritas em pacientes com uma apresentação clínica mais grave, enquanto as substituições de um único par de bases estão envolvidas em um amplo espectro de gravidade da doença (Kosuga et al. 2016; Josahkian et al. 2021).

1.2.2. Substratos acumulados em MPS

MPS é marcada pela deficiência de hidrolases envolvidas na degradação GAGs (Muenzer 2004). GAGs são polissacarídeos ácidos lineares, altamente carregados, comumente ligados covalentemente aos proteoglicanos na membrana celular ou distribuídos na matriz extracelular (MEC) (Linhardt 2003; Linhardt and Toida 2004). Duas classes de GAGs são encontradas em MPS, não sulfatado como o ácido hialurônico, e os sulfatados, incluindo HS, DS, queratan sulfato (KS), e condroitina sulfato (**Figura 3**) (Muenzer 2004).
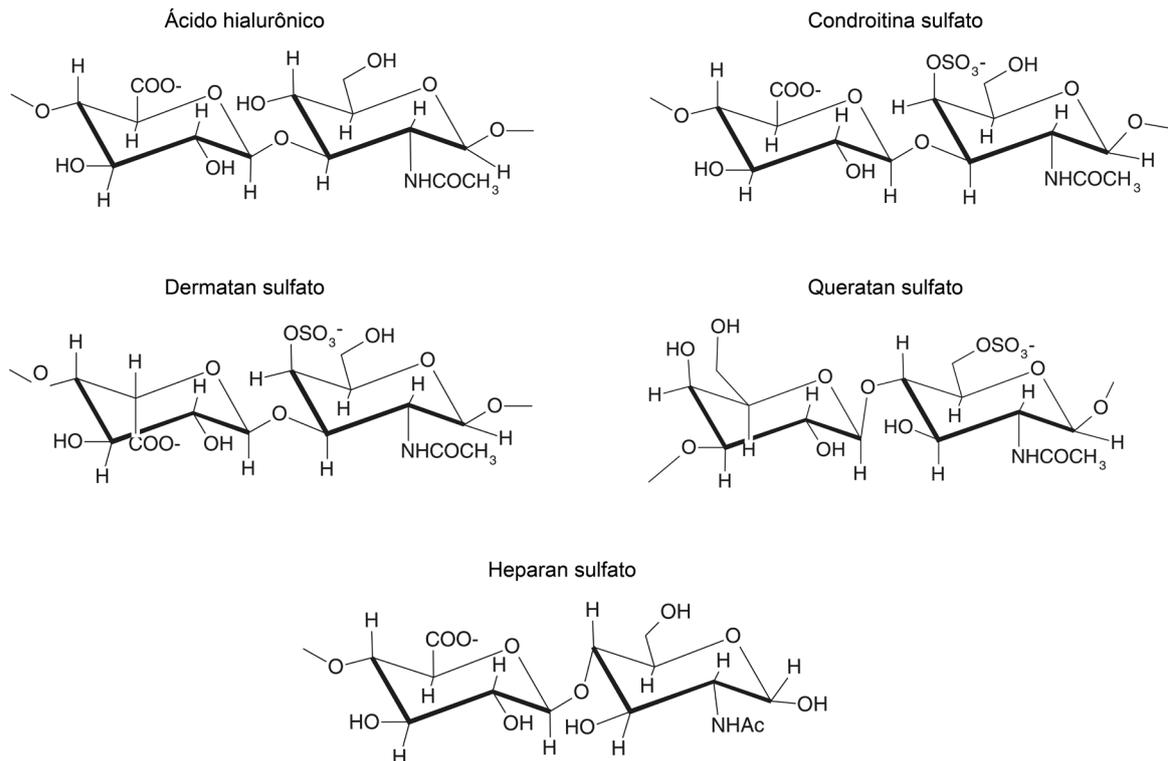
Figura 3. Tipos de glicosaminoglicanos. Adaptado de Gandhi, 2008.

As cadeias de GAGs são formadas por unidades com repetições de dissacarídeo, cada unidade contém ácido urônico (ácido D-glucorônico ou ácido L-idurônico) e amino açúcar (D-galactosamina ou D glucosamina) (Gandhi and Mancera 2008). Estruturalmente, os GAGs podem se diferenciar pelo tipo de hexosamina, hexose ou unidade de ácido hexurônico ou pela ligação glicosídica entre essas unidades (Sasisekharan and Venkataraman 2000; Gandhi and Mancera 2008). GAGs podem ser encontrados em diversos tipos de tecidos em maior ou menor nível. O ácido hialurônico é comumente encontrado no líquido sinovial, humor vítreo, e MEC do tecido conjuntivo. No caso da condroitina sulfato, é frequentemente encontrada na cartilagem, tendão, ligamento e aorta. DS é mais abundante na pele, vasos sanguíneos, e válvulas cardíacas. O polissacarídeo KS é observado na córnea e cartilagem, enquanto o HS, é amplamente difundido no espaço extracelular e nas superfícies celulares (Gandhi and Mancera 2008). Os proteoglicanos de HS (HSPGs) têm um envolvimento central na patogênese na maioria das MPS (Giugliani 2018). HSPGs são complexos moleculares que consistem em uma proteína central que carrega cadeias de HS (Kirkpatrick and Selleck 2007). Estes são categorizados em três diferentes classes: proteínas transmembranas (sindecanos); proteínas ancoradas a glicosilfosfatidilinositol (glipicans); e proteínas secretadas na MEC (perlecano,agrina e colágeno tipo XVIII) (Iozzo

and Schaefer 2015; De Pasquale and Pavone 2019). Sindecanos e glipicans são encontrados na superfície celular, no qual podem ser clivados pela enzima heparanase em oligossacarídeos de menor comprimento de cadeia (Iozzo and Schaefer 2015). HSPGs podem mediar funções entre a MEC e membrana celular em diversas vias de sinalização, influenciando na homeostase do tecido, proliferação celular, migração, adesão, diferenciação, sobrevivência, ou em processos patológicos, como mecanismos de defesa e inflamação (Kirn-Safran et al. 2009; De Pasquale and Pavone 2019) (**Figura 4**).
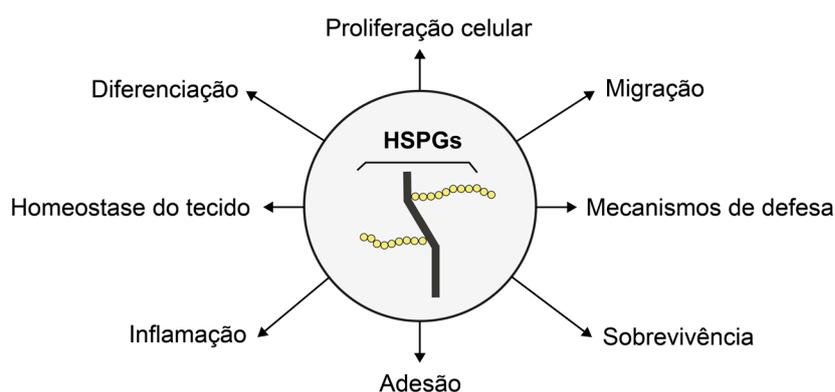
Figura 4. Processos biológicos e patológicos regulados por proteoglicanos heparan sulfato (HSPGs). Adaptado de Matsuo and Kimura-Yoshida 2014; De Pasquale and Pavone 2019.

Portanto, HSPGs realizam suas funções interagindo com diversas moléculas, incluindo quimiocinas, citocinas, fatores de crescimento, morfógenos, componentes da MEC, enzimas e proteínas de adesão (Capila and Linhardt 2002; Gandhi and Mancera 2008; Billings and Pacifici 2015). HSPGs localizados na superfície celular podem servir como co-receptores para a sinalização de fatores de crescimento, em que estas moléculas podem induzir mudanças conformacionais do ligante e/ou receptor ou servir como um molde para aproximação ligante-receptor (**Figura 5**) (Kirkpatrick and Selleck 2007; De Pasquale and Pavone 2019). Estes HSPGs podem também sofrer clivagens por metaloproteinases (MMPs) ou heparanases e provocar a liberação de cadeias de HS que contribuem no transporte ou movimento de fatores de crescimento associados ao ambiente extracelular (Matsuo and Kimura-Yoshida 2014; De Pasquale and Pavone 2019). HSPGs na superfície celular podem mediar a endocitose e o tráfego vesicular. Também podem se comportar como receptores endocíticos que sofrem endocitose induzida por ligantes, como exossomos, peptídeos, vírus, lipoproteínas, fatores de crescimento e morfógenos que podem adentrar a célula por esta via (Christianson and Belting 2014; De Pasquale and Pavone 2019). No caso de HSPGs

presentes na MEC, estes podem funcionar como reservatórios de moléculas de sinalização e liberando-as conforme a necessidade de células-alvo. Além disso, podem ser comportar como barreira para fatores de crescimento ou morfógenos, inibindo sua difusão em longas distâncias ao longo do espaço extracelular (Matsuo and Kimura-Yoshida 2014). E por conta de interações entre células adjacentes e componentes da MEC, HSPGs exercem papel na via de adesão celular.

Portanto, os HSPGs controlam tanto a formação de gradientes de morfógenos, fatores de crescimento, e outras moléculas de sinalização, quanto a sua distribuição, sinalização e tráfego intracelular. Por exemplo, HSPGs pode induzir ou inibir a sinalização de hedgehog (Hh), influenciando a formação de gradiente de Hh por meio de oscilações nos níveis de expressão de HSPGs na célula (Gallet et al. 2008; De Pasquale and Pavone 2019).
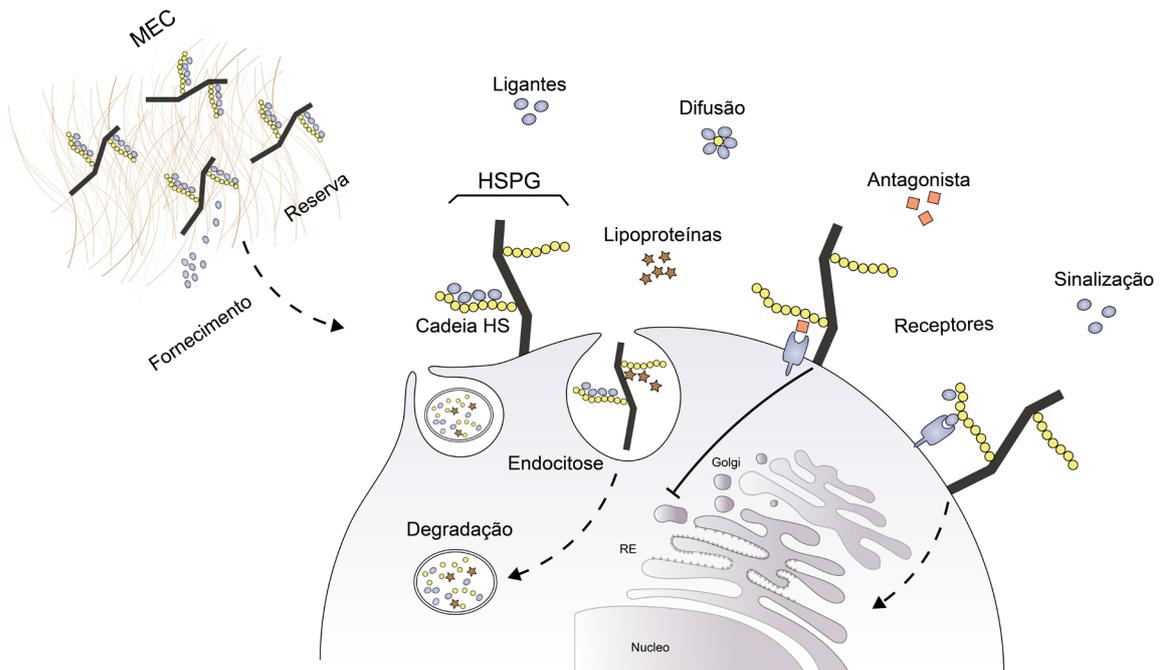
Figura 5. Diferentes funções de proteoglicanos heparan sulfato (HSPGs) no controle da transdução de moléculas sinalizadoras. HSPGs localizados na superfície celular desempenham funções de co-receptores, mediadores de endocitose, enquanto HSPGs distribuídos na MEC cumprem papéis de reservatório, barreira, ou transporte de moléculas de sinalização. Adaptado de Matsuo and Kimura-Yoshida 2014.

Desta forma, HSPGs exercem funções essenciais em diversos tecidos, inclusive no SNC, participando de processos do neurodesenvolvimento, como a neurogênese, orientação do axônio, e na formação de sinapses (Gallet et al. 2008; Poulain 2015).

1.3.   Fisiopatologia das doenças lisossômicas

DLs são desordens mendelianas causadas por mutações em um único gene, mas apresentam traços complexos, podendo gerar diferentes cursos da doença em indivíduos afetados. Os mecanismos moleculares subjacentes a deficiência proteica afeta diferentes processos associados aos lisossomos e têm sido profundamente investigados (Levine and Kroemer 2008; Ballabio and Gieselmann 2009; Schultz et al. 2011; Ballabio and Bonifacino 2020). DLs são marcadas pelo acúmulo intra-lisossomal de substratos, causa primária da doença, mas a presença de um amplo espectro clínico em indivíduos afetados indica a perturbação de múltiplas vias bioquímicas e celulares secundárias. O acúmulo de substrato pode impactar vias várias vias metabólicas, além disso, substratos secundários podem gerar defeitos em vias secundárias e terciárias e gerar um dano tecidual (Futerman and Van Meer 2004; Schultz et al. 2011; Platt et al. 2018). O impacto destas perturbações acarreta em alterações da expressão gênica que, em última análise, contribuem para o dano e morte celular (**Figura 6**). Todavia, qualquer um destes eventos pode ser a causa do prejuízo celular (Futerman and Van Meer 2004; Schultz et al. 2011). No entanto, a identificação destes mecanismos de patogênese que englobam variadas vias subjacentes e o seu real impacto em DLs, ainda é pouco esclarecido (Futerman and Van Meer 2004; Schultz et al. 2011; Fiorenza et al. 2018).

1.3.1. Defeitos na autofagia, mitofagia, homeostase lipídica e sinalização de cálcio

Ao longo dos anos, estudos demonstraram o protagonismo do lisossomo no funcionamento da célula, o que explica a gama de processos biológicos alterados em DLs. Neste sentido, inúmeros eventos patogênicos secundários podem ser desencadeados pelo acúmulo de substrato na célula. Defeitos na autofagia são amplamente reportados em DLs (Lieberman et al. 2012). O bloqueio autofágico ocorre por conta da incapacidade de fusão entre autofagossomos e lisossomos ou hiper ativação desse mecanismo, resultando no acúmulo de autofagossomos e morte celular (Vitner et al. 2010; Ballabio and Bonifacino 2020). Além disso, o acúmulo de macromoléculas em endossomos e autolisossomos pode impedir a liberação de enzimas catabólicas normais e inibir a sua atividade, resultando no acúmulo de substratos secundários (Platt et al. 2012).
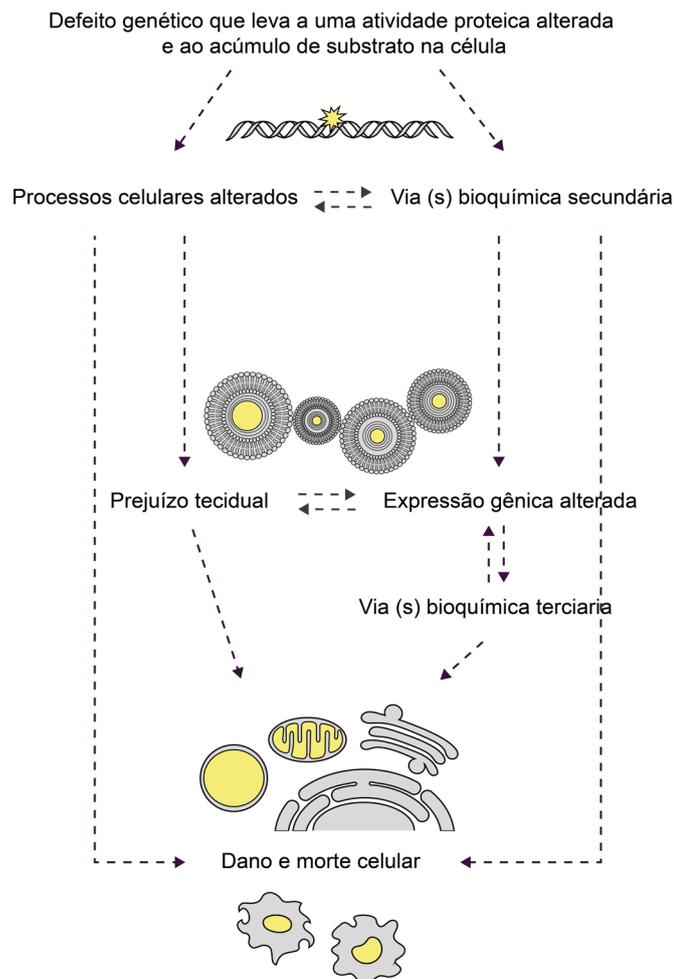
Figura 6. Modelo esquemático da fisiopatologia das DLs. O acúmulo de substrato não metabolizado pode perturbar a expressão gênica e alterar vias secundárias e terciárias, causando dano celular e tecidual. Adaptado de Futerman and Van Meer 2004; Platt et al. 2018.

O acúmulo de substratos secundários pode ser frequente em diversos DLs. Por exemplo, MPS são caracterizadas pelo acúmulo desubstrato primário de GAGs e substratos secundários, como gangliosideos, colesterol, beta amiloide, tau, e a proteína α-sinucleína (Parker and Bigger 2019). Desta maneira, além de causar dano ao sistema endossômico-autofágico lisossômico, o depósito de substratosprimários e secundários podem impedir o correto funcionamento de organelas relacionadasao lisossomo, como mitocôndrias, retículo endoplasmático e complexo de Golgi (Platt et al.2012). Por exemplo, prejuízos no sistema autofágico causam o acúmulo de mitocôndrias disfuncionais, afetando as funções lisossomais, como a geração de espécies reativas de oxigênio e o impedimento da acidificação do lúmen lisossomal pela bomba ácida V-ATPaseque depende do ATP gerado pela mitocôndria (Stepien et al. 2020). Danos mitocondriais geram excesso de cálcio

mitocondrial, diminuição do potencial de membrana e diminuição do consumo de oxigênio (Lim et al. 2015; Parenti et al. 2021). Portanto, o prejuízo da mitofagia (via de degradação das mitocôndrias) e estresse oxidativo são características comumente compartilhadas entre DLs (Stepien et al. 2020).

Algumas DLs podem mostrar o tráfego de lipídeos alterados, em que o depósito de colesterol nos lisossomos pode ser frequentemente relatado. Desequilíbrios na homeostase do colesterol são causados pelo comprometimento de rotas de exportação do colesterol lisossomal realizado por proteínas específicas, ou o acúmulo de colesterol pode ser resultado de defeitos do próprio tráfego lisossomal (Luo et al. 2017; Marques and Saftig 2019). Defeitos na sinalização intracelular de cálcio também podem estar implicados na patogênese de DLs. As causas podem depender do tipo de substrato acumulado e suas interações com canais ou bombas de cálcio específicas. As consequências para a célula, podem incluir o aumento da liberação de cálcio no RE, alteração do fluxo de cálcio na mitocôndria, e redução do depósito de cálcio no lisossomo que prejudica a fusão e o tráfego lisossomal na célula (Vitner et al. 2010; Parenti et al. 2021).

1.3.2. Disfunção em vias do desenvolvimento

Com a relevância do lisossomo no funcionamento celular, mesmo enzimas ou proteínas com funções relativamente periféricas na célula, quando deficientes são capazes de gerar distúrbios em importantes vias de sinalização envolvidas com eventos morfogênicos e de crescimento do organismo e impactar negativamente múltiplos tecidos em indivíduos afetados (Fiorenza et al. 2018). O acúmulo de substrato no ambiente intracelular e extracelular pode gerar perturbações em vias morfogênicas e de crescimento, como Hh, insulina, mTOR. Hh é envolvida em funções mitogênicas e morfogênicas, estimulando a proliferação celular e regulando eventos-chave durante processos de desenvolvimento, respectivamente. Estes processos são conservados em animais e envolvem o crescimento e padronização morfológica de embriões multicelulares (Simpson et al. 2009; Petrova and Joyner 2014). Hh é uma via de sinalização essencial na regulação da diferenciação celular e homeostase do tecido (Lee et al. 2016). Além disso, a via Hh controla a polaridade do SNC, padrão neural, e a homeostase das células-tronco em tecidos adultos (Dessaud et al. 2008; Petrova and Joyner 2014). O mau funcionamento desta via foi descrito em diferentes tipos

de MPS (Kingma et al. 2015; Peck et al. 2015; Costa et al. 2017), distúrbios cuja característica comum é o prejuízo na degradação de GAGs. Em condições normais, HS ligam-se a proteoglicanos que funcionam como co-receptores localizados adjacentes aos cílios primários, estrutura formada por uma protrusão da membrana plasmática baseada em microtúbulos necessária para a transdução de sinal de Hh durante o desenvolvimento embrionário e neuronal em vertebrados (Goetz and Anderson 2010). Portanto, GAGs associados a proteoglicanos na MEC, funcionam como co-receptores onde desempenham funções na comunicação intercelular por meio da distribuição de proteínas difusíveis e promovem a ligação e sinalização de Hh (Capila and Linhardt 2002; Linhardt and Toida 2004; Witt et al. 2013). No entanto, defeitos na sinalização desta via (**Figura 7**), consequenteao acúmulo de substratos na célula e MEC são descritos em MPS e Niemann-Pick tipo C (**Tabela 2**). Alterações da expressão gênica ou dos níveis e distribuição de proteínas Hh foram observadas e associadas a manifestações clínicas importantes nestas condições,incluindo a diminuição do crescimento ósseo e anormalidades cardiovasculares (Kingma etal. 2015; Peck et al. 2015; Costa et al. 2017).
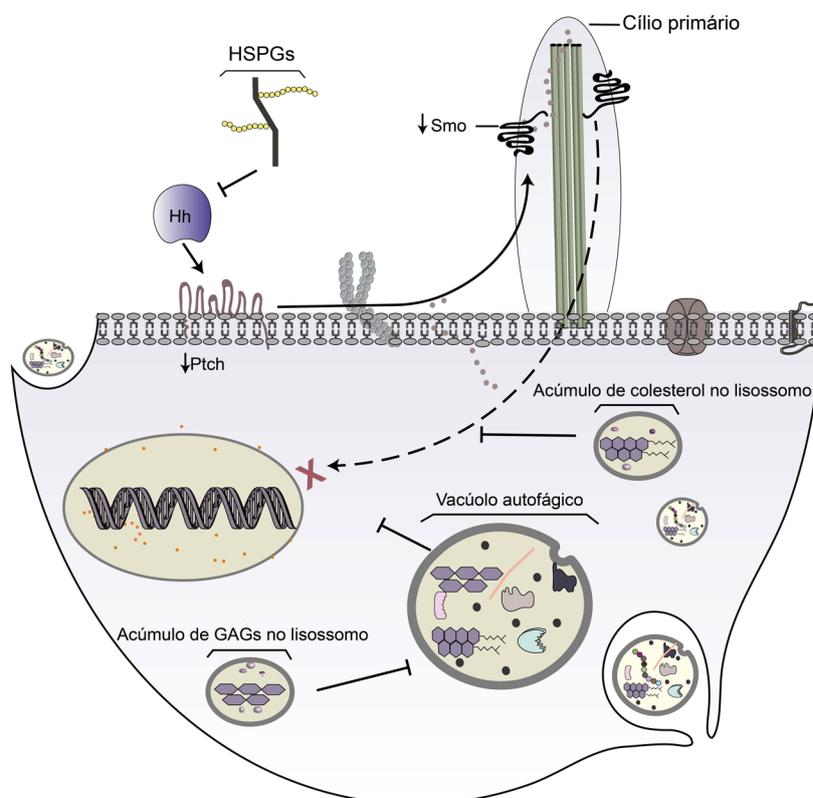

Figura 7. Perturbações na via Hh por acúmulo de colesterol e GAGs nos lisossomos e na matriz extracelular. Adaptado de Kingma et al. 2015; Costa et al. 2017.

Lisossomos podem regular o metabolismo energético da célula. Mas em condições patológicas, a deficiência da função lisossomal pode ser causada pelo acúmulo de substratos na célula e gerar perturbações nos mecanismos de autofagia e ativação de mTOR (**Figura 8**)(Ko et al. 2005; Koike et al. 2005; Fukuda et al. 2006; Settembre et al. 2008). Defeitos na reativação ou desequilíbrios na atividade de mTOR, são observados em diversas formas de DLs (**Tabela 2**), impedindo que lisossomos realizem suas funções de adaptação às condições ambientais, como a disponibilidade de nutrientes (Cao et al. 2006; Yu et al. 2010; Ivanova et al. 2015; Bartolomeo et al. 2017; Lim et al. 2017).

Tabela 2. Alterações na sinalização de Hh, mTOR e insulina.

DDL	Via desregulada	Referência
MPS I	Hh e mTOR	(Kingma et al., 2016; Yu et al., 2010).
MPS II	Hh	(Costa et al., 2017).
MPS VII	Hh e mTOR	(Peck et al., 2015; Kingma et al., 2016; Bartolomeo et al., 2017)
NPC	Hh e insulina	(Canterini et al., 2017; Formichi et al., 2018; Vainio et al., 2005).
JNCL	mTOR	(Cao et al., 2006).
FD	mTOR	(Yu et al., 2010).
Cistinose	mTOR	(Ivanova et al., 2016).
KD	mTOR.	(Inamura et al., 2018; Narayanan et al., 2009)
GD	mTOR e insulina	(Ghauharali-van der Vlugt et al., 2008; Langeveld et al., 2008; Brown et al., 2019)
PD	mTOR.	(Lim et al., 2017)

DDL (doença de depósito lisossômico); MPS I (Mucopolissacaridose tipo I); MPS II (Mucopolissacaridose tipo II); MPS VII (Mucopolissacaridose tipo VII); NPC (Niemann-Pick tipo C); JNCL (lipofuscinoses ceróides neuronais juvenil); FD (doença de Fabry); KD (doença de Krabbe); GD (doença de Gaucher); PD (doença de Pompe).

Lisossomos também podem estar envolvidos na patofisiologia de distúrbios metabólicos em algumas DLs, caracterizado por mecanismos associados ao surgimento de diabetes e obesidade (Ballabio and Bonifacino 2020). Fatores de crescimento, como a insulina podem ativar a via mTOR. A insulina é um hormônio anabólico essencial na captação de glicose e armazenamento energético no organismo. A insulina é secretada pelas células β no pâncreas e atua por meio do receptor de insulina (IR), um receptor de tirosina quinase de membrana (Tokarz et al. 2018). A autofosforilação de IR ocorre com a ligação da insulina em jangadas lipídicas na membrana celular, transduzindo o sinal para vias

metabólicas e mitogênicas (Ikonen and Vainio 2005). As jangadas lipídicas são microdomínios localizados na membrana plasmática enriquecidos em fosfolipídios saturados, esfingolipídios, glicolipídios, GAGs e colesterol (Sezgin et al. 2017). Estas estruturas na membrana são primordiais para a ativação de moléculas a jusante durante a sinalização, atuando na ativação do receptor, captação endocítica e direcionamento do IR (Simons and Gruenberg 2000; Ikonen and Hölttä-Vuori 2004; Vainio et al. 2005; Fuller 2010). Portanto, a composição modificada da membrana celular causada por perturbações nas jangadas lipídicas, pode gerar defeitos na sinalização de insulina (**Figura 8**) (Bickel 2002; Fuller 2010). Neste sentido, o acúmulo de substrato extracelular pode provocar o prejuízo na reativação de IR em Niemann-Pick tipo C (Vainio et al. 2005), e captação de glicose e resistência insulínica na doença de Gaucher (Langeveld et al. 2008).
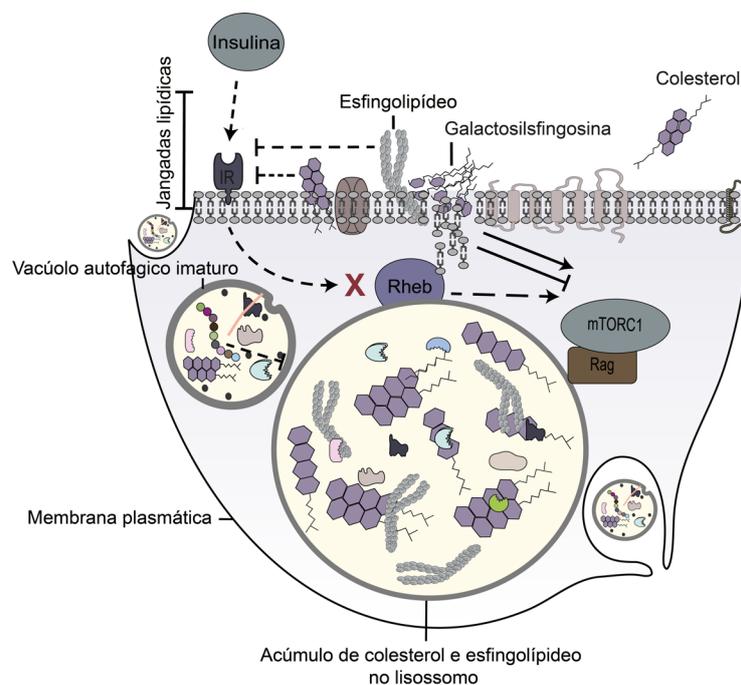
Figura 8. Defeitos na sinalização de insulina e mTOR causado pelo acúmulo de substratos no lisossomo, vacúolos autofágicos e no ambiente extracelular. Adaptado de Cao et al. 2006; Yu et al. 2010.

1.3.3. Vias inflamatórias

O sistema autofágico-lisossômico tem importante papel anti-inflamatório e de defesa na célula, mas podem estar comprometidos em DLs. Defeitos na autofagia podem provocar o acúmulo de mitocôndrias e induzir proteínas que ativam o inflamassoma por meio da produção de espécies reativas de oxigênio (ROS) e DNA mitocondrial. Também pode haver

prejuízo do mecanismo de remoção de estruturas agregadas do inflamassoma pela autofagia, que eliminam substâncias ativas e reduzem a resposta inflamatória (Shi et al. 2014; Simonaro 2016). Além disso, o dano em vias fagocíticas pode impedir a morte de bactérias ingeridas pela célula, consequentemente, aumentar a suscetibilidade a infecções bacterianas em pacientes com a doença de Gaucher, por exemplo (Marodi et al. 1995; Marques and Saftig 2019). Durante a resposta imune, a ativação de macrófagos causada pelo acúmulo de lipídeos na doença de Gaucher, pode resultar na liberação de citocinas, como TNF-a e IL-1b (Francesco, 2013). Nestas condições, os lisossomos podem regular a liberação de citocinas mediada por inflamassoma e o metabolismo de lipídeos (Simonaro 2016).

A autofagia pode ser desencadeada pela sinalização de receptores toll like-4 (TLR4) ativados por lipopolissacarídeos de bactérias gram-negativa (Simonaro 2016). A ativação desse receptor está associada com uma cascata inflamatória observada na doença de Fabry, em que a liberação de citocinas incluindo IL-6, IL-1b e TNF-a pode ser reduzida usando anticorpo bloqueador de TLR4 (De Francesco et al. 2013). Além disso, TLR4 pode se ligar a diversos outros tipos de substratos acumulados em DLs, incluindo GAGs, glicoesfingolipídeos e gangliosideos (Jou et al. 2006; Simonaro 2016). Em condições normais, diversas quimiocinas podem interagir com GAGs na MEC ou na membrana celular (Linhardt and Toida 2004; Peterson et al. 2004). No entanto, o acúmulo de GAGs pode causar a elevação de citocinas em modelos de MPS através da ligação ao receptor TLR4, e desencadear uma cascata inflamatória responsável pela liberação de múltiplas citocinas, quimiocinas e ativação do sistema complemento (Simonaro et al. 2008; Simonaro et al. 2010; Baldo et al. 2011). Estes eventos inflamatórios (**Figura 9**), podem levar a permeabilização da membrana lisossomal com a liberação de catepsina B e outras proteases, e gerar alterações na homeostase iônica. Além disso, evidências implicam que a ativação da resposta imune inata em MPS desempenha um papel na patogênese envolvida em anormalidades ósseas e cardíacas, incluindo a dilatação da aorta (Simonaro et al. 2008; Baldo et al. 2011; Parker and Bigger 2019).

No cérebro, o acúmulo de HS pode mimetizar lipopolissacarídeos, e desencadear a sinalização do receptor TLR4 na micróglia em pacientes com a forma neuropática de MPS (Ausseil et al. 2008; Wilkinson et al. 2012). No entanto, processos de neurodegeneração em MPS podem ter envolvimento de vias inflamatórias alternativas ativadas cronicamente (Ausseil et al. 2008). Portanto, o acúmulo de HS em conjunto com substratos secundários de

armazenamento e a disfunção autofágica podem desencadear outras vias neuroinflamatórias independentes de TLR4, e contribuir com a morte de células neuronais em MPS (Parker and Bigger 2019; Viana et al. 2020).
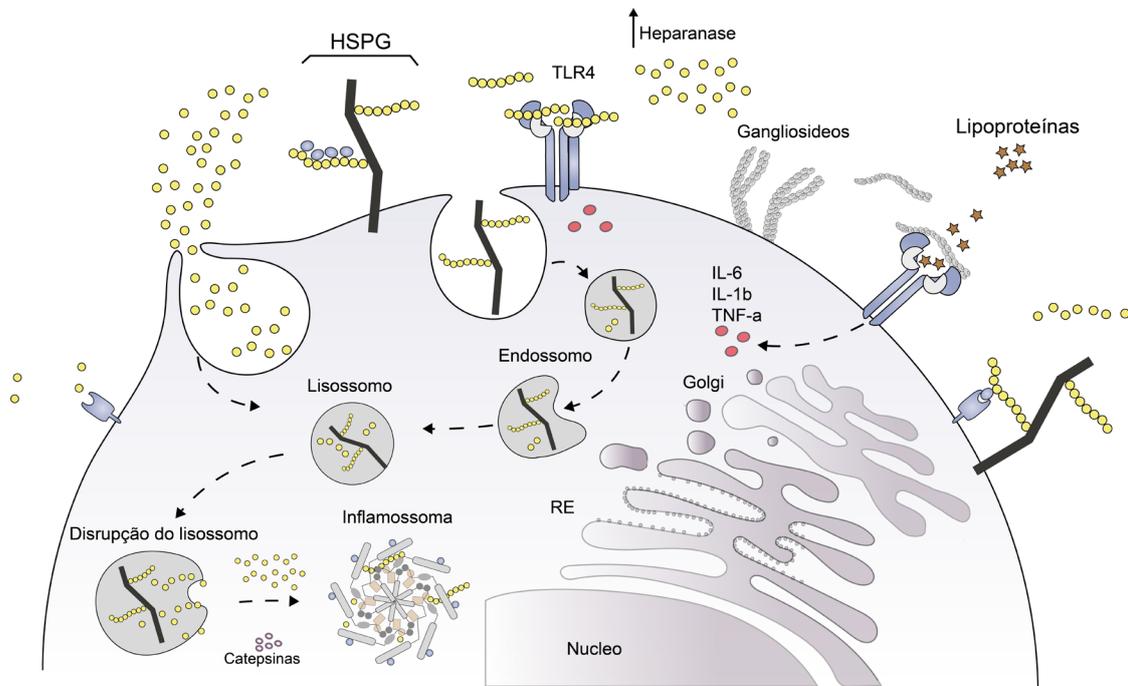
Figura 9. Processo inflamatório em DLs. HSPG pode interagir com receptores TLR4 e desencadear uma resposta inflamatória. O depósito de HS leva ao aumento da atividade da heparanase e gera fragmentos de HS que tem o potencial de se ligar a TLR4 e desencadear uma cascata inflamatória. HSPGs e fragmentos de HS são endocitados e degradados em endolisossomos. Fragmentos de HS podem ser liberados por conta da disrupção do lisossomo e ativar diretamente o inflamassoma. Adaptado de Simonaro et al. 2010; Parker and Bigger 2019.

Em resumo, o acúmulo de substratos em DLs pode desencadear diversas vias inflamatórias locais, como o cérebro, ou de forma sistêmica. O processo inflamatório pode ter como causa primária o armazenamento de macromoléculas na célula, ou ainda, ser consequente a uma disfunção secundária do sistema autofágico-lisossômico, comumente alterado em DLs (Simonaro 2016).

1.3.4. Vias do neurodesenvolvimento em MPS

GAGs ligados a proteoglicanos, exibem funções fisiológicas na superfície celular relacionadas à sinalização celular, disponibilidade de fatores de crescimento, citocinas e morfógenos. Os GAGs estão envolvidos com a motilidade e adesão celular e comunicação intercelular por meio da interação com proteínas na membrana celular (Smock and Meijers

2018). No entanto, GAGs não digeridos no tecido neural, envolvendo mecanismos inflamatórios e de neurotoxicidade, têm sido associados com alterações cognitivas graves em indivíduos com MPS (Giugliani et al. 2014). Os proteoglicanos de HS funcionam como co-receptores e a degradação anormal de fragmentos de HS afeta vias de sinalização essenciais e a atividade neuronal (Hoche et al. 2014; Bigger et al. 2018; Gaffke et al. 2020). O acúmulo de GAGs em neurônios e células gliais está associado com lesões na substância branca e atrofia cerebral, afetando o processo de mielinização. Além disso, anormalidades como o aumento dos dendritos das células de Purkinje no cerebelo, expansão do citoplasma, e diminuição de células nervosas no córtex estão associados com o depósito de GAGs (Schwartz et al. 2007; Bigger et al. 2018).

A inativação da síntese de HS ou de enzimas modificadoras de HS em modelos de camundongos, causa defeitos de desenvolvimento no prosencéfalo e erros de orientação de axônio (De Pasquale and Pavone 2019). A via de orientação do axônio ou axon guidance é responsável pelo processo de fiação neuronal, no qual os axônios são alongados e atingem seus tecidos-alvo para formar junções sinápticas (Russell and Bashaw 2018). Esses eventos ocorrem durante o neurodesenvolvimento, e são responsáveis por conectar o cérebro e a medula espinhal ao sistema nervoso periférico. Em condições normais, a interação entre moléculas de sinalização e receptores da superfície celular expressos nos cones de crescimento resulta no crescimento do axônio e no colapso do cone de crescimento. As interrupções na montagem desses circuitos neurais afetam o crescimento axonal e o desenvolvimento cognitivo em humanos (Russell and Bashaw 2018; Manzoli et al. 2021). Evidências mais recentes indicam alguns mecanismos lisossomais essenciais durante o neurodesenvolvimento. Lisossomos podem ser encontrados em todos os compartimentos neuronais, incluindo dendritos, soma e axônio. A apresentação e disponibilidade de receptores pode ser modulado pelo sistema endossomal-autofágico-lisossômico que regula a endocitose dos receptores da membrana celular e contribui para a remodelação axonal, gerando crescimento de axônio e reduzindo a estabilidade pré-sináptica (Crawley and Grill 2021). Portanto, endossomos podem regular o tráfego de receptores endocitados durante o crescimento do axônio (**Figura 10**). A via endossomal-lisossomal pode destinar estas proteínas à degradação ou reciclar estes receptores para que retornem a membrana celular (Winckler and Choo Yap 2011; Manzoli et al. 2021). Além disso, o transporte lisossomal pode percorrer longas distâncias ao longo de axônios contribuindo com a homeostase do

cone de crescimento através de sua atividade degradativa, distribuição de proteínas de sinalização e adesão celular, e transporte retrógrado de autofagossomos na porção distal do axônio (Farías et al. 2017; Manzoli et al. 2021).
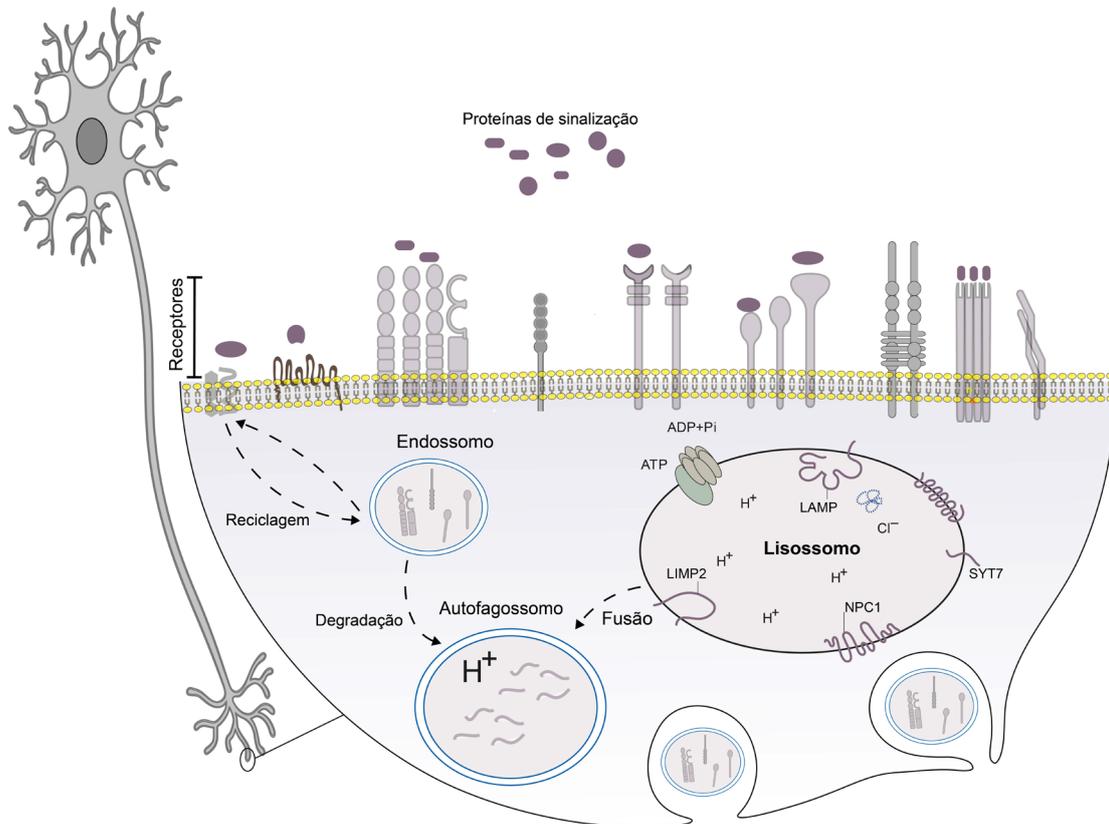
Figura 10. Sistema endossomal-autofágico-lisossômico na orientação do axônio. O sistema endossômico-lisossômico controla a disponibilidade de receptores, tráfego de receptores endocitados, e distribuição de proteínas de sinalização. Adaptado de Manzoli et al. 2021.

Portanto, disfunções da autofagia e o armazenamento de autofagossomos nos axônios podem resultar em perturbações do cone de crescimento (Crawley and Grill 2021; Manzoli et al. 2021). O transporte lisossomal ao longo do axônio com a reciclagem do conteúdo celular prejudicada ou a ausência de moléculas de sinalização e adesão disponíveis pelo sistema autofágico-endossômico afetam o desenvolvimento do axônio, levando a distúrbios do SNC em humanos (Crawley and Grill 2021; Manzoli et al. 2021). Defeitos na remodelação e orientação do axônio causam anormalidades nas conexões sinápticas e no desenvolvimento do circuito neuronal, observados em doenças congênitas com manifestações neurológicas (Manzoli et al. 2021). Danos no SNC estão presentes em formas neuropáticas de MPS. Estes indivíduos manifestam déficit cognitivo e retrocesso nos principais marcos de desenvolvimento infantil (Whiteman and Kimura 2017). Portanto,

anormalidades do sistema lisossômico-autofágico frequentemente observadas em DLs, podem gerar desequilíbrios na regulação de genes que controlam a migração e o crescimento dos axônios associados à patogênese do declínio cognitivo em MPS (Lemonnier et al. 2011; Parente et al. 2012; Salvalaio et al. 2017).

2. Objetivos

2.1. Objetivo Geral

2.1.1. Investigar os mecanismos de patogênese em doenças lisossômicas (DLs) utilizando uma abordagem de biologia de sistemas.

2.2. Objetivos específicos

2.2.1. Revisar vias morfogênicas e de crescimento envolvidas em DLs.

2.2.2. Analisar dados de expressão gênica de diferentes tipos de Mucopolissacaridose.

2.2.3. Identificar vias de regulação e patogênese envolvidas nas Mucopolissacaridoses.

2.2.4. Construção de redes de interações entre proteínas com dados do interatoma humano.

2.2.5. Investigação topológica das proteínas localizadas nas redes.

2.2.6. Análise de processos biológicos subjacentes as proteínas estudadas.

2.2.7. Geração de modelos moleculares potencialmente envolvidos na patogênese de DLs.

3. Resultados

Os resultados estão divididos em três capítulos apresentados em forma de artigos científicos.

3.1. Capítulo I: Corrêa T, Feltes BC, Giugliani R and Matte U (2021) Disruption of morphogenic and growth pathways in lysosomal storage diseases. WIREs Mech Dis 13:1–16. https://doi.org/10.1002/wsbm.1521

FOCUS ARTICLE

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Disruption of morphogenic and growth pathways inlysosomal storage diseases

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Abstract

The lysosome achieved a new protagonism that highlights its multiple cellular functions, such as in the catabolism of complex substrates, nutrient sensing, and signaling pathways implicated in cell metabolism and growth. Lysosomal storage diseases (LSDs) cause lysosomal accumulation of substrates and deficiency in trafficking of macromolecules. The substrate accumulation can impact one or several pathways which contribute to cell damage. Autophagy impairment and immune response are widely studied, but less attention is paid to morphogenic and growth pathways and its impact on the pathophysiology of LSDs. Hedgehog pathway is affected with abnormal expression and changes in distribution of protein levels, and a reduced number and length of primary cilia. Moreover, growth pathways are identified with delay in reactivation of mTOR that deregulate termination of autophagy and reformation of lysosomes. Insulin resistance caused by changes in lipids rafts has been described in different LSDs. While the genetic and biochemical bases of deficient proteins in LSDs are well understood, the secondary molecular mechanisms that disrupt wider biological processes associated with LSDs are only now becoming clearer. Therefore, we explored how specific signaling pathways can be related to specific LSDs, showing that a system medicine approach could be a valuable tool for the better understanding of LSD pathogenesis.

This article is categorized under:

   Metabolic Diseases > Metabolic Diseases>Molecular and Cellular Physiology

KEYWORDS
Hh pathway, insulin, LSDs, mTOR, systems medicine

1 | INTRODUCTION

Since first described in 1950 by Christian de Duve, the lysosome was presented as an organelle capable of degrading and recycling cellular waste (de Duve, 2005). In recent years the lysosome achieved a new status that highlights its multiple cellular functions, such as in the catabolism of complex substrates, plasma membrane repair, immune response, nutrient sensing, and signaling pathways implicated in cell metabolism and growth (Matte & Pasqualim, 2016; Parenti et al., 2015). The cross-talk between lysosomes and other related organelles is essential during these biological processes. For instance, lysosomes are necessary for the maturation of phagosomes to phagolysosomes that occurs in

pathogen phagocytosis. Moreover, the macroautophagy relies on the fusion between lysosomes and autophagosomes to generate autolysosomes (Marques & Saftig, 2019). The lysosomal biogenesis also depends on interactions with the endomembrane system, in which lysosomal proteins are synthesized in the endoplasmic reticulum and transported through the Golgi complex to the trans-Golgi network. This process is pivotal for sorting the intraluminal and membrane-bound proteins that form the lysosomes (Marques & Saftig, 2019). Such intricate interactions may suffer imbalances in pathological conditions affecting the lysosomes. This is the case of lysosomal storage diseases (LSDs) that arise when the intralysosomal accumulation of substrates or deficiency in trafficking of macromolecules occurs and subsequently impairs lysosomal function (Platt et al., 2018) (Box 1).

The molecular mechanisms by which a primary protein deficiency disrupts the biological processes associated with the lysosomes have been deeply investigated in recent years (Ballabio & Gieselmann, 2009; Levine & Kroemer, 2008; Schultz et al., 2011). The main altered biological processes in LSDs include (i) regulation of lysosomal pH; (ii) synaptic release; (iii) endocytosis; (iv) vesicular maturation; (v) autophagy; (vi) exocytosis; (vii) Ca^{2+} homeostasis; (viii) nutrient sensing; and (ix) lipid homeostasis (Bezprozvanny, 2009; Marques & Saftig, 2019; Vitner et al., 2010). Therefore, the initial substrate accumulation can impact one or several pathways which ultimately contribute to cell damage (Futerman & Van Meer, 2004). Therefore, it is necessary to expand, beyond substrate accumulation, the understanding of the pathogenic mechanisms underlying LSDs. Despite most studies so far focused on autophagy impairment and immune response, there is recent growing interest in the signaling pathways affected in the LSDs (Ballabio & Gieselmann, 2009; Fiorenza et al., 2018; Schultz et al., 2011). Deficient enzymes or proteins with relatively peripheral functions in the cell are capable of generating disturbances in the signaling pathways associated with morphogenic and growth events in the body and negatively impacting various organs in affected individuals.

In this review, we provide a brief overview of Hh, mTOR, and insulin signaling pathways, potentially involved in LSD. We describe associations between substrate accumulation and impairment in morphogenic and growth pathways and its impact on the pathophysiology of LSDs. Table 1 summarizes the key findings detailed below.

1 | HEDGEHOG (Hh) SIGNALING PATHWAY

Genetic screens identified Hh, named after the short and "spiked" appearance of the Hh mutant in *Drosophila* larvae (Varjosalo & Taipale, 2008). The Hh genes are categorized into three subgroups: (i) desert hedgehog, fundamental for testis development and peripheral nerve sheath formation; (ii) Indian hedgehog (Ihh), that coordinates differentiation, bone and cartilage growth, and is partially co-expressed with (iii) Sonic hedgehog (Shh), the most studied of the Hh proteins, which plays a role in the zone of polarizing activity in the limb bud and floor plate in the neural tube (Briscoe & Thérond, 2013; Echelard et al., 1993; Jessell, 2000). Hh proteins present *N*-terminal "Hedge" domain and a C-terminal "Hog" domain. The Hh signaling pathway is involved with mitogenic and morphogenic functions, regulating key events during developmental processes like growth and patterning of multicellular embryos (Petrova & Joyner, 2014; Simpson et al., 2009). The Hedge domain is responsible for its signaling activity, which emerges as one of the essential pathways regulating cell specification, differentiation, and tissue homeostasis (R. T. H. Lee et al., 2016). It is initially expressed in the notochord and floor plate in the specification of cell types within the neural tube (Echelard et al., 1993). In addition, the Hh pathway regulates central nervous system polarity, neural patterning, and controls stem cell homeostasis in adult tissues (Dessaud et al., 2008; Machold & Fishell, 2002; Petrova & Joyner, 2014). The deregulation of the Hh pathway may occur in LSDs and has been described in different mucopolysaccharidoses (MPS), a group of disorders whose common hallmark is an impairment on the degradation of glycosaminoglycans (GAGs). Proteoglycan-attached GAGs function as co-receptors located adjacent to the primary cilia under normal conditions, promoting Hedgehog (Hh) binding and signaling (Kingma et al., 2016; Tebani et al., 2019; Witt et al., 2013).

1.1 | GAGs catabolism failure impairing Hh pathway

The GAGs are constituents of the extracellular matrix, generally associated with proteoglycans, where they play a crucial role in the intercellular communication through the distribution of diffusible proteins and mediating ligand-receptor binding, as well as receptor recycling (Capila & Linhardt, 2002; Linhardt & Toida, 2004; Smock & Meijers, 2018). Heparan sulfate proteoglycans function as co-receptors and have been involved in the signal transduction of Hh (Witt et al., 2013), either stimulating or inhibiting its activity (Williams et al., 2010) (Figure 1). Inactivation

BOX 1　Lysosomal Storage Diseases

LSDs are a group of more than 60 rare hereditary metabolic disorders. The total or partial loss of function of a particular protein leads to the accumulation of specific macromolecules or monomeric compounds within the endosomal–lysosomal system (Platt et al., 2012). The LSDs have a progressive course, and some common clinical signs and symptoms are visceromegaly, skeletal abnormalities, cardiac problems, hearing defects, motor difficulties, and cognitive impairment. However, phenotypic characteristics depend on the specific protein affected, on the specific mutations, on the kind of stored substrate and in the tissues and organs mostly affected (Marques & Saftig, 2019; Poswar et al., 2019).

Mucopolysaccharidosis type I (MPS I). Disorder caused by the alpha-L-iduronidase deficiency leading to the accumulation of glycosaminoglycans heparan sulfate and dermatan sulfate (Giugliani, 2012). MPS I has an estimated prevalence of 1:100,000 and the median age at onset of 0.9 years in Hurler patients and 3.4 and 8.7 years in patients with Hurler–Scheie and Scheie phenotypes (Beck et al., 2014; Moore et al., 2008). The main clinical findings are facial features, hepatosplenomegaly, skeletal abnormalities, joint limitation, short stature and, in the severe form, cognitive decline (Poswar et al., 2019).

Mucopolysaccharidosis type II (MPS II). Also known as Hunter syndrome, is a rare X-linked recessive disorder caused by deficient activity of the enzyme iduronate-2-sulfatase. This enzyme is responsible for the catabolism of glycosaminoglycans dermatan sulfate and heparan sulfate. Clinical features include airway obstruction, skeletal deformities, cardiomyopathy, and neurologic impairment. The prevalence estimates in 1–9:1,000,000, and the median age at onset of the main symptoms is 6 years old (Baehner et al., 2005: Wraith et al., 2008).

Mucopolysaccharidosis type VII (MPS VII). Also known as Sly disease, presents a prevalence of <1:1,000,000 and onset infantile and late infantile. MPS VII is caused by mutations in the GUSB gene, encoding the enzyme β-glucuronidase (Muenzer, 2004). There is storage of the GAGs heparan sulfate, chondroitin sulfate, and dermatan sulfate, leading to macrocephaly, hydrocephalus, coarse facial features, hepatosplenomegaly, heart valve abnormalities, hernias and, in most cases, progressive intellectual disability (Platt et al., 2018).

Niemann-Pick type C (NPC). Neurodegenerative disorder characterized by the accumulation of non-esterified cholesterol and sphingolipids within the late endosomal/lysosomal compartment (Infante et al., 2008; Vanier, 2014). The prevalence is 1–9:100,000 and the age at onset in early infancy (<2 years) to adolescent/adult onset (≥15 years) (Patterson et al., 2013; Vanier, 2010). This disorder is caused by mutations in the NPC1 or NPC2 genes, which encode for proteins that mediate the transport of cholesterol from endosomes/lysosomes (Kwon et al., 2009; Te Vruchte et al., 2004).

Juvenile neuronal ceroid lipofuscinoses (JNCLs). A frequent cause of neurodegeneration among young patients with onset of symptoms between 5 and 10 years and with a prevalence for all forms of CCL of 1:100,000 (Mink et al., 2013; Teixeira et al., 2003). Caused by mutations in the endosomal/lysosomal membrane protein encoded by the CLN3 gene. Affected patients typically present deteriorating vision, seizures, decline of cognitive abilities, and motor failure in JNCLs (Williams et al., 2006).

Fabry disease (FD). X-linked innate error disease with a prevalence of 2–5/100,000, caused by a deficiency in glycosphingolipid catabolism (Platt et al., 2018). The defect in the enzyme alpha-galactosidase generates to systemic accumulation of glycosphingolipids (Nance et al., 2006; Pereira et al., 2007). Patients can manifest abnormalities as progressive renal failure, cardiac disease, cerebrovascular manifestations, neuropathy, and skin lesions, among other abnormalities. The mean age at the onset of this symptom in patients is before 10 years old (Branton et al., 2002; Schiffmann, 2009).

Cystinosis (CTNS). Characterized by the accumulation of cysteine within cells, due to a defect in the cysteine transport across the lysosomal membrane. The most affected organs are the kidneys and the eyes, in which children develop renal proximal tubulopathy between 6 and 12 months of age. The present a prevalence of 1–9:100,000 (Servais et al., 2008).

Krabbe disease (KD). Caused by mutations in the GALC gene, which encodes the enzyme responsible for catalyzing the breakdown of galactosylceramide, a lipid present in myelinating oligodendrocytes and Schwann cells. KD is a severe neurological condition, which results in demyelination in the nervous system and subsequent cognitive and motor decline (Graziano & Cardile, 2015; Inamura et al., 2018). The prevalence of KD is estimated at 1/100,000 and presents onset infantile and juvenile (Moser, 2006; Platt et al., 2018).

Gaucher disease (GD). Type I Gaucher disease is a glycosphingolipid storage disorder with a prevalence of 2:100,000, caused by mutations in the gene encoding for the enzyme glucocerebrosidase that causes deficiency in degradation of glucosylceramide into glucose and ceramide (Corssmit et al., 1995, Langeveld et al., 200S; Platt et al., 2018). Type I GD is the chronic non-neurological form characterized by organomegaly, bone abnormalities and cytopenia, and commonly exhibit symptoms during adolescence. Types II and III include progressive brain involvement, more severe in Type II with the onset of symptoms during early infancy (Platt et al., 2018).

Pompe disease (PD). Also known as glycogen storage disease type II, PD is a glycogen storage disease caused by deficiency of lysosomal acid-a-glucosidase encoded by the GAA gene. The disorder has a prevalence of 2.5:100,000, which leads to the intralysosomal accumulation of glycogen, mainly in the heart, skeletal, and smooth muscle. The disease presents onset infantile and adult, and the main clinical symptoms are hypotonia, progressive weakness, hepatomegaly and cardiac and respiratory failure (Dasouki et al., 2014; Platt et al., 2018).

TABLE 1 Alterations in Hh, mTOR, and insulin signaling

LSD	Molecular defect	Phenotype	Reference
MPS I	Changes in distribution of GAGs and Ihh protein levels in chondrocytes.	Decreased bone growth.	Kingma et al. (2016)
	Impaired mTOR reactivation and defective lysosome reformation.	Enlarged and long-lasting autolysosomes.	Yu et al. (2010)
MPS II	Reduction of Shh signal transduction.	Defects in heart ventricle development and trabeculation.	Costa et al. (2017)
MPS VII	Abnormal expression of Ihh.	Shortening of bones.	St-Jacques et al. (1999), Metcalf et al. (2009). Peck et al. (2015), Kingma et al. (2016)
	Increased mTORC1 activity.	Impaired chondrocyte function and bone growth.	Bartolomeo et al. (2017)
NPC	Reduction of Ptch1 and Smo protein levels.	Reduced number and length of primary cilia.	Canterini et al. (2017), Formichi et al. (2018)
	Changes in composition of lipid rafts.	Receptor activation of insulin is impaired.	Vainio et al. (2005)
JNCL	Increase in autophagy and decrease of mTOR activity	Maturation of autophagic vacuoles and lysosomes are impaired.	Cao et al. (2006)
FD	Impaired mTOR reactivation and defective lysosome reformation.	Enlarged and long-lasting autolysosomes.	Yu et al. (2010)
Cystinosis	Delay in mTORC1 reactivation.	Late endosomes/lysosomes enlarged and clustered in the perinuclear area.	Ivanova et al. (2016)
KD	Disruption of lipid rafts and deficiency of the activation of mTOR.	Developmental defects and impaired myelin formation.	Inamura et al. (2018), Narayanan et al. (2009)
GD	Accumulation of GM3 in lipid rafts.	Lower insulin-mediated glucose uptake and insulin resistance.	Ghauharali-van der Vlugt et al. (2008), Langeveld et al. (2008)
	mTOR hyperactivity.	Defect in autophagic flux.	Brown et al. (2019)
PD	Defect in activation of mTOR.	Autophagic build-up.	Lim et al. (2017)

Note: FD, Fabry disease; GD, Gaucher disease; JNCL, juvenile neuronal ceroid lipofuscinoses; KD, Krabbe disease; LSD, lysosomal storage disease; MPS I, mucopolysaccharidosis type I; MPS II, mucopolysaccharidosis type II; MPS VII, Mucopolysaccharidosis type VII; NPC, Niemann-Pick type C; PD, Pompe disease.
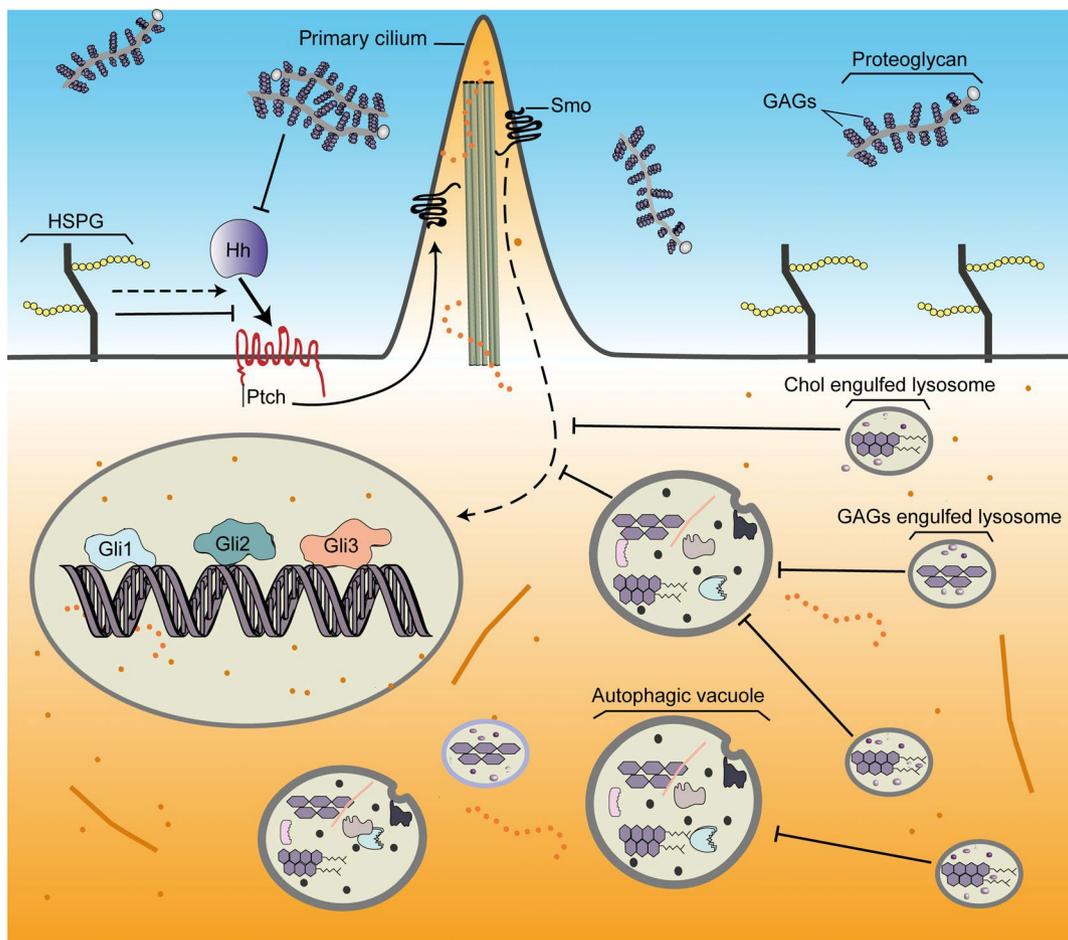
FIGURE 1 Model for the theoretical contribution of macromolecules in the Hedgehog (Hh) pathway alterations. The accumulation of glycosaminoglycans and heparan sulfate proteoglycans in the extracellular matrix, as well as lysosomal cholesterol (chol) and GAGs engorgement, may disturb the Hh pathway (see text for more details)

of HS biosynthetic or HS modifying enzymes affects Hh activity in mouse models resulting in developmental defects of the forebrain, in axon guidance errors, and in abnormalities of bone development (Bandari et al., 2015; De Pasquale & Pavone, 2019).

MPS patients exhibit severe spine deformities, including progressive kyphoscoliosis and spinal cord compression (Lampe & Lampe, 2018; Muenzer et al., 2009). An imbalance of chondrocyte hypertrophic differentiation rate during the process of cartilage to bone transition was shown in MPS VII dogs, leading to a delay in the endochondral bone formation (Smith et al., 2012). It is important to note that the timing and rate of chondrocyte differentiation during bone formation are regulated by secreted growth factors, such as bone morphogenetic proteins and the Ihh pathway (Vortkamp et al., 1996). Ihh is also a key regulator of chondrocyte proliferation (Long et al., 2001), and abnormal expression has been reported in the pathophysiology of shortening of bones seen in MPS VII mice (Kingma et al., 2016; Metcalf et al., 2009; Peck et al., 2015; St-Jacques et al., 1999).

The transcript factor Sox9 is regulated by Ihh and is considered essential in chondrocyte differentiation (Sugita et al., 2013). mRNA analysis shows the aberrant persistence of Sox9 protein in MPS VII dogs (Peck et al., 2015). Kingma et al. (2016) observed changes in the distribution of GAGs and Ihh protein levels in chondrocytes from MPS I patients and growth plates in mice with decreased bone growth (Kingma et al., 2016). Therefore, these studies suggest that GAG alters Ihh signaling cascade and impair chondrocytes to progress from proliferation to hypertrophy, causing a delay in the formation and consequent bone abnormalities in MPS I and VII (Akiyama et al., 2002; Kingma et al., 2016; Peck et al., 2015).

Shh is an essential signaling factor during the heart's development and regeneration (Dyer & Kirby, 2009; Wang et al., 2015). In the MPS II mouse model, the loss of iduronate-2-sulfatase activity may cause a reduction of Shh signal

transduction. These animals present defects in heart ventricle development and trabeculation with electrophysiological abnormalities such as prolonged P-R interval at postnatal stages before any considerable accumulation of GAGs in the lysosomes (Costa et al., 2017).

1.1.1 | Primary cilium and cholesterol homeostasis

The primary cilium is a microtubule-based plasma membrane protrusion and constitutes an organelle required for optimal Hh signal transduction during embryonic and neuronal development in vertebrates (Goetz & Anderson, 2010) (Figure 1). Mutations that lead to defects in the composition and structure of the primary cilia are associated with abnormal cerebellar morphogenesis and intellectual disability (Aguilar et al., 2012; J. H. Lee & Gleeson, 2010). Cholesterol plays a key role in Shh modulation and in the formation and maintenance of primary cilia. Indeed, a modification by covalent addition of cholesterol is required during Shh protein activity. The absence of this modification by cholesterylation results in stimulation delay and fails to activate signaling in downstream molecules in the Shh pathway (Porter et al., 1996; Xiao et al., 2017). Moreover, the deficiency in lipid metabolism and traffic result in problems in the formation of primary cilia, including the proliferation of cerebellar granule neuron precursors in Niemann-Pick type C (NPC), a disease caused by intracellular cholesterol trafficking (Formichi et al., 2018; Vanier, 2014; Willemarck et al., 2010).

Reduced number and length of primary cilia are seen in human NPC patient's fibroblasts when compared to controls, with a reduction of Ptch1 and Smo protein levels (Canterini et al., 2017; Formichi et al., 2018). In normal conditions, these proteins are located on the primary cilium and in the absence of Ptch, Smo inhibition occurs, inactivating the Shh signaling pathway. However, during pathway activation, the Shh protein binds to Ptch allowing the activation of Smo that in turn liberates the Gli transcription factor from its inhibitory protein Sufu, leading to the activation of downstream genes (Jiang & Hui, 2008; R. T. H. Lee et al., 2016). Therefore, cilia abnormalities in NPC may disrupt Shh pathway that ultimately contributes to the cerebellar hypoplasia seen in patients (Canterini et al., 2017; Formichi et al., 2018).

2 | mTOR SIGNALING PATHWAY

Mammalian or mechanistic target of rapamycin (mTOR) displays serine/threonine-protein kinase activity that controls biosynthetic and catabolic processes in response to extracellular and intracellular signals, including nutrients, oxygen availability, and growth factor signaling (Sebgupta et al., 2010). The recruitment of mTOR in the lysosomal surface is necessary to regulate its activation/inactivation, evidencing a linear relationship between mTOR and the lysosomal function (Lim et al., 2017; Sancak et al., 2010). The mTOR pathway is formed by Complex 1 (mTORC1) and 2 (mTORC2) (D. H. Kim et al., 2002; Pearce et al., 2007; Saxton & Sabatini, 2017). While mTORC1 regulates cell growth and metabolism, promoting the synthesis of proteins, nucleotides, lipids, and glucose metabolism, mTORC2 is associated with the control of proliferation and survival, regulating cytoskeletal rearrangement, ion transport and cell migration (Jacinto et al., 2004; Porstmann et al., 2008; Saxton & Sabatini, 2017).

mTORC1 signaling is closely related to cellular anabolic activities, suppressing protein turnover pathways such as autophagy, ubiquitin-proteasome systems, and lysosomal biogenesis (J. Kim et al., 2011; J. Zhao et al., 2015). Moreover, mTORC1 can promote the maintenance of mitochondrial oxidative function, enhance respiration by generating a complex with the transcription factors PPARγ coactivator 1α, and regulate the expression of mitochondrial genes coordinating their transcriptional programs (Cunningham et al., 2007; Zoncu et al., 2011). A more comprehensive review of mTOR's importance in mitochondrial functions can be found in Morita et al. (2015). Lysosome biosynthesis requires a coordinated transcription of the gene network regulating lysosomal biogenesis. These genes have a common motif, called coordinated lysosomal expression and regulation element, which is a target of transcription factor EB (TFEB) (Sardiello et al., 2009). mTORC1 phosphorylates and inhibits the nuclear translocation of the TFEB, which promotes the expression of a dozen genes involved in lysosomal biogenesis and the autophagy process (Saxton & Sabatini, 2017; Settembre et al., 2012). In normal conditions, under nutrient deprivation or in the absence of growth factors, TFEB promotes perinuclear lysosomal repositioning, repressing mTORC1 activity and stimulating autophagy (Saxton & Sabatini, 2017; Schultz et al., 2011). The disturbance of lysosomal function caused by substrate accumulation can generate perturbations in cellular networks that spread through proteins, causing dysfunctions in autophagy and mTOR, frequently seen in LSDs (Fukuda et al., 2006; Ko et al., 2005; Koike et al., 2005; Settembre et al., 2008).

2.1 | Dysfunction of mTOR in LSDs

Studying lymphoblastoid and cerebellar cells established from homozygous *Cln3* knock-in mice in juvenile neuronal ceroid lipofuscinosis (JNCL), Cao et al. (2006) observed an increase in autophagy and a significant decrease in mTOR activity. Besides that, morphological analyses showed that the maturation of autophagic vacuoles and lysosomes is impaired when compared to wild-type organelles (Cao et al., 2006). mTORC1 regulates the termination of autophagy and reformation of lysosomes, but this control is disrupted in cell lines derived from patients with MPS I, and Fabry disease (Yu et al., 2010). Studies in epithelial cells obtained from cystinotic patients show that the delay in mTORC1 reactivation is due to its change of cellular location during starvation (Ivanova et al., 2016). In normal conditions, mTORC1 is dissociated into the cytoplasm, where it remains inactive during starvation, but in cells from patients with cystinosis mTORC1 does not dissociate from the late endosomal/lysosomal vesicles clustered in the perinuclear area (Ivanova et al., 2016; Sancak & Sabatini, 2009).

Possible answers to the delay in reactivation of mTOR in LSDs involve the loss of function of proteins as the battenin, deficient in JNCL, which plays a role in autophagic vacuole maturation toward the lysosome, leading to a deficiency in the maturation of vacuoles to degradative autolysosomes. The inefficiency in autophagic vacuole cargo turn over in the cell can stimulate nutrient deprivation and suppress mTOR signaling, leading to upregulation of autophagy (Cao et al., 2006; Ivanova et al., 2016) (Figure 2). In this sense, the reduced availability of amino acids is a result of the deregulation of its production via autophagic degradation of proteins and subsequent decrease of mTORC1 activity, or the increased autophagic process could inhibit mTORC1 activity, since autophagy and mTORC1 pathways are mutually antagonistic (Sancak et al., 2010; Wong et al., 2012). Otherwise, substrates may be directly involved in the deficiency of mTOR activity. For instance, the accumulation of galactosylsphingosine can disrupt lipid rafts during development of membrane microdomains and affect the activation of mTOR in oligodendrocytes isolated from mouse brain with
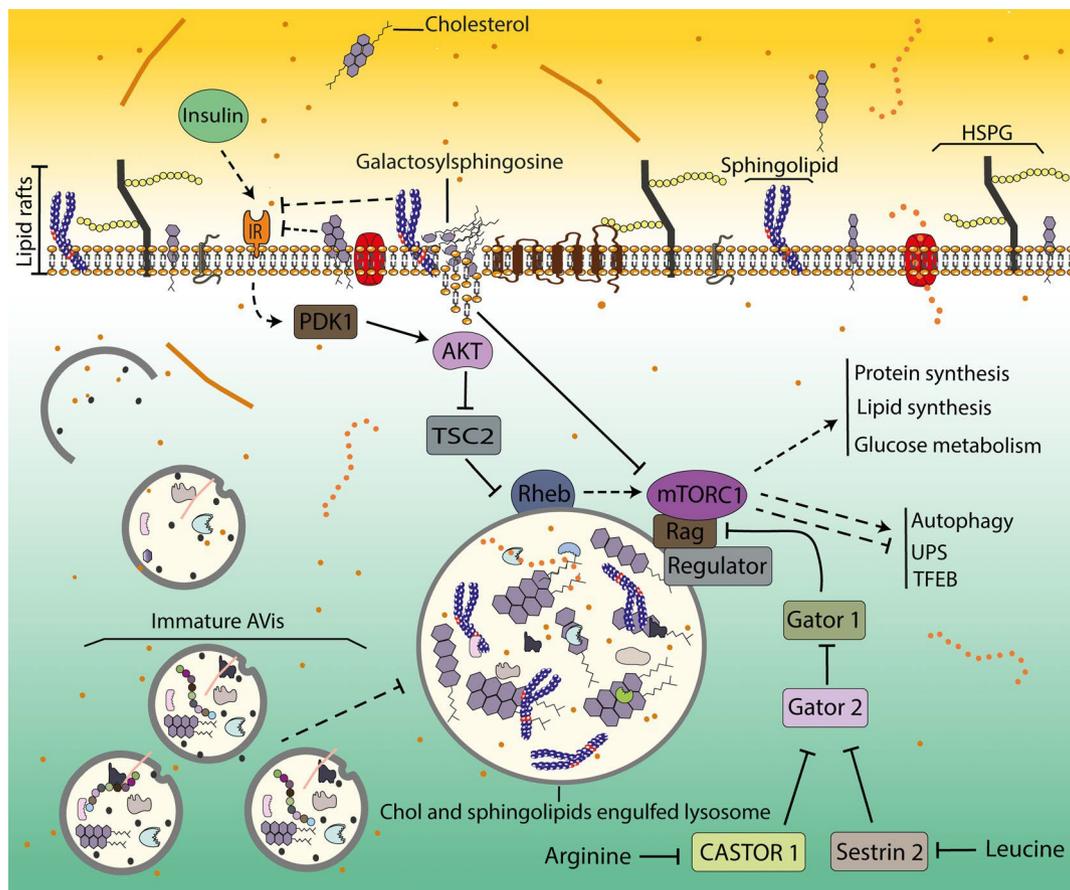

FIGURE 2 Model for the possible involvement of macromolecules in the mTOR and insulin pathway alterations. The accumulation of sphingolipids and cholesterol in the extracellular matrix may affect insulin signaling. Also, the increased autophagy induction due to decreased mTORC1 appears to be common in LSDs (see text for more details)

Krabbe disease (Inamura et al., 2018; Narayanan et al., 2009) (Figure 2). Moreover, one study brought impressive results involving Rag GTPases, proteins that mediate amino-acid signals, and subsequent activation of mTORC1 (E. Kim et al., 2008). Amino acids induce the translocation of mTORC1 to lysosomal membranes, where the Rag GTPases are anchored (Sancak et al., 2010). Knock-out of Rag proteins in cardiomyocytes was associated with LSDs phenocopies, including the increase of autophagy initiation, but with a defective autophagy flux (Y. C. Kim et al., 2014). Therefore, studies indicate that the deregulation of mTOR and the defect in autophagy can be a common finding among many LSDs. Possible new strategies in therapeutic intervention through mTORC1 modulation begin to be further investigated (Ivanova et al., 2016). Examples demonstrate that the reactivation of mTOR inhibits autophagy and initiates lysosome reformation in normal rat kidney cells (Yu et al., 2010). Also, the reactivation of mTOR by knockdown of tuberous sclerosis 2 protein or arginine was able to reverse muscle atrophy and remove the autophagic build-up in muscle cells from a mice model of Pompe disease (Lim et al., 2017) (Box 1).

In contrast, the increased mTORC1 activity was observed in chondrocytes of the MPS VII model with β-glucuronidase deleted. The enhanced mTORC1 activity and lysosomal dysfunctions were associated with impaired chondrocyte function and bone growth in a mouse model MPS VII (Bartolomeo et al., 2017). Furthermore, mTOR hyperactivity has been identified in the pluripotent stem cell model of Gaucher disease. Interestingly, pharmacological inhibition of glucosylceramide can reverse mTORC1 hyperactivation, indicating that the increase in mTORC1 signaling is mediated by the accumulation of glycosphingolipids (Brown et al., 2019). Therefore, these data support the idea that the uncontrolled mTORC1 activity in pathological conditions is not only a consequence of the dysregulation of autophagy, but also the abnormal deposit of substrates in the cell can directly affect its activity.

3 | INSULIN SIGNALING AND SPHINGOLIPIDS

Insulin is the primary anabolic hormone that promotes the deposition of carbon energy in the body, and one of its primary functions is glucose uptake and energetic storage for the cells in the body (Tokarz et al., 2018). Insulin is a peptide hormone secreted by β-cells in the pancreas, and it acts through the insulin receptor (IR), a membrane tyrosine kinase receptor. The binding of insulin to the cell membrane allows the autophosphorylation of its receptor on tyrosine residues, transducing the signal to metabolic and mitogenic pathways (Ikonen & Vainio, 2005; Saltiel & Kahn, 2001). Insulin signal transduction can be hampered by altered membrane composition due to disturbances in lipid rafts (Bickel, 2002; Fuller, 2010). Lipid rafts are plasma membrane microdomains required to effectively activate downstream molecules by transmembrane receptors, influencing hitting receptor activation, endocytic uptake, and receptor routing (Fuller, 2010; Ikonen & Vainio, 2005; Simons & Gruenberg, 2000; Vainio et al., 2005). These highly dynamic and transient entities are enriched in saturated phospholipids, sphingolipids, glycolipids, GAGs, and cholesterol (Sezgin et al., 2017).

3.1 | Insulin resistance in LSDs

GM3 is a ganglioside constituent of lipid rafts that modulate receptor-mediated signal transduction (Kabayama et al., 2007; Sekimoto et al., 2012). In cellular and animal models of TNF-α induced insulin resistance, an increase in GM3 synthesis has been observed (Kabayama et al., 2007; Tagami et al., 2002). Besides, pharmacological depletion of GM3 reverted the effect of TNF-α on insulin-dependent tyrosine phosphorylation of IRS-1 signaling (Tagami et al., 2002). Moreover, the addition of exogenous GM3 led to the suppression of tyrosine phosphorylation of IRS-1 and reduced glucose uptake, demonstrating that GM3 mimics the effects of TNF-α on insulin signaling (Kabayama et al., 2007). Finally, mice unable to synthesize GM3 show an increase in insulin sensitivity, indicating that GM3 can negatively regulate insulin signaling (Yamashita et al., 2003; H. Zhao et al., 2007) (Figure 2). An increase in plasma concentration of GM3 is observed in type I Gaucher disease, a sphingolipidosis caused by the accumulation of glucosylceramide due to glucocerebrosidase deficiency. The secondary increase of GM3 in glucocerebrosidase deficiency may be a compensatory mechanism with consequent lower insulin-mediated glucose uptake and higher peripheral insulin resistance in Gaucher patients, probably caused by the accumulation of GM3 in lipid rafts of myocytes and adipocytes (Ghauharali-van der Vlugt et al., 2008; Langeveld et al., 2008).

The *NPC1* gene was previously associated with early-onset and morbid adult obesity by GWAS study (Meyre et al., 2009). Indeed, weight gain and metabolic changes associated with insulin resistance were supported in a mouse model with haploinsufficiency of *NPC1* (Jelinek et al., 2011). Moreover, the receptor activation of insulin is also

FIGURE 3   Systems biology strategies for studying lysosomal storage disease. Multiple methodologies are used to produce omics data and generate networks that allow the investigation of biological aspects of these disorders. Algorithms can infer disease genes, pathways that contribute to disease pathophysiology, and new pharmacological targets. Study model adapted from Argmann et al., (2016)
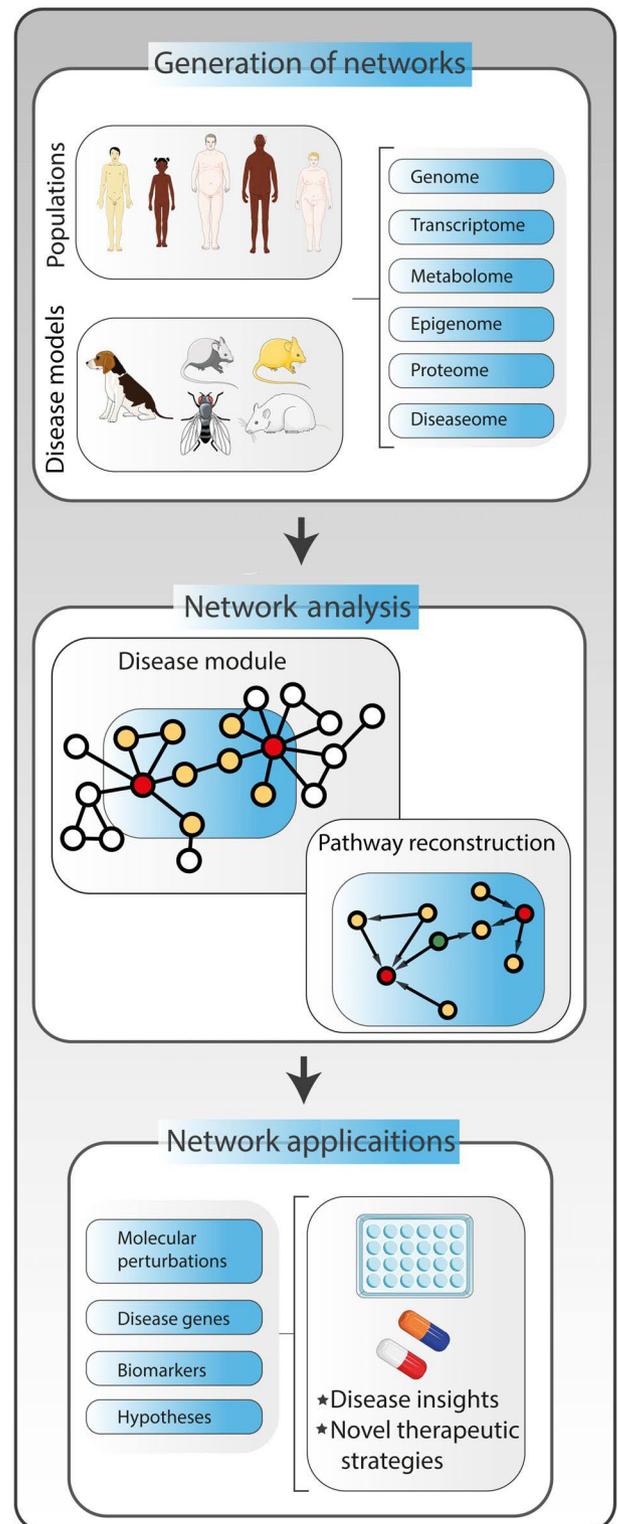
impaired in *Npc1^{−/−}* hepatocytes, and IR levels are upregulated in these mice, probably due to changes in the composition of lipid rafts compared to control (Vainio et al., 2005) (Figure 2).

3.2 | Perspective

While the genetic and biochemical bases of deficient proteins in LSDs are well understood, the secondary molecular mechanisms that disrupt broader biological processes associated with LSDs are only now becoming more apparent.

Substrate accumulation can impact primary and secondary pathways to alter gene expression and tertiary biochemical pathways (Futerman & Van Meer, 2004). Questions about how lysosomal function is affected by storage, independent of the primary substrate, or how the substrates deposit affects cell signaling flow, are still open and need to be investigated (Platt et al., 2012). Thus, investigating altered signaling pathways during pre- and post-deposition of the substrate will allow new insights to understand their impact on lysosomal functions. This paper explored how specific signaling pathways can be related to specific LSDs, showing that a systematic approach could be a valuable tool for a better understanding of LSD pathogenesis.

The use of network science in medicine allows the analysis and the understanding of various biological processes, with nodes identifying a biological entity as a protein and edges representing the relationships between entities (Loscalzo et al., 2017). Therefore, systems biology tools allow identifying new genes responsible for different diseases by examining their cellular networks' relationships (Loscalzo et al., 2017). Topological analyses show significant molecular similarities as gene co-expression, shared clinical and pathobiological characteristics, and high comorbidity in the overlap of diseases in the interactome (Loscalzo et al., 2017; Menche et al., 2015). The analysis demonstrated that proteins associated with inborn errors of metabolism (IEM) tend to locate in the same network neighborhood in the human interactome (Woidy et al., 2018). Furthermore, proteins associated with specific disease groups, such as lysosomal disorders, form homogeneous clusters distant from other proteins related to IEM (Woidy et al., 2018). If there is a proximity between LSDs in the human interactome, systems biology approaches will allow integrating multi-omics data, and the knowledge about lysosomal diseases could help to identify molecular perturbations associated with pathways and new biomarkers for the development of novel therapeutic strategies (Argmann et al., 2016; Caldera et al., 2017; Woidy et al., 2018) (Figure 3).

The endosomal-autophagic-lysosomal system's engorgement alone cannot explain the clinical spectrum of manifestations found in LSD patients. Studies discussed here show that abnormalities in signaling pathways can affect lysosome reformation, autophagic flow, glucose uptake, and tissue morphogenesis in several LSDs. In the last years, the idea that pathogenicity was only derived from a mutation in a single gene leading to the storage of a specific single substrate has been changed to a more comprehensive view that allows a better knowledge of the complex underlying mechanisms by which the accumulation of substrates broadly affects the cellular function in these disorders. A broad understanding of these processes may help develop more effective therapeutic strategies for the LSDs.

CONFLICT OF INTEREST
The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS
Thiago Correa: Conceptualization; data curation; formal analysis; methodology; writing-original draft; writing-review and editing. Bruno C. Feltes: Conceptualization; formal analysis; methodology; supervision; writing-original draft; writing-review and editing. Roberto Giugliani: Supervision; writing-review and editing. Ursula Matte: Conceptualization; supervision; writing-review and editing.

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ORIGINAL RESEARCH ARTICLE

Network Analysis Reveals Proteins Associated with Aortic Dilatationin Mucopolysaccharidoses

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Abstract

Mucopolysaccharidoses are caused by a deficiency of enzymes involved in the degradation of glycosaminoglycans. Heart diseases are a significant cause of morbidity and mortality in MPS patients, even in conditions in which enzyme replacement therapy is available. In this sense, cardiovascular manifestations, such as heart hypertrophy, cardiac function reduction, increased left ventricular chamber, and aortic dilatation, are among the most frequent. However, the downstream events which influence the heart dilatation process are unclear. Here, we employed systems biology tools together with transcriptomic data to investigate new elements that may be involved in aortic dilatation in Mucopolysaccharidoses syndrome. We identified candidate genes involved in biological processes related to inflammatory responses, deposition of collagen, and lipid accumulation in the cardiovascular system that may be involved in aortic dilatation in the Mucopolysaccharidoses I and VII. Furthermore, we investigated the molecular mechanisms of losartan treatment in Mucopolysaccharidoses I mice to underscore how this drug acts to prevent aortic dilation. Our data indicate that the association between the TGF-b signaling pathway, Fos, and Col1a1 proteins can play an essential role in aortic dilation's pathophysiology and its subsequent improvement by losartan treatment.

Keywords Aortic dilatation · MPS I · MPS VII · TGF-b · *Fos* · *Col1a1*

1 Introduction

Mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders caused by a deficiency of enzymes involved in glycosaminoglycans' degradation (GAGs). Although there are nine types of MPS [1], all of them share

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a common abnormality of progressive GAG accumulation, leading to a multisystemic dysfunction [2]. MPS patients' clinical manifestations include intellectual disability, skeletal deformities, dysmorphic facial characteristics, impairment of the central nervous system, hearing deficit, organomegaly, pulmonary, and cardiac dysfunction [3, 4]. However, the disease's clinical findings and severity depend on the type of accumulated GAG and the specific genetic mutation present in the individual [5–7].

Except for MPS IX, cardiac anomalies were reported in all MPS types, with the potential to cause sudden death, death by heart failure, and coronary occlusion [8–11]. In MPS, cardiovascular manifestations are common and include heart hypertrophy, cardiac function reduction, increased left ventricular chamber, and aortic dilatation [12]. The overall incidence of aortic root dilatation (ARD) was estimated to be as high as 40% in MPS patients [11, 13].

Heart diseases are a significant cause of morbidity and mortality in MPS patients [11, 13], even in conditions in which enzyme replacement therapy is available [14–16]. Impairment in the GAG degradation pathway leads to aortic and valve thickening, macrophage infiltration,

collagen alteration, and elastin laminae fragmentation [12, 17]. The proposed pathogenesis model consists of the binding of GAG to the Toll-like receptor 4 (TLR4), triggering an inflammatory cascade that culminates in the liberation of several cytokines, chemokines, and activation of the complement system [18–20]. However, the downstream events which influence the heart dilatation process are unclear [14, 16]. Hence, the elucidation of common mechanisms shared between cardiac anomalies in MPS and other heart pathologies may increase these patients' therapeutic options.

In previous studies, it was shown that aortic dilatation is present in animal models MPS I and VII, and it was established that the process of aortic dilation shares common features among both disorders [20, 21]. Furthermore, losartan treatment seems to prevent aortic dilation in MPS I mice, but the molecular mechanisms by which losartan acts were not investigated [22].

In this study, we applied an integrative analysis using data from the MPS VII mouse model that can be considered a model of aortic dilation in other forms of MPS. Using systems biology tools and transcriptomic data, we investigated potential new elements involved during aortic dilatation in MPS syndrome. Our network analysis brings new insights for identifying relevant genes/proteins and suggests the identification of new possible therapeutic targets.

2 Materials and Methods

2.1 Gene Expression Data

Transcriptomic data were gathered from the matrix file GSE30657 (available at Gene Expression Omnibus (GEO) [http://www.ncbi.nlm.nih.gov/geo]), published by [20], from microarray analysis of ascending aortas of healthy vs. MPS VII mice at six months of age. Groups were formed by three normal mice, three MPS VII mice with dilated aortas, and two MPS VII mice with non-dilated aortas. We used only differentially-expressed genes (DEGs) of normal vs. dilated aorta of MPS VII mice among the different groups.

The *GEOquery* package [23] for the R platform was employed to download the raw data. The dataset was submitted to background correction and normalization preprocessing using the packages *lumi* [24] and *beadarray* [25]. After preprocessing, the dataset was analyzed by the *arrayQualityMetrics* [26] package to access the sample quality information. Finally, the package *limma* [27] was employed to find DEGs analysis. DEGs were obtained by applying a filter of logFC>1 and p-value<0.05, with the Benjamini–Hochberg test for FDR correction.

3 Network Design

The protein–protein interaction (PPI) metasearch engine STRING 10.0 [http://string-db.org/] was used to create PPI networks based on the DEGs obtained from the previous step. The parameters used in STRING were, as follows: (i) medium degree of confidence of 0.400, with 1.0 being the highest level of confidence; (ii) 500 proteins in the first and second shell; and (iii) all prediction methods enabled, except for text mining and gene fusion. The STITCH database 5.0 [http://stitch.embl.de] was used to identify predictive interactions among different proteins and losartan. The parameters used were: (i) medium degree of confidence of 0.400; (ii) first shell no more than ten interactors and second shell: none; and (iii) all prediction methods enabled. The final PPI network obtained by STRING and STITCH was analyzed in Cytoscape V.3.7.2 [28].

4 Centrality Analysis

A centrality analysis was conducted to access the most topologically relevant genes and identify new pivotal targets in the PPI network. Tree significant parameters of network centralities (node degree, closeness, and betweenness) were calculated to determine hub-bottlenecks (H-B) nodes from the PPI network using the Cytoscape plugin CentiScaPe 3.2.1 [29]. Betweenness measures the numbers of shortest paths that pass through a node, which are intermediate between neighbors rank higher. Nodes with high betweenness values are considered bottlenecks [30]. Closeness Centrality identifies relevant nodes that can communicate quickly with other nodes of the network. Degree centrality is defined by the number of links directly connected to a node. Nodes with high degree values are called hubs and are involved in many network interactions [31, 32]. Equations (1), (2), and (3) describe how the node degree, closeness, and betweenness centralities are calculated, respectively:

$$Cd(i) = \deg(i), \tag{1}$$

where the degree centrality is calculated by node i [30].

$$\text{Cclo}(i) = \frac{1}{\sum_{t \in V} \text{dist}(i,j)}, \tag{2}$$

where dist (i, j) represents the distance or the shortest path p between the nodes i and j [30].

$$C(i) = \sum_{j,k} \frac{\sigma_{j,k}(i)}{\sigma_{j,k}}, \tag{3}$$

where $\sigma_{j,k}(i)$ is the number of shortest paths between nodes j and k that pass node i and $\sigma_{j,k}$ is the total number of shortest paths between nodes j and k [33].

5 Clustering and Gene Ontology (GO) Analysis

The MCODE algorithm was employed to identify densely connected regions in the final Cytoscape network [34]. The algorithm uses a vertex-weighting scheme based on the clustering coefficient, Ci, which measures 'cliquishness' of the neighborhood of a vertex. Equation (4) describes how the cluster score is calculated:

$$Ci = \frac{2n}{ki(ki-1)}, \tag{4}$$

where ki is the vertex size of the neighborhood of vertex i and n is the number of edges in the neighborhood [34]. The PPI modules generated by MCODE were studied by focusing on influential bioprocesses using the Biological Network Gene Ontology (BiNGO) 3.0.3 Cytoscape plugin [35]. The degree of functional enrichment for a given cluster and category was quantitatively assessed using a hypergeometric distribution. Multiple test correction was implemented by applying the algorithm [36] at a significance level of $p < 0.05$.

6 Construction H-B Subnetwork

We selected our H-B in protein–protein interaction data from a previously curated human interactome [37]. To generate the subnetwork, we extracted only the H-B in the interactome and its first direct neighbors. Network measures were calculated using the Cytoscape tool Network Analyzer. Cytoscape V.3.7.2 was used for the drawing and coloring of both networks.

7 Molecular Pathway Reconstruction

The Cytoscape plugin PathLinker 1.4.2 [38] was employed to identify and reconstruct the signaling pathway of interest. The app computes the k shortest paths that connect any source to any target in the network and create a table with the shortest paths' rank. The H-B (Fos, Hadc5, Plau, Fcer1g, Col1a1, Pdgfb, Cfd) in the chemo-biology network was selected as source and target in paths, and the protein–protein interactome [39] was used as a background network. The parameters used in Pathlinker were: (i) k: 50 (number of paths the user seeks); (ii) edge penalty: 1; and (iii) edge weight: weight probabilities that consider the edge weights as a multiplicative, resulting result in the k highest cost paths [38].

8 Results

8.1 Networks and Topological Analysis

Gene expression analysis of the dilated aorta group resulted in 202 DEGs (Supplement Table 1). However, the STRING database presented data for 199 proteins for the current input. Finally, the PPI network was composed of 1149 nodes (proteins) and 23,424 edges (interactions). Centrality analyses were carried out to identify the most topologically relevant H-B nodes, in which 217 were identified. Out of these, 16 (Lgals3bp, Igf2, Lpxn, Pdgfb, Tyrobp, Myh9, Cfd, Col1a1, Plau, Ccnd1, Fos, Acsl1, Aldh3b1, Fcer1g, Hspa2, and Dac5) are DEGs. A typical workflow employed in this study can be found in Fig. 1.

9 Clustering and GO Analysis

Once generated, clusters were selected according to bioprocesses, mostly related to immunological processes and the cardiovascular system. The most relevant GO terms (Supplement Tables 3–5) were associated with the immune system, such as (i) leukocyte activation; (ii) complement activation; and (iii) chemotaxis. Additionally, the bioprocesses related to the cardiovascular system were: (i) heart development; (ii) myofibril assembly; (iii) positive regulation of smooth muscle cell proliferation and; (iv) striated muscle cell differentiation. We excluded GO terms unrelated to the studied disease or that were too general (e.g., regulation of the biological process, signaling process, or response to endogenous stimulus) and anything below a significance value of 10^{-3}.

10 H-B Subnetwork and Human Interactome

Different measures were calculated and compared between the H-B subnetwork and the human interactome curated by Menche et al. (2015) (Fig. 2a). The clustering coefficient (Cl) indicates which nodes tend to cluster together, allowing the detection of densely connected regions in sizeable protein–protein interaction networks. Therefore, the Cl defines the tendency of proteins to form clusters or modules in the interactome [40]. The H-B subnetwork's average clustering coefficient was 0.35, higher than the human interactome (cl, 0.17) (Fig. 2b). The subsequent analysis measured the diameter that determines the distance between two nodes [41]. We found a diameter of 7 for the H-B subnetwork and 13 for the human interactome. Lastly, the average shortest path length was used to detect the expected distance between all pairs of nodes that

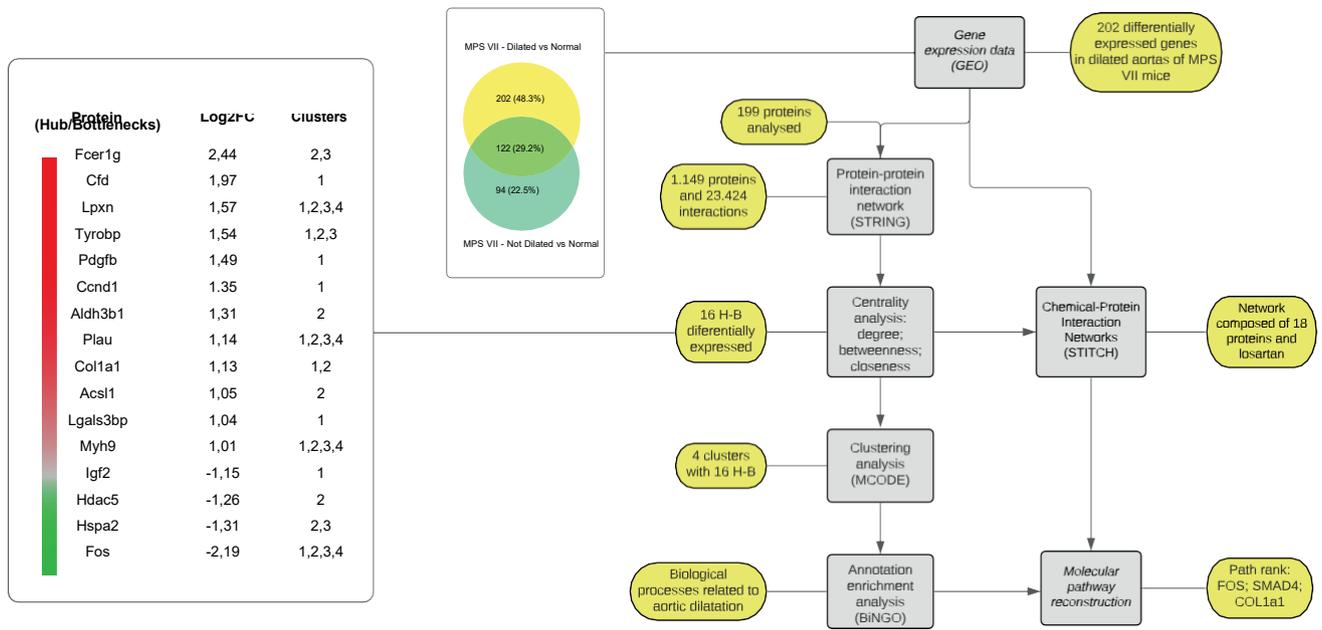| Protein (Hub/Bottlenecks)	Log2FC	Clusters
Fcer1g	2,44	2,3
Cfd	1,97	1
Lpxn	1,57	1,2,3,4
Tyrobp	1,54	1,2,3
Pdgfb	1,49	1
Ccnd1	1.35	1
Aldh3b1	1,31	2
Plau	1,14	1,2,3,4
Col1a1	1,13	1,2
Acsl1	1,05	2
Lgals3bp	1,04	1
Myh9	1,01	1,2,3,4
Igf2	-1,15	1
Hdac5	-1,26	2
Hspa2	-1,31	2,3
Fos	-2,19	1,2,3,4

Fig. 1 System biology analysis workflow with differentially expressed genes of dilated and non-dilated aortas in mice and differentially expressed H-B proteins

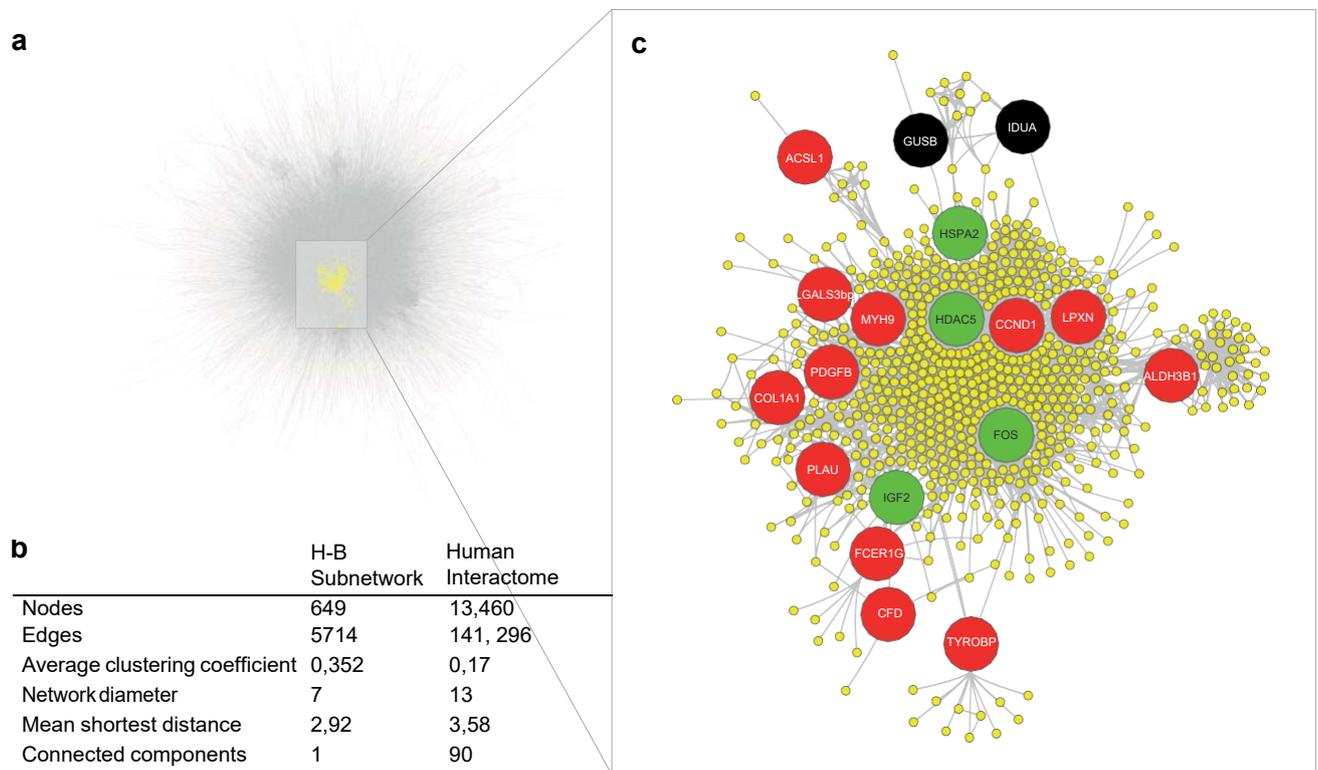| b	H-B Subnetwork	Human Interactome
Nodes	649	13,460
Edges	5714	141, 296
Average clustering coefficient	0,352	0,17
Network diameter	7	13
Mean shortest distance	2,92	3,58
Connected components	1	90

Fig. 2 Analysis H-B subnetwork in the human interactome. **a** The human interactome with all known physical interactions within the cell. **b** Network measures of the human interactome and the H-B subnetwork. **c** H-B subnetwork extracted from human interactome. H-B upregulated in red and downregulated in green. Black nodes are deficient proteins of MPI and MPS VII. The first direct neighbors of H-B in yellow

offers a measure of a network's navigability [40]. The average shortest path length was 2, 92 for the H-B subnetwork and 3, 58 for the human interactome. These results support that H-B DEGs tend to locate close to each other in a specific region in the human interactome.

11 Molecular Pathway Reconstruction

In an attempt to investigate the connections between H-B and losartan, interaction data from the STITCH database were used, allowing the identification of the interactions between Fos, Col1a1, and losartan in the chemo-biology network (Fig. 4a). Investigating connections between our H-B and associated signaling pathways, we identified Fos, Smad4, and Col1a1 with the highest path score (Fig. 4b).

12 Discussion

This study sought to identify genes and molecular pathways that could contribute to aortic dilatation in MPS using a systems biology approach and expression analysis. To measure the importance of DEGs in MPS in the PPI network, we examined the network's topological properties using centrality and clustering analyses. The consequence of a disease phenotype is not limited to the deficient protein, but the perturbations that spread through connections with other proteins located in underlying cellular networks [42–44]. Hence, systemic approaches allow looking beyond the deficient protein that causes lysosomal storage diseases.

Moreover, disease-associated proteins are not located randomly in the interactome. Still, they tend to interact with each other creating a disease module with perturbations resulting from a pool of genes associated with the disease [37, 45, 46]. In this sense, network measures of the H-B subnetwork (Fig. 2c) indicate that our H-B tend to locate to the same network neighborhood in the human interactome [45], which implies that the H-B are more likely to be involved in regular cellular functions, and its disruption results in the pathophysiology of the disease [43]. Many H-Bs also were identified with high closeness centrality value in the network (Supplement Table 2). The model described in Network Medicine supports our hypothesis, where proteins usually found interacting with each other in the same illness happens more frequently than expected by chance [37, 43, 47].

13 H-B Associated with the Recruitment of B Cells, Production of Inflammatory Mediators, Adherence and Migration Cellular

Once the disease module has been identified, we investigated candidate pathways involved in cardiac dysfunction in MPS. Genes from the immune system are frequently related to cardiovascular diseases in MPS I and MPS VII mice models [20, 48, 49]. The accumulation of undegraded GAGs in the heart can trigger an inflammatory process through the recruitment of macrophages. Macrophages secrete enzymes that degrade the extracellular matrix (ECM) in pathological situations, leading to aortic dilatation [20, 22, 50]. In this sense, processes involving plasma activation proteins in acute inflammation, complement activation, B cell-mediated immunity, regulation of leukocyte activation, regulation of interleukin-6 production, and response to lipopolysaccharide were some of the GO terms present in our protein–protein network (Supplement Tables 3–5).

Topological analyses allow identifying proteins in the network, which can play an essential role in a biological process [29, 31, 51]. From the H-B identified, *Fcer1g*, *Ccnd1*, and *Plau* were overexpressed while *Fos* underexpressed in dilated aortas of MPS VII mice (Fig. 1). Fos is a well-recognized AP-1 transcription factor with an influential role in signal transduction, cellular proliferation, and phospholipids synthesis [52]. Macrophages lacking Fos exhibit enhanced production of proinflammatory cytokines and increased apoptosis [53]. The absence of Fos led to the reduction of IL-10 and enhanced TNF and IL-6 in response to LPS [53]. Interestingly, the TLR4 activation by GAG generates an inflammatory response through the activation of the LPS signaling pathway, which results in processes involving the production of TNF and IL-6 in aortas from MPS VII mice and dogs [18, 48]. Therefore, the downregulation of *Fos* could lead to the production of inflammatory mediators associated with aortic dilatation in MPS.

Fcer1g, Plau, Ccnd1, Mmp2, and Mmp3 proteins are the first direct neighbors of Fos in the PPI-network (data not shown), which were also genes overexpressed in the expression analysis. Mmps are proteases with known collagen and elastase activity, resulting in aortic dilatation in MPS [21]. In this sense, the H-B Plau, a plasminogen activator urokinase, controls ECM by activating Mmps by converting plasminogen into plasmin, regulating cell migration and adhesion in inflammatory processes [21, 54]. Besides, the recruitment of B cells involves *Fcer1g*, which encodes the Fc receptor, and its depletion reduces the infarct region in mice [55, 56]. Cardiac alterations are also

Fig. 3 Summary of the main findings involving H-B and heart diseases. The black rectangles indicate a bioprocess associated with DEG; gray rectangles are associated with cardiac findings. Ellipses in red are upregulated genes, in green, downregulated. **a** H-B is associated with the recruitment of B cells, production of inflammatory mediators, adherence, and migration cellular. **b** Complement activation and lipid accumulation. **c** Cardiovascular remodeling and deposition of collagen

Fig. 4 Chemo-biology network and signaling pathway. **a** Interaction data from STITCH were used to construct networks using Cytoscape software. The network comprises 19 nodes and 70 edges and shows connections between angiotensin receptor blockade (Losartan) and H-B. **b** Reconstruction signaling pathway with H-B from the chemo-biology network. Upregulated genes in red and downregulated in green

related to the overexpression of *Ccnd1* that triggers the hyperplasia of cardiomyocytes in the embryonic mutant hearts [57] and cardiac hypertrophy in rats [58]. Therefore, the proteins mentioned above interact directly with each other, are located in the disease module indicated by our topological measures (Fig. 2c), share inflammatory bioprocesses involved in aortic dilatation in MPS. Figure 3a summarizes the suggested mechanism for such interaction.

14 Complement Activation and Lipid Accumulation

GAGs can directly activate complement proteins C3 and C5 through the signal transduction of TLR4 [20, 59]. The H-B complement factor D (Cfd) was overexpressed in aortic dilatation. Cfd is essential for activation of the complement system's alternative pathway, and it is considered a risk marker for coronary heart disease [60]. The contribution of Cfd in the emergence of cardiac abnormalities may be associated with a pathologic process that leads directly to elastin fragmentation or indirectly produces aortic dilatation in MPS VII [20].

Moreover, cardiomyopathies are related to the accumulation of lipids in the heart [61]. The H-B Cfd is inversely correlated with HDL cholesterol ratio, positively associated with insulin levels, glucose, and diastolic pressure [60], which are bioprocesses identified in our GO analyses (Supplement Tables 3–5). In this sense, the H-B *Acsl1,* found overexpressed in aortic dilatation in mice (Fig. 1), catalyzes the conversion of long-chain fatty acids into acyl-coAs [61]. Expression of *Acsl1* was increased in patients' peripheral blood with acute myocardial infarction, which can be considered a molecular marker for this condition [62]. The H-B Cfd and Acsl1 suggest functional relations between activation of the complement system and lipid accumulation, necessary conditions found in heart dysfunction [63] (Fig. 3b). Overexpression of *Cfd* in the aortic tissue contributes to cardiac remodeling during the inflammatory process and, together with Acsl1, may be associated with marked lipid accumulation in aortic dilatation [20, 60, 64].

15 Cardiovascular Remodeling and Deposition of Collagen

During the cardiac tissue's morphogenesis and maintenance, an adequate balance between ECM degradation and synthesis is required since the lack of control in the ECM maintenance can lead to cardiac dilatation [65]. While the *Col1a1* and *Pdgfb* were overexpressed, *Hdac5* was underexpressed in dilated aortas. Collagen, type I, alpha 1

(Col1a1), was associated with heart dysfunction in a heart failure rat model when overexpressed [66]. In mice, both mRNA and protein expression of Col1a1 and Mmps in heart tissues were enhanced by continuous infusion of angiotensin II, whereas cardiac fibrosis was attenuated by angiotensin II type I receptor blockade. Therefore, effects, such as the increase of collagen deposition in the mouse heart, by angiotensin II were inhibited with losartan [67, 68].

Furthermore, the overexpression of the *Pdgfb* in transgenic mice was associated with fibrosis and cardiac hypertrophy [69]. The increase of hypertrophy also was seen in response to pressure overload in knockout mice for *Hdac5* [70]. In this sense, the H-B Hdac5 was observed to repress the expression of genes that promote cardiac hypertrophy [71] (Fig. 3c). Xu and collaborators demonstrated that *Hdac2* was overexpressed, and the *Hdac5* and *Hdac9* down-regulated in the myocardial hypertrophy rat model. However, differences in expression levels were attenuated with valsartan use, causing a reduction of myocardial hypertrophy compared with controls [72].

Interestingly, it has been demonstrated that the use of another angiotensin II type 1 receptor antagonist, such as losartan, can reduce aortic dilation and normalize cardiac hypertrophy in mice MPS I [22, 73]. Therefore, the data suggest a possible perturbation in the interactions between the H-B located in a specific region of the human interactome, culminating in hypertrophy and dilation of the aorta in MPS. The modulation of expression of these genes could be further investigated as potential targets involved in losartan's therapeutic effect in MPS [72].

16 Angiotensin Receptor Blockade and H-B

Losartan is a blocker of the angiotensin receptor that has been shown to improve aortic dilation in MPS I mice [22]. Data from the STITCH database showed losartan interactions with Fos and Col1a1 (Fig. 4a), which is interesting since treatment with losartan causes a decreased expression of *Col1a1* mRNA in atrial myocyte and fibroblast from mice [74, 75]. Besides, Fos levels are increased in the paraventricular nuclei in the rat brain after losartan administration [76].

The beneficial effects of losartan are supposedly by antagonism of transforming growth factor TGF-β [22]. TGF-β is one pathway of multiple transcriptional programs with effects in ECM homeostasis, vascular remodeling, blood pressure regulation, and immune response [77]. TGF-β can modulate the expression of the transcription factor *Fos* and the corepressor *Hdac5* through Ras/ERK/MAPK signaling [78, 79]. Positive regulation of MAPKKK and regulation of ERK1 and ERK2 cascade were identified by GO analysis in clusters encompassing these

proteins (Supplement Tables 3–4). Moreover, TGF-β and BMP signaling exerts crosstalk with cytokine signaling and induces JAK-STAT signaling [80]. Markers associated with elastin fragmentation and aortic dilation are probably overexpressed with the activation of STAT proteins, induced by the accumulation of GAGs in lysosomes [21]. JAK-STAT cascade and STAT proteins are seen in cluster 3 (Supplement Table 5).

Investigating connections between our H-B and their associated signaling pathways, we identified Fos, Smad4, and Col1a1 with the highest path score (Fig. 4b). Smad4 is a member of signal transduction proteins activated by TGF-b family receptors and coreceptors family as BMP ligands. Smad4 forms complexes that translocate to the nucleus to control target genes' activity [80, 81]. Smad4 is essential for cardiac mesoderm formation in human embryonic stem cells, and the knockout in mouse cardiomyocytes results in cardiac hypertrophy and heart failure [82, 83]. Therefore, our data indicate that the association between the TGF-b signaling pathway, Fos, and Col1a1 proteins could play an essential role in the pathophysiology of aortic dilation in MPS VII and its subsequent improvement by losartan treatment in MPS I [22].

17 Conclusion

This work allowed the refining and prediction of genes with potential involvement in aortic dilatation in MPS VII through network analysis. We identified candidate genes with potential contributions to the aortic dilatation in MPS VII with a systemic approach to gene expression data by the PPI and chemo-biology network. Moreover, pathways involved in cardiac remodeling during the inflammatory process, deposition of collagen, and lipid accumulation affect the cardiac tissue's morphogenesis and maintenance in the MPS I and VII. The scarcity of expression data in databases has generated few studies focusing on MPS until now. However, the limitation of available expression data can be partially solved using system biology strategies that may provide a better understanding of the pathophysiological mechanisms underlying these disorders, allowing them to investigate new therapeutic approaches.

Compliance with Ethical Standards

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3.3. Capítulo III: Corrêa T, Poswar F and Santos-Rebouças CB (2021) Convergent molecular mechanisms underlying cognitive impairment in mucopolysaccharidosis type II. Metab Brain Dis. https://doi.org/10.1007/s11011-021-00872-8

Convergent molecular mechanisms underlying cognitive impairment in mucopolysaccharidosis type II

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Abstract

Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disorder caused by pathogenic variants in the iduronate- 2-sulfatase gene (*IDS*), responsible for the degradation of glycosaminoglycans (GAGs) heparan and dermatan sulfate. IDS enzyme deficiency results in the accumulation of GAGs within cells and tissues, including the central nervous system (CNS). The progressive neurological outcome in a representative number of MPSII patients (neuronopathic form) involves cognitive impairment, behavioral difficulties, and regression in developmental milestones. In an attempt to dissect part of the influence of axon guidance instability over the cognitive impairment presentation in MPS II, we used brain expression data, network propagation, and clustering algorithm to prioritize in the human interactome a disease module associated with the MPS II context. We identified new candidate genes and pathways that act in focal adhesion, integrin cell surface, laminin interactions, ECM proteoglycans, cytoskeleton, and phagosome that converge into functional mechanisms involved in early neural circuit formation defects and could indicate clues about cognitive impairment in patients with MPSII. Such molecular changes during neurodevelopment may precede the morphological and clinical evidence, emphasizing the importance of an early diagnosis and directing the development of potential drug leads. Furthermore, our data also support previous hypotheses pointing to shared pathogenic mechanisms in some neurodegenerative diseases.

Keywords MPS II · Cognitive impairment · Axon guidance · Extracellular matrix · Signaling pathways

Introduction

Mucopolysaccharidosis type II (MPS II; Hunter syndrome; OMIM 309900) is a rare X-linked lysosomal storage disorder caused by pathogenic variants in the iduronate-2-sulfatase gene (*IDS*), responsible for the degradation of the glycosaminoglycans (GAGs) heparan and dermatan sulfate (Beck 2011). Deficiency of enzyme activity results in the accumulation of partially digested GAGs within cells and tissues, including the central nervous system (CNS) (Beck 2011). Hunter syndrome is the most frequent type of MPS in Brazil

(Josahkian et al. 2021b), with an incidence of 1/92,000 to 1/500,000 live births, depending on the geographical location (Wraith et al. 2008; Kingma et al. 2015; Mohamed et al. 2020). The *IDS* gene, located at Xq28, contains nine exons that covering approximately 24 kb (Wilson et al. 1993). More than 670 *IDS* pathogenic variants have been registered in the Human Gene Mutation Database, including missense, nonsense and splicing variants, as well as indels, gross deletions, and complex rearrangements (HGMD, 2021).

The huge molecular heterogeneity of *IDS* variants partially explains the clinical variability in individuals with MPS II, which present multisystemic and progressive symptoms (Mohamed et al. 2020). The main clinical manifestations include short stature, hepatosplenomegaly, umbilical/inguinal hernias, skeletal abnormalities, upper respiratory tract dysfunction and recurrent respiratory infections, cardiac valve disease, and neurological dysfunctions, such as carpal tunnel syndrome, hydrocephalus, deafness, and spinal cord compression (Beck 2011; Giugliani et al. 2014; Whiteman and Kimura 2017). The onset of symptoms occurs between 12 to

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18 months (severe form) or from 2 to 4 years old (attenuated form) (Wraith et al. 2008). Severe forms can be caused by a change in the tertiary structure of the IDS protein with loss of IDS enzyme activity, while pathogenic variants in attenuated forms tend to affect partially the protein structure with the maintenance of residual enzyme activity (Kato et al. 2005; Mohamed et al. 2020). However, it should be noted that clinically, enzymatic activity is not reliable to differentiate the severe form from the attenuated one, as also seen in MPSI. Moreover, it is difficult to predict the phenotype in MPS II. For example, large structural alterations are frequently described in patients with a more severe clinical presentation, while single base pair substitutions are involved with a broad spectrum of disease severity (Kosuga et al. 2016; Josahkian et al. 2021a).

There are two clinical forms in MPS II, according to the presence or absence of progressive neurological involvement: non-neuronopathic and neuronopathic with the presence of cognitive impairment, behavioral difficulties, and regression in developmental milestones (Whiteman and Kimura 2017). Multifocal or diffuse white matter lesions are commonly seen in MPS II patients with cognitive impairment. Dilated perivascular spaces are observed in periventricular and subcortical white matter. Furthermore, brain atrophy with enlargement of the cortical *sulci* and increase of cerebrospinal fluid production are recurrent features in patients with MPS II (Bigger et al. 2018). White matter lesions and brain atrophy are the result of GAG storage in neurons and glial cells, which can lead to myelination abnormalities. At the cellular level, the brain accumulation of GAGs results in enlargement of Purkinje cell dendrites in the cerebellum, expansion of neuronal cytoplasm, reduction of nerve cells in the cortex, and development of meganeurites in the cerebral cortex (Schwartz et al. 2007; Bigger et al. 2018). The cognitive impairment in individuals with the severe neuronopathic form has been associated with undigested GAGs in neural tissue, involving inflammatory and neurotoxicity mechanisms (Giugliani et al. 2014).

The accumulation of GAGs alters the regulation of lysosomal pH, synaptic release, endocytosis, autophagy, and exocytosis. The GAGs reside on the extracellular matrix and are generally associated with proteoglycans, which display physiological functions on the cell surface related to cell signaling, availability of growth factors, cytokines, and morphogens. GAGs are involved with cell motility and adhesion, and intercellular communication through the spatial distribution of diffusible proteins (Smock and Meijers 2018; Corrêa et al. 2021). Inactivation of heparan sulfate (HS) biosynthetic or HS modifying enzymes in mouse models leads to developmental defects in the forebrain and axon guidance errors (De Pasquale and Pavone 2019). HS proteoglycans function as co-receptors and the abnormal degradation of HS fragments affects essential signaling pathways and neuronal activity (Hoche et al. 2014; Bigger et al. 2018; Gaffke et al. 2020; Corrêa et al. 2021).

Evidence about the influence of axon guidance defects in the onset of neurological dysfunction in patients with MPS II is increasingly emerging. The axon guidance pathway is responsible for the neuronal wiring process, in which axons are elongated and reach their target tissues to form synaptic junctions (Russell and Bashaw 2018). These events connect the brain and spinal cord with the peripheral nervous system during neurodevelopment. The interaction between signaling molecules and cell surface receptors expressed on growth cones results in axon outgrowth and growth cone collapse. Disruptions in the assembly of these neural circuits affect axonal growth and cognitive development in humans (Russell and Bashaw 2018; Manzoli et al. 2021).

Recent findings also indicate essential lysosomal mechanisms during neurodevelopment. The endosomal autophagy system contributes to axonal remodeling, generating axon growth, and reducing presynaptic stability (Crawley and Grill, 2021). The presentation and availability of receptors can be modulated by the endosomal-lysosomal pathway, which regulates the endocytosis of cell membrane receptors. However, abnormal autophagy and storage of autophagosomes in the axons can result in growth cone perturbations (Crawley and Grill, 2021; Manzoli et al. 2021). Inhibition of lysosomal transport along the axon with impaired cell content recycling or the absence of signaling and adhesion molecules available by the endosomal-autophagy system affect axon development, leading to CNS diseases in humans (Crawley and Grill 2021; Manzoli et al. 2021). Differentially expressed genes (DEGs) in the brains of MPS II and MPS VII mice indicate a contribution of axon orientation defects in neurological abnormalities (Parente et al. 2012; Salvalaio et al. 2017). In this sense, the four molecule families that participate in the axon guidance pathway exhibit altered brain expression in MPS II mice, compared to controls. Therefore, imbalances in the fine-tuning regulation of genes that control axon migration and growth can cause cognitive decline in MPS II (Salvalaio et al. 2017).

Here, we investigated the impact of axon guidance instability over the cognitive impairment presentation observed in the neuronopathic form of MPS II. Through brain expression data, each DEG was analyzed from the perspective of its interactions in the human interactome. We identified new candidate genes and pathways that converge into functional aspects involved in the strict relationship between defects in neural circuit formation and cognitive impairment in MPS II.

Methods

Differentially expressed genes in MPS II

There are few transcriptomic studies in MPS II reported so far. However, due to the evolutionary gene conservation between humans and mice, *Ids*- knockout mice models are considered useful to investigate the pathogenesis of the disease and evaluate treatment in preclinical studies (Gaffke et al. 2020). Therefore, DEGs were recovered from brain RNA-seq data in an MPS II mouse model at 9 months old (Salvalaio et al. 2017). We considered upregulated genes and downregulated genes (Supplementary Tables S1 and S2) in the cerebral cortex of *Ids* knockout mice versus the cerebral cortex of wild-type mice and genes in the midbrain, diencephalon, and hippocampus of Ids knockout mice versus the midbrain, diencephalon, and hippocampus of wild-type mice. A total of 1,611 DEGs with a p-value < 0.05 and $\log^2$ ratio ± 0.7 were recovered.

Human Interactome and ITGA5 Subnetwork construction

The human interactome, which contains 17,185 nodes and 420,534 edges, was generated using the STRING – Human Protein Links – High Confidence (Score > = 0.7) pro- tein–protein interaction network. The human interactome was recovered from the Network Data Exchange (NDEx) (Pratt et al. 2015). The *ITGA5*-subnetwork was created by extracting interactions concerning its first neighbors. Cytoscape V.3.8.2. software (Shannon et al. 2003) was used for data visualization and calculation of centrality parameters.

Gene-disease associations and centrality parameters

To measure the impact of DEGs in human diseases, we used DisGeNET, which integrates data from UNIPROT, CGI, ClinGen, Genomics England, CTD (human sub-set), PsyGeNET, and Orphanet databases (Piñero et al. 2021). The Z-scores for the observed values (V_{total}) of the absolute number of genes and CNS/mental diseases were obtained by comparing the brain network and random networks. The calculation was performed according to the equation:

$$Z - score = \frac{V_{total} - mean \ V_{Random}}{stand \ deviation \ V_{Random}}$$

The equation points to the mean (V_{random}) and standard deviation (V_{random}) of the random expectation for each random network. Z-scores > 1.6 (p-value < 0.05) were considered significant, as described by Woidy and colleagues

(2018). Spearman's correlation coefficient was used to calculate the statistical relationship between centrality parameters (degree and betweenness) from the human interactome and the number of associated diseases for each gene in the brain network.

Hierarchical ontology network and enrichment analysis

CyCommunityDetection V1.12.0 (Singhal et al. 2020) was employed to detect multiscale communities and functional enrichment for brain network by identification of densely connected regions with the Community Detection APplication and Service (CDAPS). We used the HiDeF algorithm for community detection and generation of a hierarchical network, whereas gProfiler was used for functional enrichment analysis with a minimum overlap (p-value < 0.05). The communities of the hierarchical network were classified by biological processes, and the orthogonal edge router layout was employed using yFiles V1.1.1. CTD:genes-pathways associations (Davis, 2019) through NDEx Integrated Query (Pratt et al. 2015) and the webserver Enrichr (Kuleshov et al. 2016) were employed in the *ITGA5*-subnetwork to identify enriched pathways with adjusted p-value < 0.05. The gene-set libraries included KEGG, Reactome, and WikiPathways databases.

Gene expression during brain development and neurodevelopmental/neurodegenerative conditions

RNA-seq and expression microarray data on human brain development were collected from BrainSpan (Tebbenkamp et al. 2014). Data throughout the course of human brain development were recovered from dorsolateral prefrontal cortex (DFC), ventrolateral prefrontal cortex (VFC), anterior (rostral) cingulate (medial prefrontal) cortex (MFC), and orbital frontal cortex (OFC). Statistical analysis was initially performed using the Shapiro–Wilk test to investigate the distribution of values. Parametric data were treated using RM one-way ANOVA and paired t-test. Friedman and Wilcoxon's tests were applied to nonparametric data. Only p-values < 0.05 were considered statistically. Additionally, we used the GEO signatures of DEGs for diseases via Harmonizome platform available at https://maayanlab.cloud/Harmonizome/ (Rouillard et al. 2016) to retrieve altered genes in neurodevelopmental/neurodegenerative conditions available in the datasets with a fold change cutoff of 0.6 (upregulated) and -0.6 (downregulated) for the 23 genes in the network of genes-pathways associations.

Prioritization of candidate genes and disease modules

We selected candidate genes related to the term "cognitive impairment", according to the Human Phenotype Ontology (HPO) database (Köhler et al. 2021). These genes were used as a seed to recover the most relevant neighborhood proteins by propagating the network through the diffusion algorithm (V. 1.6.1) (Carlin et al. 2017). Network propagation can estimate the distance between different proteins and recognize a community with closely related nodes (Carlin et al. 2017). The proximity between genes related to cognitive impairment and neighborhood nodes was evaluated using 60 as a maximum diffusion rank value.

Candidate disease modules were identified using the Cytoscape ClusterMaker V.1.3.1 plugin (Morris et al. 2011). Modules were recognized by the k-medoid algorithm, a method associated with k-means clustering that minimizes the distance between points by partitioning the dataset into k groups or modules (Morris et al. 2011). Minkowsky's distance metric algorithm was used in conjunction with brain development RNA-seq and microarray data as network node attributes for cluster generation.

DEGs-miRNA and transcription factor networks

The experimentally validated DEGs-miRNA interactions data were collected from miRTarBase V.8.0 and TarBase V.8.0 and transcription factors from ENCODE via the NetworkAnalyst web interface (Xia et al. 2014). The DEGs-mirRNA network contained 664 nodes (25 seed genes; 859 has-mirRNAs) and 1,927 interactions. Only the mirRNAs of the hub (> 14 connections) were filtered. The transcription factors network was composed of 252 nodes (19 seed genes; 233 transcription factors) and 553 interactions. Only TFE3 and its first neighbors were collected due to its prominence in lysosomal functions. A workflow of the methodologies used in the study can be seen in Supplementary Figure S1.

Results and Discussion

Neurological conditions were representative in the brain network

The brain network resulting from DEGs in MPS II comprised 1,321 proteins and 3,421 interactions from the human interactome. To measure the impact of the brain network in human diseases, we used random networks to compare the number of diseases and genes associated with CNS diseases and mental disorders. The number of genes related to CNS and mental disorders in the brain network was larger than in random expectations with an average z-score of 9.1, indicating enrichment of genes with an essential role in the brain (Fig. 1A). Proopiomelanocortin or beta-melanocyte stimulating hormone (*POMC*) and acetylcholinesterase (*ACHE*) were the genes with the highest number of associated diseases with 98 and 70 connections, respectively.

Fig. 1 Gene-disease associations and centrality analysis. **A** The number of genes and **B** CNS diseases were compared with random networks. Only z-scores > 1.6 (p-value < 0.05) were considered significant. The statistical relationship between **C** degree and **D** betweenness with the number of associated diseases for each gene, calculated by Spearman's correlation coefficient
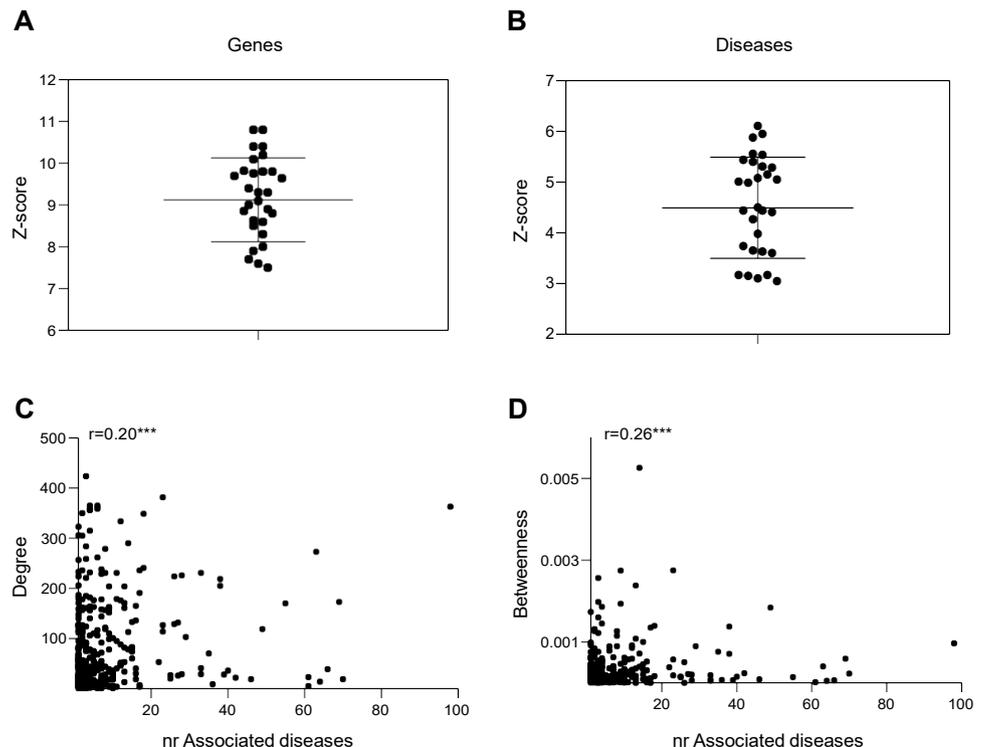
The complete list of genes can be found in Supplemental Table S3. Similarly, the frequency of CNS and mental disorders was higher in the brain network than in random networks with an average z score of 4.5 (Fig. 1B). Schizophrenia was the disease with the highest number of associated genes (97). Brain disorders, in which alterations in the axon guidance process have been reported, were also evidenced, such as gaze palsy, familial horizontal, with progressive scoliosis; Joubert syndrome; and Duane retraction syndrome (Manzoli et al. 2021). The configuration of the brain network indicates a representative expression dataset with many disease genes involved in CNS function and development that could not have arisen by chance.

Centrality parameters, such as degree and betweenness, are commonly used to identify candidate disease genes. The degree is defined by the number of connections of a specific node (protein) in the network, which may display multiple cellular functions. Proteins with a high degree are considered hubs in the network. Betweenness represents the number of nonredundant shortest paths that pass through a node of interest with the capacity to create a bridge for communication between distant nodes, thus these proteins are known as bottlenecks (Girvan and Newman 2002). We identified a low positive correlation between topological characteristics in the brain network and the number of CNS and mental diseases with a degree value of r = 0.20 (Fig. 1C) and a betweenness value of r = 0.26 (Fig. 1D). Essential human genes tend to encode hub proteins with relevant expression in most tissues. Contrariwise, disease genes are more often located in peripheral regions of the network (Goh et al. 2007). This evidence supports our findings of the lack of correlation between disease genes and topological analysis over an evolutionary perspective. Pathogenic variants in genes with central topological parameters (hubs and bottlenecks) can impact regions that are extensively interconnected in the network, affecting neurodevelopment or essential physiological functions in humans (Goh et al., 2007; Yu et al., 2007).

A hierarchical neuro-network model of MPS II

To better elucidate the functional context in which DEGs affect neurodevelopment in MPS II, we used multiscale community and functional enrichment methods for the identification of densely connected regions in the brain network, composed of high-confidence protein–protein interactions and biological processes in hierarchical relationships. We recognized densely interconnected protein clusters represented by nodes with significant enrichment in neurological processes (Fig. 2). This approach allowed predicting clusters of proteins in the network related to specific cellular functions and mechanisms.As expected, we found significant pathways related to brain function and development. The

axon guidance process (p-value = 5.70 X 10^{-24}) (Fig. 2A) showed strong associations with pathways involved in various steps of axonal growth and pathfinding processes, including netrin-activated signaling (p-value = 1.13 X 10^{-9}), ephrin signaling (p-value = 1.15 X 10^{-13}), and neural crest cell migration (p-value = 6.20 X 10^{-8}). There is also a connection between the axon guidance processes and the synaptic transmission module (Fig. 2B) through posterior midgut development (p-value = 2.44 X 10^{-6}). Chemical synaptic transmission (p-value = 4.43 X 10^{-127}) cluster connected with bioprocesses involved in neuronal development (Fig. 2C), such as neuroblast division (p-value = 9.84 X 10^{-6}) and Wnt signaling (p-value = 1.80 X 10^{-19}). Furthermore, chemical synaptic transmission interacts with the neurotransmitter activity module (Fig. 2D) by neuronal system (p-value = 1.92 X 10^{-82}) and with the vesicle pathways (Fig. 2E), through the clathrin-coated endocytic vesicle (p-value = 2.24 X 10^{-9}). Endocytosis and trafficking of axon guidance receptors are essential for human neurological development (Pasterkamp, 2021). The clathrin-coated endocytic vesicle (p-value = 2.24 X 10^{-9}) was connected with clathrin binding (p-value = 1.02 X 10^{-7}), which participates in the endosomal trafficking pathway. Evidence is emerging on the importance of autophagy mechanisms for human neurodevelopment. Autophagy contributes to the growth of axons and reduces presynaptic stability (Crawley and Grill 2021). Genes involved in the cellular autophagy-phagosome process (*TUBA8*, *PRKAA1*, *RRAGB*, *ITGA5*) are present in most of the clusters described above. The proper neurodevelopment depends on the maintenance of these mechanisms. Activated receptors can be endocytosed and sorted into other compartments in neurons by endosomes; the content is forwarded to degradation or return to the cell surface (Pasterkamp and Burk 2021). Therefore, this hierarchical network suggests that secondary pathways triggered by the accumulation of GAGs may alter the internalization and transport of neurotransmitters, as observed in the decrease of glial glutamate transporters in human brain tissue with MPS II (Hamano et al. 2008). Moreover, highlights the close relationship between the endosomal-autophagy system and neurodevelopment pathways.

Disease module from axon guidance network

There are several DEGs involved in the axon guidance pathway (Salvalaio et al. 2017), but identifying a specific subset of genes that interact with each other and, consequently, contribute to cognitive impairment in this context is an approach that has not yet been carried out. The interface between functional and topological data retrieved by network analysis may suggest a relevant phenotype associated with a specific region in the human interactome. The axon guidance network (Fig. 3A) was composed of 122
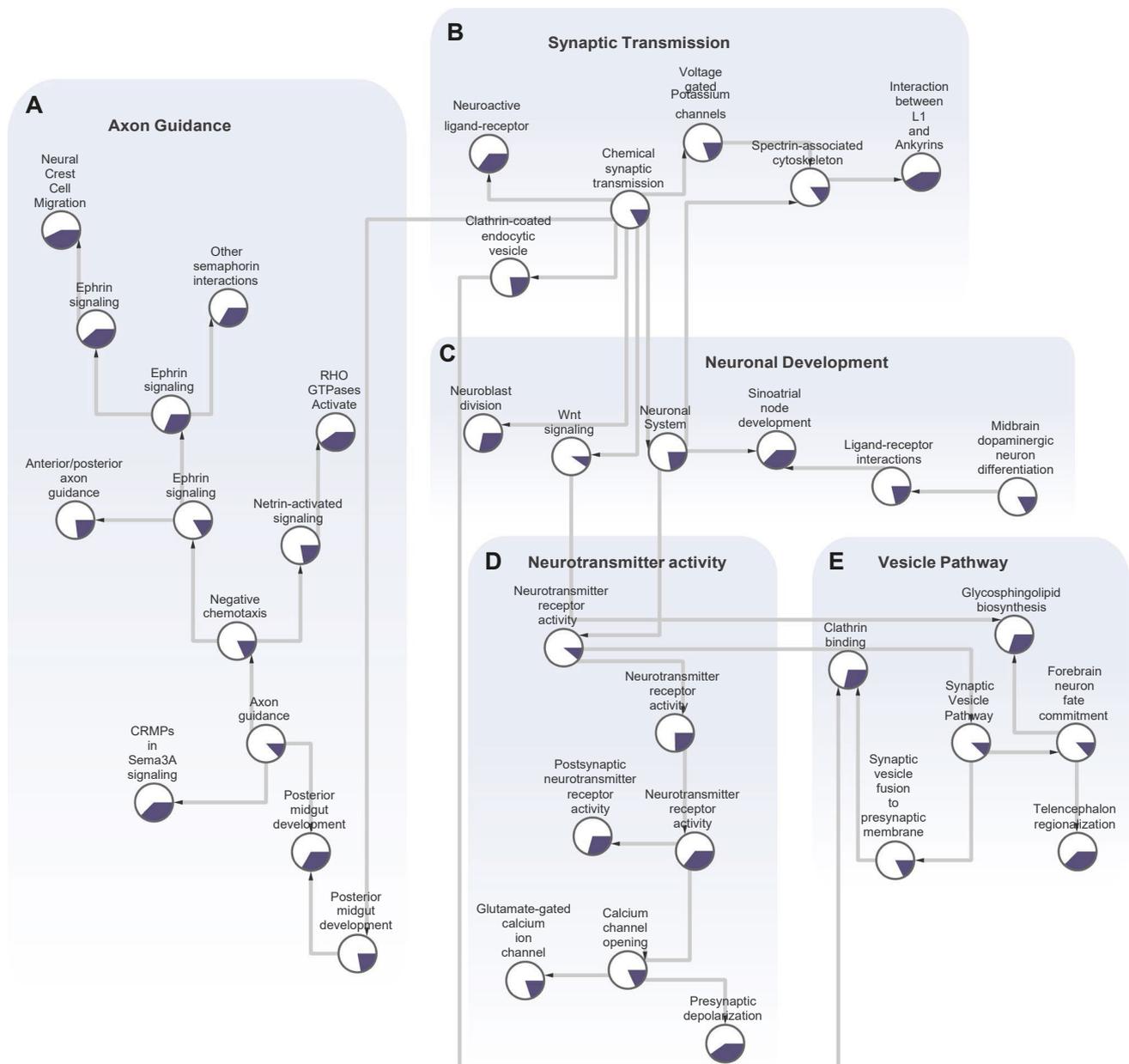
Fig. 2 Hierarchical ontology network. Communities in the hierarchical network **A** axon guidance; **B** synaptic transmission; **C** neuronal development; **D** neurotransmitter activity; and **E** vesicle pathway are represented by biological processes with a minimum overlap (*p*-value < 0.05). The orthogonal edge router layout was employed using yFiles V1.1.1

proteins and 273 interactions in the axon guidance module (Fig. 2A). To identify proteins with greater influence on neurological aspects, we applied a supervised propagation network method with genes previously associated with cognitive impairment (*ROBO3, NIPAL4, ALOX12B, RTN4R, A2M, TTPA, PDGFB*) within the network, according to the HPO database (Köhler et al. 2021) (Fig. 3B). Among these genes, *PDGFB* (platelet-derived growth factor subunit b) is the most connected, and *ROBO3* (roundabout guidance receptor 3) belongs to the axon guidance pathway.

Importantly, there is a significant increase in HS concentrations in individuals with the neuronopathic form, when compared to patients with the attenuated phenotype in MPS II (Okuyama et al. 2021). The accumulation of partially digested GAGs within cells and the extracellular matrix can cause perturbations in crucial biological functions, affecting the cellular networks responsible for the development of the cerebral cortex. Therefore, we recovered expression data from cortex structures during brain development to identify candidate genes involved in the pathophysiology
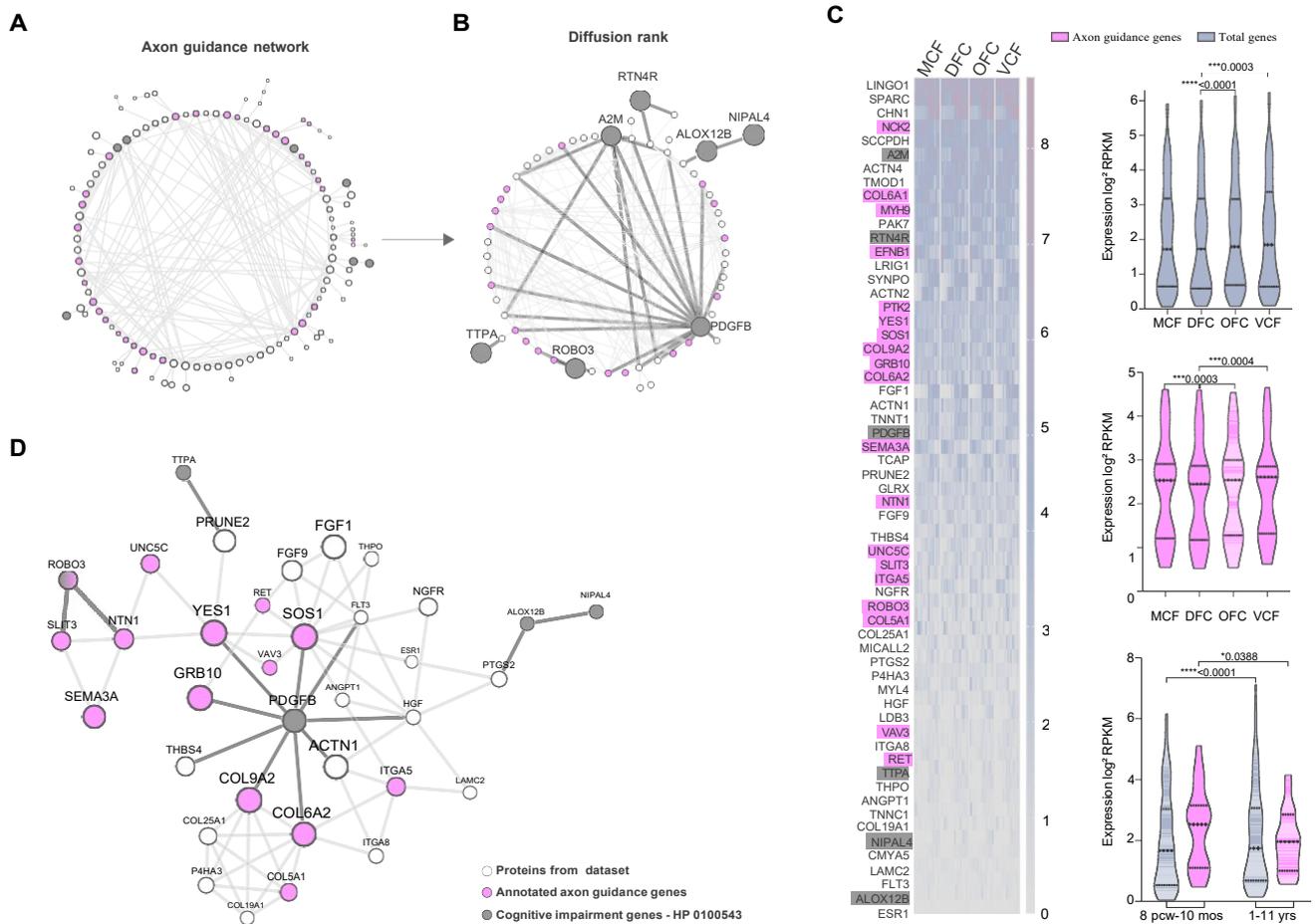
Fig. 3 Prioritization of candidate module and genes. **A** axon guidance network with 122 nodes and 273 edges. In the network, proteins are nodes connected by interactions or edges. **B** Diffusion rank through cognitive impairment proteins. **C** RNA-seq and expression microarray during the human brain development and statistical analysis by cortex regions and age. Values are shown in RPKM (reads per kilobase of exon model per million mapped reads). **D** Disease module recognized by the k-medoid algorithm. Cytoscape V.3.8.2. software was used for the visualization of networks. DFC: dorsolateral prefrontal cortex; VCF; ventrolateral prefrontal cortex; MFC: anterior (rostral) cingulate (medial prefrontal) cortex; OFC: orbital frontal cortex; PCW: postconceptional week; MOS: months

of cognitive impairment in MPS II (Fig. 3C). *NCK2* (NCK adaptor protein 2) and *A2M* (alpha-2-macroglobulin) were the most expressed among the axon guidance and cognitive impairment genes throughout this developmental period, respectively. We found statistical significance when comparing mean expression between different regions of the cortex. The dorsolateral prefrontal (MCF) showed a difference in the expression levels of all genes when compared to the orbital frontal (OFC; $p < 0.001$) and ventrolateral prefrontal (VCF; $p = 0.0003$) region. Statistical differences in cortical regions were also observed when we limited the analysis to axon guidance genes. Finally, we compared the expression of both groups at two periods during brain development. As expected, genes responsible for neural circuits formation have a more prominent expression in the early stages of development ($p = 0.0388$). Furthermore, an inverse pattern was identified during the analysis of total genes ($p < 0.0001$;

Fig. 3C). These data indicate that genes expressed during early stages of human cortex development have potential involvement in the pathogenesis of MPS II. The gene expression imbalance could lead to impairment of axon guidance and be irreversible under treatment. The use of the k-medoid algorithm, in conjunction with brain development gene expression data as network node attributes, resulted in the identification of a potential disease module in the interactome (Fig. 3D).

This module consists of many nodes associated with the axon guidance process (*COL9A2*, *ROBO3*, *ITGA5*, *GRB10*, *SEMA3A*, *YES1*, *SOS1*, *COL5A1*, *COL6A2*, *NTN1*, *UNC5C*, *SLIT3*, *RET*, *VAV3*) and cognitive impairment (*TTPA*, *PDGFB*, *ALOX12B*, *NIPAL4*, *ROBO3*). Many of these genes participate in adhesion mechanisms, organizing matrix components, and cell-surface mediated signaling that direct axon extension, cell growth, and migration

during neurodevelopment (Manzoli et al. 2021). In particular, *ITGA5* (integrin subunit alpha 5) encodes a receptor that mediates cell adhesion to the extracellular matrix or other cells by cytoskeleton activity and several signaling molecules (Wang et al. 2018). *ITGA5* is an integrin that plays a role in axon guidance and phagosome processes. Integrins are intermediates in cell–cell and cell–matrix adhesive interactions from early stages to mature tissue, in which glycosaminoglycans are their substrate for signaling in the cell–matrix (De Pasquale and Pavone 2019; Swinehart et al. 2020). Disease modules may suggest hypothetical models that explain the condition heterogeneity, capturing genes and pathways, even in Mendelian diseases such as MPS II. In this sense, *ITAG5* could indicate pathways and other genes affected by GAG accumulation within cells and the extracellular matrix, commonly affected in MPS.

Disturbances in extracellular matrix structure and cell surface signaling

To better understand the impact of *ITGA5* gene deregulation, a subnetwork was generated to identify its first neighbors in the interactome (Fig. 4A). The *ITGA5* subnetwork comprised 87 nodes and 1,220 edges in a highly stringent protein–protein interaction network, indicating strongly connected proteins. Several biological processes related to the development of axons and signaling pathways originating on the cell surface and extracellular matrix have been interconnected in a functional network (Fig. 4B). As expected, various integrins (*ITGB1, ITGB3, ITGA2, ITGAV, ITGA9, ITGA10, ITGA1*) were present in the subnetwork, as well as a large number of proteins that exhibit different functions in extracellular matrix glycoproteins (*L1CAM, LAMC1, LAMA1, FLNA, LAMA2, FBN1, LAMB1*) (Stelzer et al. 2016). Furthermore, many genes are associated with cognitive impairment (*COL18A1, COL4A1, LAMA1, FLNA, L1CAM, LAMA2, FBN1*), according to the HPO database (Köhler et al. 2021).

These results indicate that the functions performed by these genes may be compromised in patients with the neuronopathic form of MPS II, thus affecting cell adhesion, differentiation, neuronal migration, signaling, and neurite outgrowth due to the influence of GAG accumulation in the cell membrane during neurodevelopment. Therefore, our data suggest that the accumulation of GAGs in the cell can cause perturbations in the interactome and consequently affect
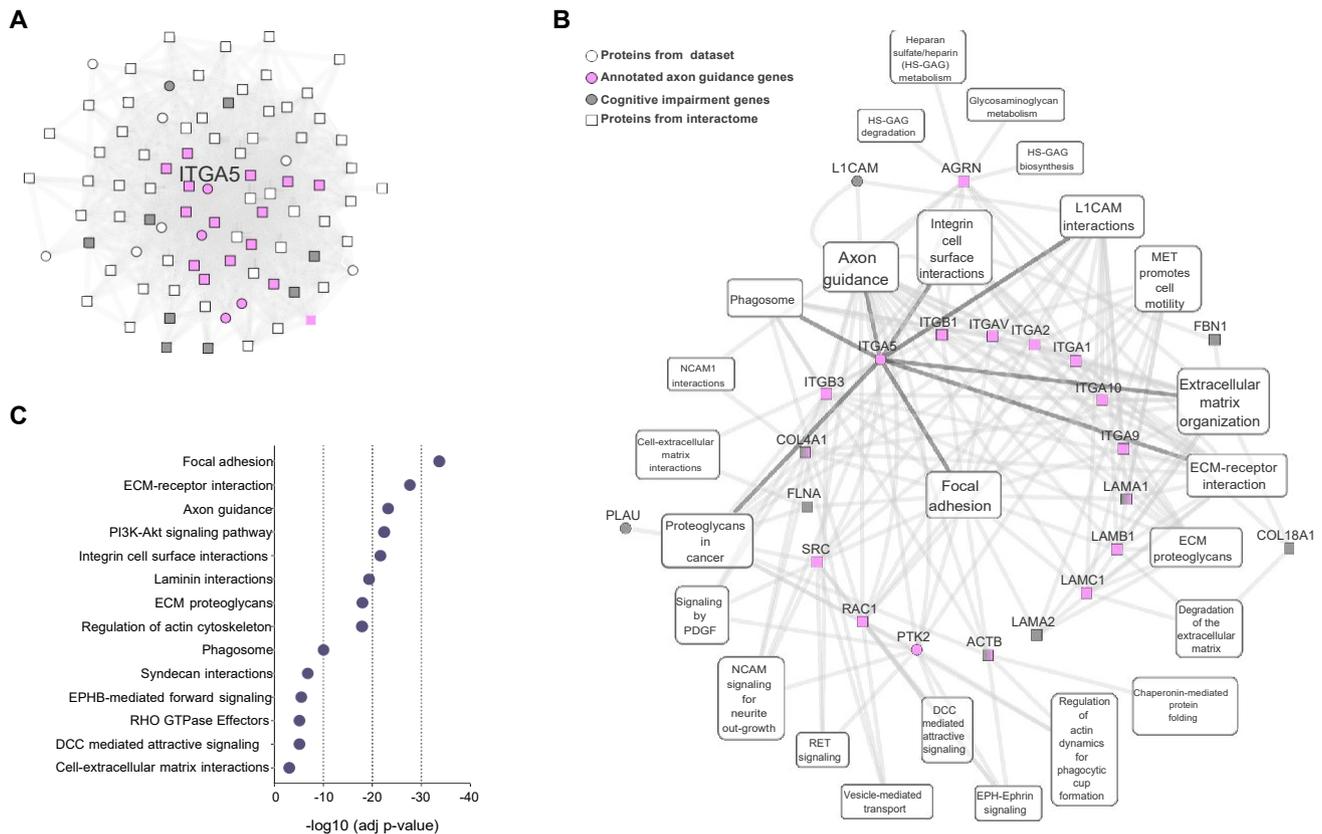

Fig. 4 *ITGA5* in the context of human interactome. **A** *ITGA5* subnetwork with 87 nodes/proteins and 1,220 edges/interactions. **B** Genes-pathways associations network. **C** Enriched pathways with an adjusted p-value < 0.05

essential pathways, such as focal adhesion (*p*-value = 2.20 X 10^{-34}), integrin cell surface interactions (p-value = 2.13 X 10^{-22}), laminin interactions (p-value = 4.70 X 10^{-20}), ECM proteoglycans (*p*-value = 9.91 X 10^{-19}), regulation of actin cytoskeleton (p-value = 1.27 X 10^{-18}), and phagosome (*p*-value = 8.39 X 10^{-11}) (Fig. 4C). Exposure to exogenous HS fragments leads to activation of integrin-based focal adhesions (FA) in neural stem cells of MPS III type B. FAs cause cell anchoring in the extracellular matrix, and its stimulation by HS can impair cell polarization and oriented migration in MPS III type B (Bruyère et al. 2015). In addition, alterations of FAs and ECM gene expression were associated with migration and neurite outgrowth defects in a pluripotent stem cell model from a patient with mucopolysaccharidosis type I-Hurler (Lito et al. 2020). HS proteoglycans function as co-receptors and are crucial components in the cell surface and ECM in the brain (Maeda et al. 2011). Furthermore, extracellular matrix dysfunctions have been identified in various types of MPS (Gaffke et al. 2020), for example, during bone development in the mucopolysaccharidosis type I model before morphological modifications in bone and joints (Heppner et al. 2015). Moreover, the primary storage in neurons causes neuroinflammation and may contribute to the progression of neurodegeneration in MPS (Fecarotta et al. 2020). Therefore, the abnormal accumulation of HS fragments associated with impairment of biological processes related to the development of axons and signaling pathways on the cell surface and extracellular matrix suggests that these substrates affect essential steps of the early stages of neurodevelopment in the neuronopathic forms of MPS II.

Common disturbances between MPS II and central nervous system diseases

We recovered DEG signatures to determine whether genes from the disease module or within the *ITGA5* subnetwork could exhibit disturbances in transcriptomic studies of conditions associated with impaired autophagy–phagosome mechanisms, frequently reported in MPS. We found several DEGs in Huntington's disease, multiple sclerosis, bipolar disorder, and Rett syndrome when compared to controls. Importantly, all these diseases can show impairment of autophagic-phagosome mechanisms and the presence of common phenotypes, such as cognitive impairment, in some patients (Sbardella et al. 2017; Croce and Yamamoto 2019; Scaini et al. 2019; Misrielal et al. 2020). The most frequent dysregulated gene (GRB10) is a growth factor receptor-bound protein that interacts with several signaling molecules, and receptor tyrosine kinases, including the Eph receptor involved in axon guidance processes (Stein et al. 1996; Wick et al. 2003). *GRB10* is differentially expressed in Huntington's disease (Fig. 5A), multiple sclerosis (Fig. 5B),

and bipolar disorder (Fig. 5C). In the disease module, we found many genes that encode collagen proteins: *COL9A2*, *COL5A1*, and *COL6A2*. Collagens are structural tissue components that can act as cell-binding proteins in the extracellular matrix, including covalent bonds to GAGs during the axonal outgrowth (Erdman et al. 2002). Furthermore, *ITGA5* is differentially expressed in multiple sclerosis and Rett syndrome (Fig. 5D). Integrins are cell adhesion molecules implicated in the transport of cells of the immune system with key functions in immune cell migration in the inflamed nervous system during multiple sclerosis (Kawamoto et al. 2012). Integrins also play a role in dendritic development (Swinehart et al. 2020) and abnormalities in dendritic morphology were observed in a Rett syndrome mice model (Rietveld et al. 2015). *ROBO3* and *TTPA* were the genes related to cognitive impairment that were up-regulated in multiple sclerosis and bipolar disorder, respectively. *ROBO3* is a member of the roundabout (ROBO) family composed of four major components (ROBO1–4) that regulate neurite outgrowth, growth cone, and axon fasciculation (Jen et al. 2004). ROBO proteins are regulated by lysosomal degradation, and signal transduction can occur through interaction with HS proteoglycans that act as co-receptors in Slit-Robo signaling (Fukuhara et al. 2008; Manzoli et al. 2021). Pathogenic variants in *ROBO3* involve abnormal axon guidance and cognitive impairment in horizontal gaze palsy and progressive scoliosis syndrome (Jen et al. 2004; Chan et al. 2006; Volk et al. 2011; Manzoli et al. 2021). Therefore, we hypothesized that the accumulation of HS proteoglycans could affect ROBO protein signaling during essential phases of neurodevelopment in MPS II.

Perturbations in gene expression were observed in the same diseases of the CNS seen in the brain network, when we analyzed the *ITGA5* subnetwork. All differentially expressed genes are involved in axon guidance mechanisms. Furthermore, many genes associated with cognitive impairment were identified: *LAMA1*, *FLNA*, and *FBN1* were dysregulated in Huntington's disease (Fig. 5A), *COL4A1* and *ACTB* in multiple sclerosis (Fig. 5B), *PLAU* and *LAMA2* in bipolar disorder (Fig. 5C), and *ACTB* again in Rett syndrome (Fig. 5D). As expected, many integrins interact with *ITGA5* in the subnetwork, but some of them show altered expression in the following conditions: *ITGB1* (Huntington's disease and Rett syndrome), *ITGAV* and *ITGA9* (Huntington's disease), and *ITGA5* (multiple sclerosis and Rett syndrome). The members of the Integrin family are receptors that play a role in cell adhesion and perform functions in axon growth and pathfinding through interaction with several signaling molecules (Manzoli et al. 2021). Integrins encoded by these genes participate in autophagy-phagosome-axon guidance pathways (Jassal et al. 2020), indicating a strict contribution to the pathogenesis of neurodevelopmental abnormalities. In this sense, the degradative dysfunction of the lysosomal
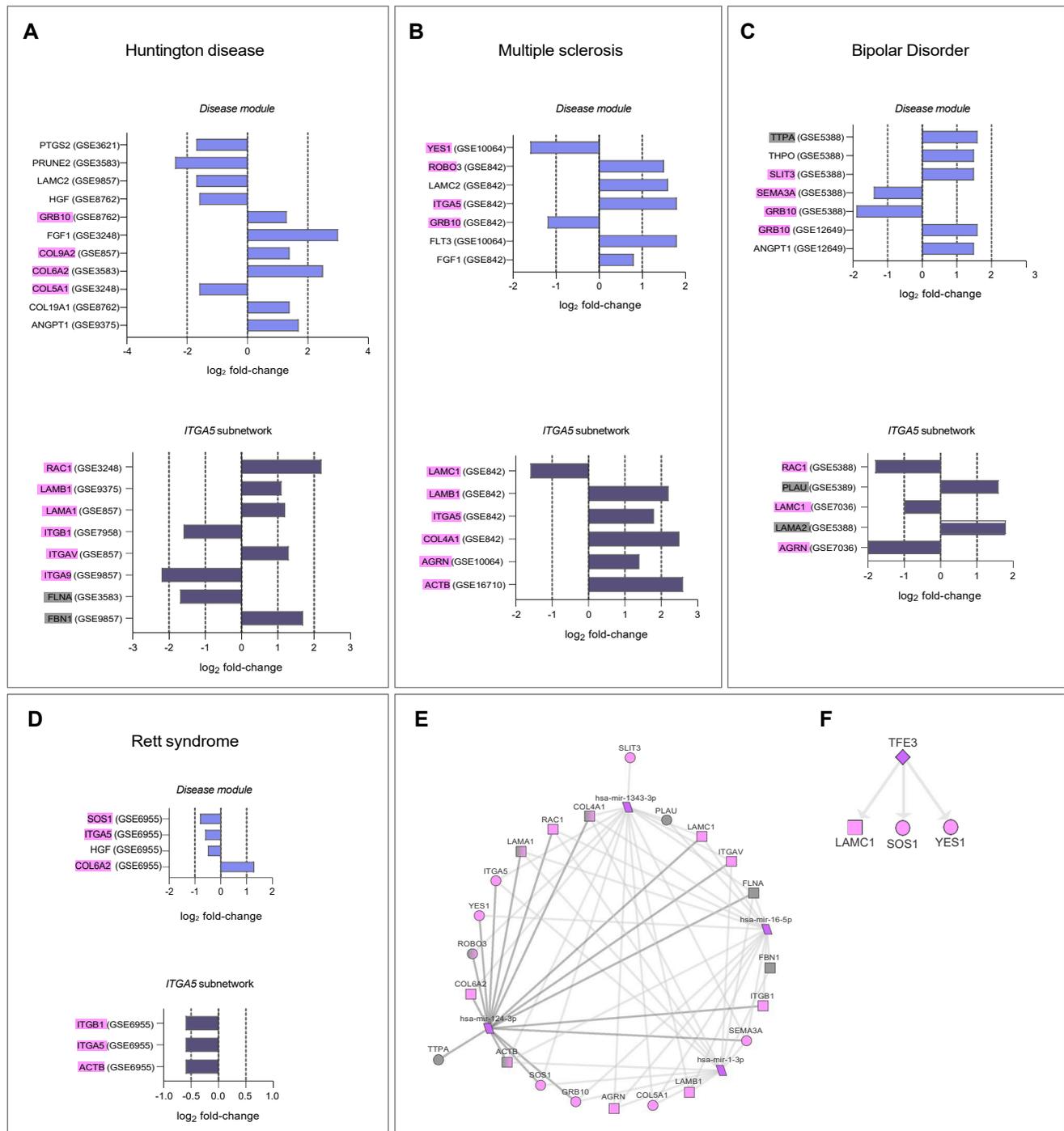
Fig. 5 Genes and regulators in four neurodegenerative conditions. Differentially expressed genes with a fold change cutoff of 0.6 (upregulated) and -0.6 (downregulated) in **A** Huntington's disease; **B** multiple sclerosis; **C** bipolar disorder; **D** Rett syndrome. Genes from the axon guidance network are represented by rectangles in light blue, and the *ITGA5* subnetwork in dark blue. **E** Network of the DEG-miRNA interactions with 22 nodes and 60 edges. **F** TFE3 and their interactions with axon guidance proteins

or the absence of lysosomal-mediated delivery of morphogens, growth factors and adhesion molecules can affect lysosomal transport within the axon and alter the stability of the growth cone and turnover (Farías et al. 2017; Manzoli et al. 2021). Besides that, we found altered expression in several genes that encode laminins: *LAMB1* (Huntington's disease and multiple sclerosis), *LAMA1* (Huntington's disease), *LAMC1* (multiple sclerosis and bipolar disorder), and

LAMA2 (bipolar disorder). Laminins belong to a group of glycoproteins and, through interaction with components of the extracellular matrix, play a role in cell adhesion, signaling, neurite growth, migration, and organization of the architecture of the cerebral cortex during embryonic development (Virtanen et al. 2003).

Taken together, our data corroborate previous studies (Hamano et al. 2008; Fecarotta et al. 2020; Manzoli et al. 2021), pointing to commonly altered mechanisms between MPS II and neurodegenerative diseases. Furthermore, we indicate collagens, integrins, and laminins necessary for axonal outgrowth, dendritic development, and migration of immune cells with potential involvement in these pathogenic conditions.

Potential key regulators involved in the neurologic phenotype in the MPS II

We investigated the miRNAs that target the DEGs in neurological diseases as a potential therapeutic strategy for MPS II. Among the 22 DEGs, 16 were targeted by hsa-mir-124-3p, 15 by hsa-mir-1343-3p and hsa-mir-16-5p, and 14 by hsa-mir-1-3p (Fig. 5E). The degree of centrality indicated that the genes *ACTB*, *SEMA3A*, *FLNA*, and *COL4A1* were connected by the four miRNAs. Furthermore, *TTPA* and *SLIT3* were targeted only by hsa-mir-124-3p and hsa-mir-1343-3p, respectively.

Hsa-mir 124-3p is involved in the negative regulation of microglial cell activation and the neuroinflammatory response associated with the pathogenesis of neurodegenerative disorders, including amyotrophic lateral sclerosis and Huntington's disease (Han et al. 2020). Interestingly, the inhibition of hsa-mir 1343-3p was capable of upregulating the expression of *ATG7* and promote autophagy in thyroid cancer cells (Qin et al. 2020). Autophagy can also be regulated by hsa-mir 1-3p and cause a decrease in the accumulation of toxic protein in mammalian cells (Nehammer et al. 2019). These findings converge with the idea of disruption in autophagy regulators during axonal development and not necessarily loss of central components of this mechanism (Crawley and Grill 2021). Already, hsa-mir -16 is an inhibitor of amyloid precursor protein, β-amyloid peptides production, and Tau phosphorylation in cells (Parsi et al. 2015). The overexpression of hsa-mir 16-5p decreases amyloid β-induced loss of viability and generation of apoptosis in a cell model of Alzheimer's disease (Zhang et al. 2020). Biomarkers, such as α-synuclein, an important component of Lewy bodies in Parkinson's disease, and whose accumulation is also seen in MPS II brains, is a putative target of hsa-mir -16 in vivo (Hamano et al. 2008; Parsi et al. 2015; Fecarotta et al. 2020). Finally, we found interactions between *TFE3* and *LAMC1*, *SOS1*, and *YES1* filtered in the transcription factors network (Fig. 5F). *TFE3* is a transcription factor

capable of regulating lysosomal homeostasis and immune response, stimulating autophagy, lysosomal biogenesis, and clearing of cellular detritus in a cellular model of a lysosomal storage disorder (Martina et al. 2014). Altogether, these data point to key regulators involved in the pathogenesis of MPS II that could act as potential therapeutic targets to prevent neurological consequences in MPSII. Furthermore, these results ratify the close relationship between axon guidance genes, regulators of lysosomal mechanisms, and the neuroinflammatory response associated with the neurologic findings in MPS II.

Conclusion

Our data indicate that undigested GAGs in the extracellular matrix can lead to impairment of the integrin involved in signaling axon guidance and vesicular pathways that integrate the development of neural circuitry during the early stages of neurodevelopment. These alterations can precede the morphological and clinical presentation in the neuronopathic form, highlighting the importance of neonatal screening. We also identified gene expression imbalances in a specific cluster in the interactome that can be associated with cognitive impairment in MPS II patients. Furthermore, we pointed out the shared pathogenic mechanisms between MPS II and neurodegenerative diseases and explored potential therapeutic targets involved in autophagic regulation that could delay neuronal dysfunction in the initial stages of the disease. Experimental studies are needed to validate these findings.

Declarations

Consent for publication Not applicable.

Conflict of Interest The authors have no conflict of interest to declare.

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4. Discussão

Como aspectos da função lisossômica são afetados pelo depósito, ou como o acúmulo de macromoléculas afeta o fluxo de sinalização celular e o seu impacto no organismo, são questões ainda em aberto a serem exploradas. Portanto, investigar vias de sinalização envolvidas na função lisossômica e do seu papel central na célula no contexto da patofisiologia das DLs, poderá trazer novos insights capazes de compreender e modular a função lisossômica de forma sensível e seletiva (Ballabio 2016). Estudos focados em estabelecer correlações fenótipo-genótipo em DLs, restritos às mutações descritas, demonstram-se incapazes de obter uma visão global dos efeitos destas desordens. Portanto, é importante notar que abordagens mais amplas, utilizando ferramentas de biologia de sistemas, possam oferecer um entendimento mais coerente da patogênese em DLs, uma vez que a função lisossômica também pode ser delineada por redes de proteínas por meio de suas interações (Ballabio 2016). A patofisiologia em DLs não pode ser considerada apenas o resultado de mutação em um único gene que leva ao acúmulo de substrato primário, mas passa pelo reconhecimento de mecanismos complexos subjacentes pelos quais o acúmulo de substratos afeta amplamente a função celular nesses distúrbios. Além disso, as opções terapêuticas atualmente disponíveis são pouco eficientes, sobretudo quando há prejuízo do SNC (Futerman and Van Meer 2004; Ballabio 2016). Logo, aprofundar na compreensão dos mecanismos relacionados à patogênese em DLs, pode ser um importante recurso para suportar novas estratégias terapêuticas.

4.1. Vias morfogênicas e de crescimento

A fisiopatologia das DLs vem sendo investigada ao longo de décadas, tendo emergido associações entre acúmulo de substrato e prejuízo em vias morfogênicas e de crescimento, com evidências que tentam justificar alguns dos fenótipos presentes neste grupo de doenças. Por exemplo, deformidades da coluna, como a cifoescoliose progressiva e compressão da medula espinhal são frequentemente observadas em MPS (Muenzer et al. 2009; Lampe and Lampe 2018).

A via de sinalização Hh estimula a proliferação celular e o desenvolvimento tecidual, regulando eventos fundamentais durante processos de desenvolvimento, como crescimento e padronização de embriões (Simpson et al. 2009; Petrova and Joyner 2014). Alterações na expressão de Ihh estão associadas ao encurtamento ósseo em camundongos com MPS VII (Metcalf et al. 2009; Peck et al. 2015; Kingma et al. 2016), visto que proteínas Ihh regulam a proliferação e a taxa de diferenciação de condrócitos (Vortkamp et al. 1996; Long et al. 2001). O estudo de Kingma et al. (2016) também observou mudanças nos níveis da proteína Ihh em condrócitos de pacientes e em camundongos com MPS I com crescimento ósseo diminuído (Kingma et al. 2016). Desta maneira, estes trabalhos sugerem que GAGs possam alterar a cascata de sinalização de Ihh e afetar o desenvolvimento dos condrócitos durante a progressão entre proliferação e hipertrofia, resultando em anormalidades ósseas em MPS I e VII (Akiyama et al. 2002; Peck et al. 2015; Kingma et al. 2016). Além disso, o colesterol também pode desempenhar um papel na sinalização da via Hh, especificamente na modulação da atividade da proteína sonic hedgehog (Shh) e na formação e manutenção dos cílios primários (Xiao et al. 2017). O cílio primário é uma estrutura localizada na membrana plasmática envolvida na transdução de sinal da via Hh (Goetz and Anderson 2010). A diminuição dos níveis das proteínas Ptch1 e Smo, envolvidas na sinalização desta via, foi observada em fibroblastos de indivíduos com NPC (Niemann-Pick tipo C) que expressaram redução do número e comprimento dos cílios primários (Canterini et al. 2017; Formichi et al. 2018).

A sinalização de vias de crescimento como mTOR e insulina podem ser afetadas pelo acúmulo de substratos na célula em diversas DLs. Perturbações em lipofuscinose ceróide neuronal juvenil (JNCL) são marcadas pelo aumento da autofagia, mas com vacúolos e lisossomos autofágicos imaturos e uma diminuição na atividade de mTOR (Cao et al. 2006). O controle de mTOR sobre mecanismos de autofagia também pode ser prejudicado em células de indivíduos com MPS I e doença de Fabry (Yu et al. 2010). Evidências em pacientes com cistinose indicam que a perda desse controle pode ocorrer por não dissociação de mTORC1 de vesículas endossômicas/lisossomais concentradas na região perinuclear (Sancak and Sabatini 2009; Ivanova et al. 2016).

Outras possíveis respostas para o atraso na reativação de mTOR envolvem a perda de função de proteínas como a battenina, deficiente em JNCL, associada com a maturação do vacúolo autofágico, resultando no prejuízo na maturação destes vacúolos em autolisossomos degradativos. A ineficiência destes mecanismos pode levar à privação de nutrientes e suprimir a sinalização de mTOR, gerando uma regulação positiva da autofagia (Cao et al. 2006; Ivanova et al. 2016). Por outro lado, há a possibilidade de substratos poderem estar diretamente envolvidos na deficiência da atividade de mTOR em DLs. Por exemplo, o depósito anormal de galactosilsfingosina em oligodendrócitos de camundongo com doença de Krabbe pode alterar as jangadas lipídicas e afetar a ativação de mTOR (Narayanan et al. 2009; Inamura et al. 2018). Além disso, o knock-out de proteínas Rag, que controlam a atividade de mTORC1, é capaz de gerar fenocópias de DLs em cardiomiócitos, incluindo o aumento da iniciação da autofagia, mas com prejuízo do fluxo de autofágico (Kim et al. 2014).

O acúmulo de substratos como os gangliosideos pode prejudicar a sinalização de insulina em DLs. O gangliosídeo GM3 presente em jangadas lipídicas pode modular a transdução de sinal mediado por receptores em diversas condições fisiológicas (Kabayama et al. 2007; Sekimoto et al. 2012). Todavia, em condições patológicas, é observado um aumento da síntese de GM3 em modelos animais de resistência à insulina (Tagami et al. 2002; Kabayama et al. 2007). A adição de GM3 exógeno pode levar à supressão da fosforilação da tirosina do substrato do receptor de insulina (IRS-1) e à redução da captação de glicose (Kabayama et al. 2007). O papel de GM3 na regulação negativa da sinalização da insulina foi demonstrada por camundongos incapazes de sintetizar GM3 que expressaram um aumento na sensibilidade à insulina (Yamashita et al. 2003; Zhao et al. 2007). Portanto, o acúmulo de GM3 em jangadas lipídicas de miócitos e adipócitos em pacientes com a doença de Gaucher é associado a menores taxas de captação de glicose mediada pela insulina e resistência à insulina (Langeveld et al. 2008). Além disso, a ativação do receptor de insulina (IR) também é defeituosa em hepatócitos Npc1-/- de camundongos, onde os níveis de IR são regulados positivamente. Estas alterações possivelmente ocorrem devido a mudanças na composição das jangadas lipídicas, fundamental para a correta transdução de sinal da insulina (Vainio et al. 2005).

4.2. Vias inflamatórias

Perturbações em processos imunológicos também podem ser vistas em DLs. Anormalidades em vias inflamatórias são observadas em pacientes com dilatação da aorta em MPS. Defeitos na via de degradação dos GAGs levam ao espessamento da aorta, infiltração de macrófagos, alteração do colágeno e fragmentação das lâminas de elastina (Braunlin et al. 2011). O mecanismo de patogênese sugerido consiste na ligação de GAGs ao receptor TLR4, desencadeando uma cascata inflamatória que culmina na liberação de diversas citocinas, quimiocinas e ativação do sistema complemento (Simonaro et al. 2008; Baldo et al. 2011). Portanto, o acúmulo de GAGs não degradados no coração pode desencadear um processo inflamatório por meio do recrutamento de macrófagos. Estas células secretam enzimas que degradam a MEC em condições patológicas, resultando na dilatação da aorta (Sleeper et al. 2004; Baldo et al. 2011; Gonzalez et al. 2017).

Com a integração de dados de expressão e interações proteína-proteína, nosso trabalho permitiu identificar potenciais genes e processos biológicos subjacentes a estes mecanismos envolvidos na disfunção cardíaca em MPS. Neste sentido, nós identificamos um enriquecimento funcional de genes diferencialmente expressos em MPS VII em processos que envolvem proteínas de ativação plasmática na inflamação aguda, ativação do complemento, imunidade mediada por células B, regulação da ativação leucocitária, regulação da produção de interleucina-6 e resposta ao lipopolissacarídeo. Com as análises topológicas, identificamos nós com alta centralidade com altos valores de degree e betweenness na rede. Estas proteínas podem ter um grande número de conexões, ou ainda, serem essenciais para a transmissão do fluxo de informação na rede (Newman 2006; Yu et al. 2007; Scardoni et al. 2009). Dentre estas proteínas, investigamos o fator de transcrição AP-1 (Fos) com papel na transdução de sinal, proliferação celular e síntese de fosfolipídios(Alfonso Pecchio et al. 2011), subexpresso em aortas dilatadas de camundongos MPS VII. Macrófagos sem *Fos* exibem liberação de citocinas pró-inflamatórias e apoptose aumentada(Maruyama et al. 2007), sugerindo que a regulação negativa de *Fos* pode levar à produção de mediadores inflamatórios associados à dilatação da aorta em MPS.

Além disso, Fos interagiu diretamente com diversas outras proteínas (Fcer1g, Plau, Ccnd1, Mmp2 e Mmp3) em nossa sub-rede presente no interatoma humano. Todas estas proteínas são codificadas por genes superexpressos em nossos dados. As proteases Mmps apresentam atividade colagenase e elastase, e seus desequilíbrios gênicos estão associados com a dilatação da aorta na MPS (Ma et al. 2008). Plau controla a MEC por meio da ativação do Mmps ao converter o plasminogênio em plasmina, regulando a migração e adesão celular em processos inflamatórios (Alfano et al. 2005; Ma et al. 2008). Além disso, Fcer1g está envolvido no recrutamento de células B, em que a sua depleção diminui a região de infarto em camundongos (Alfano et al. 2005; Srikakulapu et al. 2016). As alterações cardíacas também estão relacionadas à superexpressão de Ccnd1 que desencadeia a hiperplasia de cardiomiócitos em corações mutantes embrionários (Araújo et al. 2014) e hipertrofia

cardíaca em ratos (Giraud et al. 2005).

Portanto, as proteínas descritas acima, interagem diretamente entre si em um módulo específico na rede e podem compartilhar bioprocessos inflamatórios envolvidos na dilatação da aorta em MPS. GAGs podem desencadear uma resposta inflamatória por meio da ativação da via de sinalização do LPS, a qual resulta em processos que envolvem a produção de TNF e IL-6 em aortas de camundongos MPS VII e cães (Simonaro et al. 2008; Metcalf et al. 2010), e liberação de proteínas do complemento C3 e C5 por meio da ativação de TLR4 (Hajishengallis and Lambris 2010; Baldo et al. 2011). O gene CFD (fator D do complemento) superexpresso é fundamental para a ativação da via alternativa do sistema complemento e é considerado um marcador de risco para doença cardíaca coronária (Prentice et al. 2013). Desta forma, Cfd pode estar associado a um mecanismo patológico que gera a fragmentação da elastina ou produz dilatação aórtica de forma indireta (Baldo et al. 2011).

Em condições fisiológicas, é necessário um equilíbrio adequado entre a degradação e a síntese da MEC durante a morfogênese e manutenção do tecido cardíaco, mas alterações neste controle podem contribuir para a dilatação do tecido cardíaco (Cleutjens and Creemers 2002). O colágeno tipo I alfa 1 (*Col1a1*) é associado com a disfunção cardíaca em modelo de rato com insuficiência cardíaca quando superexpresso (Yim et al. 2018).

Além disso, a superexpressão de *Pdgfb* em camundongos transgênicos foi associadacom fibrose e hipertrofia cardíaca (Gallini et al. 2016). O aumento da hipertrofia também foiobservado em resposta à sobrecarga de pressão em camundongos knockout para *Hdac5*, subexpresso em nossa análise (Chang et al. 2004), indicando que Hdac5 pode reprimir a expressão de genes que promovem a hipertrofia cardíaca (Monovich et al. 2009). No entanto, a normalização dos níveis de expressão de *Hdac5* com o uso de valsartan, pode levar a umaredução da hipertrofia miocárdica em modelo animal (Xu et al. 2015). Além disso, é observado que o uso de outro antagonista do receptor da angiotensina II tipo 1, como o losartan, pode reduzir a dilatação aórtica e normalizar a hipertrofia cardíaca em camundongos com MPS I (Gonzalez et al. 2017; Osborn et al. 2017).

Redes compostas de proteínas e moléculas químicas mostraram interações do losartan com Fos e Col1a1, o que é interessante uma vez que o tratamento com losartan causauma diminuição da expressão do mRNA de *Col1a1* no miócito atrial e fibroblasto de camundongos (Chen et al. 2004; Tsai et al. 2008). Além disso, os níveis de *Fos* estão aumentados nos núcleos paraventriculares do cérebro de ratos após a administração de losartan (Crews and Rowland 2005). Os efeitos benéficos do losartan são sugeridos pelo antagonismo do fator transformador de crescimento TGF-β (Gonzalez et al. 2017). TGF-β éuma via que influencia a homeostase da MEC, remodelação vascular, regulação da pressão arterial e resposta imunológica (Zacchigna et al. 2006). O TGF-β pode modular a expressãodo fator de transcrição *Fos* e do co-repressor *Hdac5* por meio da sinalização Ras/ERK/MAPK (Kang et al. 2005; Molinaand Adjei 2006), indicando alvos e seu papel namelhora do tratamento com o losartan.

4.2.Vias do neurodesenvolvimento

Prejuízos em vias do SNC são frequentemente observados em DLs, portanto, com o uso de dados de expressão cerebral sob a perspectiva de suas interações no interatoma humano, nós investigamos o impacto de vias do neurodesenvolvimento no comprometimento do SNC observado em formas neuropáticas de MPS II.

Neste trabalho, identificamos novos genes candidatos e vias que convergem em aspectos funcionais envolvidos na estreita relação entre defeitos na formação do circuito neural e comprometimento cognitivo em MPS II. Em nossas análises topológicas, medimospossíveis correlações entre dados de centralidade e número de doenças mentais para cada gene em nossa rede cerebral. Identificamos uma correlação positiva baixa para degree r=0,20 e betweenness r=0,26. Isto pode ser explicado pelo modelo de que genes essenciais tendema codificar proteínas em posições centrais e com expressão relevante na maioria dos tecidos. Por outro lado, genes de doenças estão mais frequentemente localizados em regiões periféricas da rede (Goh et al. 2007). Sob uma perspectiva evolutiva, variantes patogênicas em genes com parâmetros topológicos centrais podem impactar regiões que estão amplamente interconectadas na rede, afetando o neurodesenvolvimento ou funções fisiológicas vitais em humanos (Goh et al. 2007; Yu et al. 2007).

Vias associadas à função e desenvolvimento cerebral foram enriquecidas em nossos dados, mostrando a presença de mecanismos endossomal-autofágicos e de orientação de axônios. A autofagia contribui para o crescimento dos axônios e reduz a estabilidade pré-sináptica (Crawley and Grill 2021). Os genes envolvidos nos processos de autofagia- fagocitose celular (*TUBA8*, *PRKAA1*, *RRAGB*, *ITGA5*) estão presentes na maioria dos clusters formados por bioprocessos, sendo que o neurodesenvolvimento depende da manutenção desses mecanismos. Os receptores ativados podem ser endocitados e classificados em outros compartimentos nos neurônios pelos endossomos; o conteúdo é encaminhado para degradação ou retorno à superfície celular (Pasterkamp and Burk 2021). Portanto, nossos dados sugerem que vias secundárias desencadeadas pelo acúmulo de GAGs podem alterar a internalização e o transporte de neurotransmissores, conforme observado nadiminuição dos transportadores de glutamato glial no cérebro de indivíduos com MPS II (Hamano et al. 2008). Identificamos um módulo específico na rede com diversos nós relacionados com processo de orientação do axônio e deficiência cognitiva.

Muitos desses genes participam de mecanismos de adesão, organização de componentes da matriz e sinalização mediada pela superfície celular que direciona a extensão do axônio, o crescimento celular e a migração durante o neurodesenvolvimento (Manzoli et al. 2021). Em particular, *ITGA5* (subunidade alfa 5 da integrina) codifica um

receptor que medeia a adesão celular à matriz extracelular ou outras células pela atividade do citoesqueleto e várias moléculas de sinalização (Wang et al. 2018). ITGA5 é uma integrina que desempenha um papel na orientação dos axônios e nos processos do fagossomo. As integrinas são intermediárias nas interações entre célula-célula e matriz celular desde os estágios iniciais até o tecido maduro, no qual GAGs são seu substrato para a sinalização na matriz celular (De Pasquale and Pavone 2019; Swinehart et al. 2020).

Estes módulos específicos podem ser associados à doença e sugerir modelos hipotéticos que explicam a heterogeneidade da condição, capturando genes e vias, mesmo em doenças mendelianas como a MPS II. Desta maneira, ITAG5 poderia indicar mecanismos afetados pelo acúmulo de GAGs na matriz extracelular, comumente prejudicada em MPS. ITGA5 e seus vizinhos imediatos no interatoma humano foram enriquecidos para vias como a adesão focal, interações de integrina da superfície celular, e atividade de proteoglicanos na MEC. A exposição a fragmentos de HS exógenos leva à ativação de aderências focais baseadas em integrina (FA) em células-tronco neurais de MPS III tipo B. FAs causam ancoragem celular na matriz extracelular, e sua estimulação por HS pode afetar a polarização celular e a migração orientada em MPS III tipo B (Bruyère et al. 2015). Além disso, alterações da expressão gênica de FAs e MEC foram associadas a defeitos de migração e crescimento de neuritos em um modelo de células-tronco pluripotentes de um paciente com mucopolissacaridose tipo I-Hurler (Lito et al. 2020). Disfunções da matriz extracelular foram identificadas em vários tipos de MPS (Gaffke et al. 2020). Portanto, o acúmulo anormal de fragmentos de HS associado ao comprometimento de processos biológicos relacionados ao desenvolvimento de axônios e vias de sinalização na superfície celular e na matriz extracelular sugere que esses substratos possam afetar etapas essenciais dos estágios iniciais do neurodesenvolvimento em formas neuropáticas de MPS II.

Perturbações no transcriptoma foram encontradas em condições neurológicas com distúrbios em mecanismos de autofagia-endossomos e prejuízo cognitivo, incluindo doença de Huntington, esclerose múltipla, transtorno bipolar e síndrome de Rett. Múltiplos genes diferencialmente expressos associados com o declínio cognitivo em humanos foram identificados em comum nestas condições. Neste sentido, nossos resultados corroboram estudos anteriores, os quais descrevem mecanismos comumente

alterados entre MPS II e doenças neurodegenerativas (Hamano et al. 2008; Fecarotta et al. 2020; Manzoli et al. 2021).Nós identificamos perturbações na expressão de colágenos, integrinas e lamininas necessárias para crescimento axonal, desenvolvimento dendrítico e migração de células do sistema imunológico, no qual podem apresentar potencial envolvimento nessas condições. Além disso, alguns destes genes regulados por miRNAs específicos, podem atuar como potenciais alvos terapêuticos para prevenir parte das consequências neurológicas em MPS II.

5. Conclusões

Neste trabalho usamos abordagens de biologia de sistemas para investigar os mecanismos de patogênese em doenças lisossômicas (DLs). Nós sugerimos modelos englobando genes e vias celulares para explicar parte dos processos fisiopatológicos em DLs. Inicialmente, verificamos o envolvimento de vias morfogênicas e de crescimento impactadas pelo acúmulo de substrato celular em diferentes DLs em um artigo de revisão. Revisitamos evidências indicando perturbações na sinalização de insulina, Hedgehog e mTOR em modelos animais e humanos. Além disso, geramos modelos de patogênese associados com processos inflamatórios e dilatação da aorta em MPS I e MPS VII. Por fim, identificamos genes e vias envolvidas em defeitos na formação do circuito neural que podem convergir em processos biológicos potencialmente envolvidos com o declínio cognitivo em MPS II. No entanto, a falta de dados de expressão gênica em humanos e a baixa cobertura do interatoma humano são importantes limitações deste trabalho.

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7. Anexos

Nesta seção estão incluídas produções bibliográficas produzidas durante o período do doutorado de autoria principal, mas não diretamente ligadas ao tema principal da tese.

7.1.Artigo I. Corrêa T, Feltes BC and Riegel M (2019) Integrated analysis of the critical region 5p15.3–p15.2 associated with cri-du-chat syndrome. Genet Mol Biol 42:186–196. https://doi.org/10.1590/1678-4685-GMB-2018-0173

Research Article

Integrated analysis of the critical region 5p15.3–p15.2 associated with cri-du-chat syndrome

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Abstract

Cri-du-chat syndrome (CdCs) is one of the most common contiguous gene syndromes, with an incidence of 1:15,000 to 1:50,000 live births. To better understand the etiology of CdCs at the molecular level, we investigated theprotein–protein interaction (PPI) network within the critical chromosomal region 5p15.3–p15.2 associated with CdCs using systemsbiology. Data were extracted from cytogenomic findings from patients with CdCs. Based on clinical findings, molecular characterization of chromosomal rearrangements, and systems biology data, we explored possible genotype–phenotype correlations involving biological processes connected with CdCs candidate genes. We identified biological processes involving genes previously found to be associated with CdCs, such as *TERT*, *SLC6A3,* and *CTDNND2*, as well as novel candidate proteins with potential contributions to CdCs phenotypes, including CCT5, TPPP, MED10, ADCY2, MTRR, CEP72, NDUFS6, and MRPL36. Although further functional analyses of these proteins are required, we identified candidate proteins for the development of new multi-target genetic editing tools to study CdCs. Further research may confirm those that are directly involved in the development of CdCs phenotypes and improve our understanding of CdCs-associated molecular mechanisms.

Introduction

Cri-du-chat syndrome (CdCs, OMIM 123450) is one of the most common contiguous gene syndromes, with an incidence of 1:15,000 to 1:50,000 live births (Niebuhr, 1978; Duarte *et al.*, 2004). Although 5p deletion is clinically and genetically well described, the phenotypic variability observed among patients with the deletion suggests that additional modifying factors, including genetic and environmental factors, may impact patients' clinical manifestations (Nguyen *et al.*, 2015). The classic phenotype of CdCs encompasses a cat-like cry, facial dysmorphism, microcephaly, psychomotor delays, and intellectual disability (Overhauser *et al.*, 1994). However, the clinical spectrum and severity of the disease depend of the size of the deleted chromosomal region (Smith *et al.*, 2010). Around 80% of individuals with CdCs exhibit *de novo* terminal deletions, and 5% exhibit interstitial deletions, where the deletion is most commonly inherited (Mainardi, 2006). In this sense, approximately 10–15% of the deletions result from an un-

balanced parental translocation (Mainardi, 2006), whereas complex genomic rearrangements, such as mosaicism, *de novo* translocation, or ring chromosomes, comprise fewer than 10% of cases (Perfumo *et al.*, 2000).

Previous studies looking for phenotype–genotype correlations through determination of deleted regions on 5p have described critical regions related to increased susceptibility for cat-like cry, speech delay, facial dimorphism, and intellectual disability (Overhauser *et al.*, 1994; Church *et al.*, 1997; Marinescu *et al.*, 1999; Mainardi *et al.*, 2001; Zhang *et al.*, 2005; Elmakky *et al.*, 2014). Although studies differ in the actual contribution of these critical regions to a particular phenotype, they allow that refinement of genes under hemizygous conditions may contribute to the pathogenesis of CdCs (Mainardi, 2006; Damasceno *et al.*, 2016). Candidate genes, such as *TERT*, *MARCH6*, *CTNND2*, and *SLC6A3,* are considered dose-sensitive or conditionally haploinsufficient (i.e., a single copy of these genes is insufficient to ensure normal functioning in individuals with CdCs) (Nguyen *et al.*, 2015). Haploinsufficiency of the genes mentioned above has been implicated in telomere maintenance dysfunction, cat-like cry, intellectual disability, and attention-deficit/hyperactivity disorder, respective-

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ly (Wu *et al.*, 2005; Du *et al.*, 2007; Hofmeister *et al.*, 2015; Tong *et al.*, 2015).

Even with the increasing resolution of cytogenetic techniques and the large amount of information available in databases, the investigation of contiguous gene syndromes remains a challenge. Studies have attempted to characterize genomic rearrangements and establish genotype–phenotype correlations through the identification of critical regions of susceptibility to CdCs, candidate genes, and haploinsufficiency-related altered mechanisms implicated in CdCs phenotypes (Lupski and Stankiewicz, 2005; Nguyen *et al.*, 2015). Therefore, in this study, to better understand the etiology of CdCs at the molecular level, we applied an integrative approach that combines conventional cytogenetic techniques, chromosomal microarray analysis (CMA), and systems biology tools to elucidate the probable molecular mechanisms underlying the clinical conditions present in CdCs.

Subjects and Methods

Study design and sample selection

This is a retrospective cytogenomic integrative analysis involving results of a series of cases. Clinical and cytogenomic data were extracted from six patients with CdCs enrolled in the Brazilian Network of Reference and Information in Microdeletion Syndromes (RedeBRIM) project (Riegel *et al.*, 2014, 2017; De Souza *et al.*, 2015; Dorfman *et al.*, 2015). The patients were regularly reevaluated over several years. Psychomotor development assessments were based on personal observations, school performance, and parent information. Daily abilities and skills, such as language, social interactions, concentration/attention, impulsiveness, motor control, perception, and learning and memory were recorded and published by our group elsewhere (Damasceno *et al.*, 2016). The five most frequent groups of clinical findings were selected and registered in the present study. This study has been approved by the Ethics Research Committee of Hospital de Clínicas de Porto Alegre (HCPA), followed the Declaration of Helsinki, and the standards established by the author's Institutional Review Board.

Cytogenomic Small Region of Overlap (SRO)

The deletions were mapped by whole genome array-CGH using a 60-mer oligonucleotide-based microarray with a theoretical resolution of 40 kb (8 60K, Agilent Technologies Inc., Santa Clara, CA). Labeling and hybridization were performed following the protocols provided by Agilent 2011. The arrays were analyzed using a microarray scanner (G2600D) and Feature Extraction software (version 9.5.1) (both from Agilent Technologies). Image analyses were performed using Agilent GenomicWorkbench Lite Edition 6.5.0.18 with the statistical algorithm ADM-2 at a sensitivity threshold of 6.0. The detailed cytogenomic

profiles of the patients analyzed in this study were presented by our group elsewhere (Damasceno *et al.*, 2016). Based on it, the chromosomal SRO was determined.

Network design

The protein–protein interaction (PPI) metasearch engine STRING 10.0 (http://string-db.org/) was used to create PPI networks based on genes located in the SRO. The list of genes was obtained from the human assembly of February 2009 (GRCh37/hg19) (Kent *et al.*, 1976; von Mering *et al.*, 2005). The parameters used in STRING were: (i) degree of confidence, 0.400, with 1.0 being the highest level of confidence; (ii) 500 proteins in the 1st and 2nd shell; and (iii) all prediction methods enabled, except for text mining and gene fusion. The final PPI network obtained through STRING was analyzed using Cytoscape 3.5 (Shannon *et al.*, 2003). Non-connected nodes from the networks were not included.

Clustering and GO analysis

The MCODE tool was used to identify densely connected regions in the final Cytoscape network. The analysis was based on vertex weighting by the local neighborhood density and outward traversal from a locally dense seed protein to isolate the highly clustered regions (Bader and Hogue 2003). The PPI modules generated by MCODE were further studied by focusing on major biology-associated processes using the Biological Network Gene Ontology (BiNGO) 3.0.3 Cytoscape plugin (Maere *et al.*, 2005). The degree of functional enrichment for a given cluster and category was quantitatively assessed *(p*-value) using a hypergeometric distribution. Multiple test correction was also implemented by applying the false discovery rate (FDR) algorithm (Benjamini and Hochberg 1995) at a significance level of $p < 0.05$.

Centralities

Two major parameters of network centralities (node degree and betweenness) were used to identify H-B nodes from the PPI network using the Cytoscape plugin CentiScaPe 3.2.1 (Scardoni *et al.*, 2009). The node degree centrality indicates the total number of adjacent nodes that are connected to a unique node. Nodes with a high node degree are called hubs and have central functions in a biological network (Scardoni *et al.*, 2009). Furthermore, we also analyzed the betweenness score, which corresponds to the number of shortest paths between two nodes that pass through a node of interest. Thus, nodes with high betweenness scores, compared to the average betweenness score of the network, are responsible for controlling the flow of information through the network topology (Newman, 2006; Scardoni *et al.*, 2009). These nodes are called bottlenecks and are normally related to the control of information between groups of proteins (Scardoni *et al.*, 2009).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files (Tables S1 - S17).

Results

The main clinical findings of six patients with CdCs selected to this study are presented in Figure 1. Intellectual disability (6/6 patients), learning difficulties (6/6 patients), multiple congenital abnormalities (6/6 patients), hyperactivity/impulsiveness (5/6 patients), and heart defects (4/6 patients) were the most frequent findings (Figure 1). Among the samples, three were from males, with ages ranging from 6 to 38 years, and three were from females, with ages ranging from 7 to 20 years.

Cytogenomic data analysis MR

Six *de novo* terminal deletions that ranged in size from approximately 11.2 Mb to 28.6 Mb, with breakpoints from 5p15.2 to 5p13 were mapped. The analysis of CMA profile data revealed a small region of overlap (SRO) of 10.8 Mb encompassing the bands 5p15.33–p15.2. The approximate genomic position of the SRO is chr5:527552–11411700, comprising 44 genes according to the UCSC genome browser human assembly of February 2009 (GRCh38/hg19) (Figure 2).

Networks and topological analysis

Overall, the scale-free network was composed of 2284 nodes (proteins) and 83340 edges (interactions) (Figure 3). Centrality analyses were carried out to identify hub-bottlenecks (H-B), the most topologically relevant no-
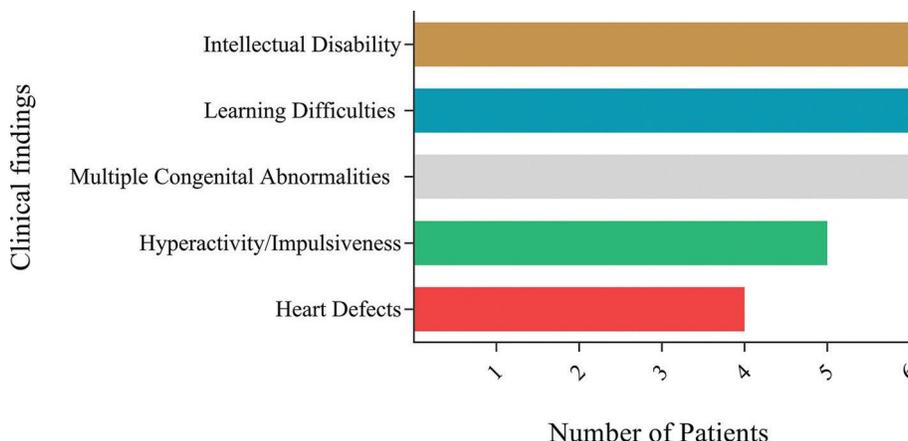
Figure 1 - Summary of clinical findings of the six individuals in the study according to Damasceno *et al.* (2016).
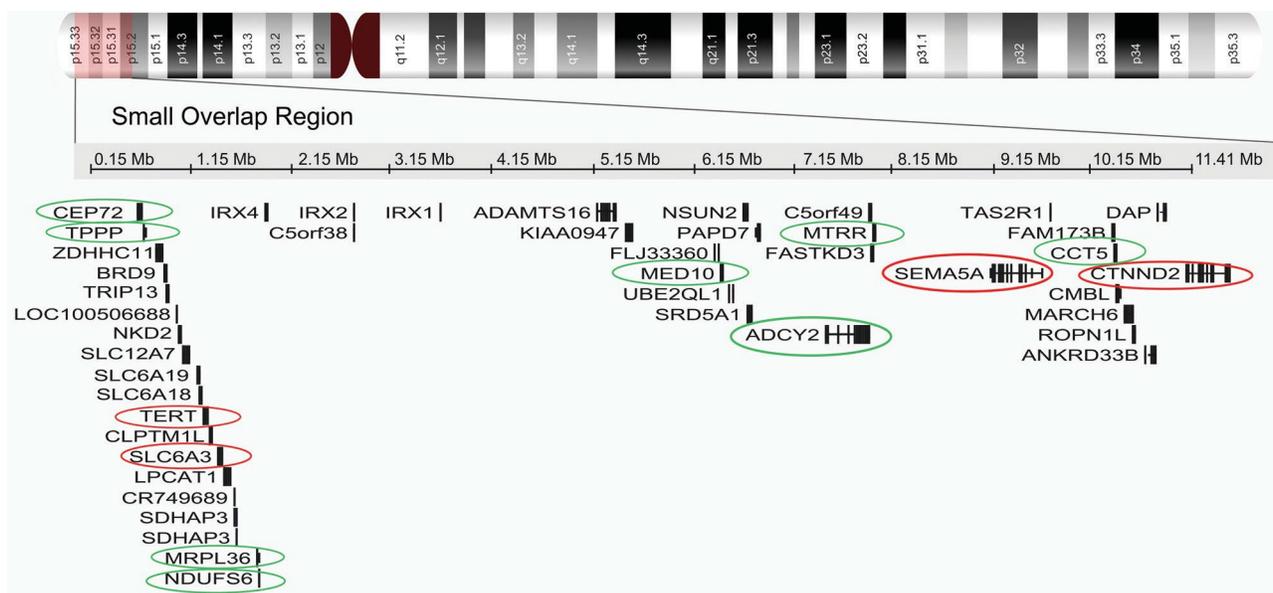
Figure 2 - Cytogenomic profile of chromosome 5. Chromosomal critical region of 5p15.33–p15.2. Genes localized to the critical region were obtained from the human assembly of February 2009 (GRCh37/hg19). Red circles show genes already associated with CdCs. Green circles show candidate genes from this study for contributing to the phenotype in CdCs.
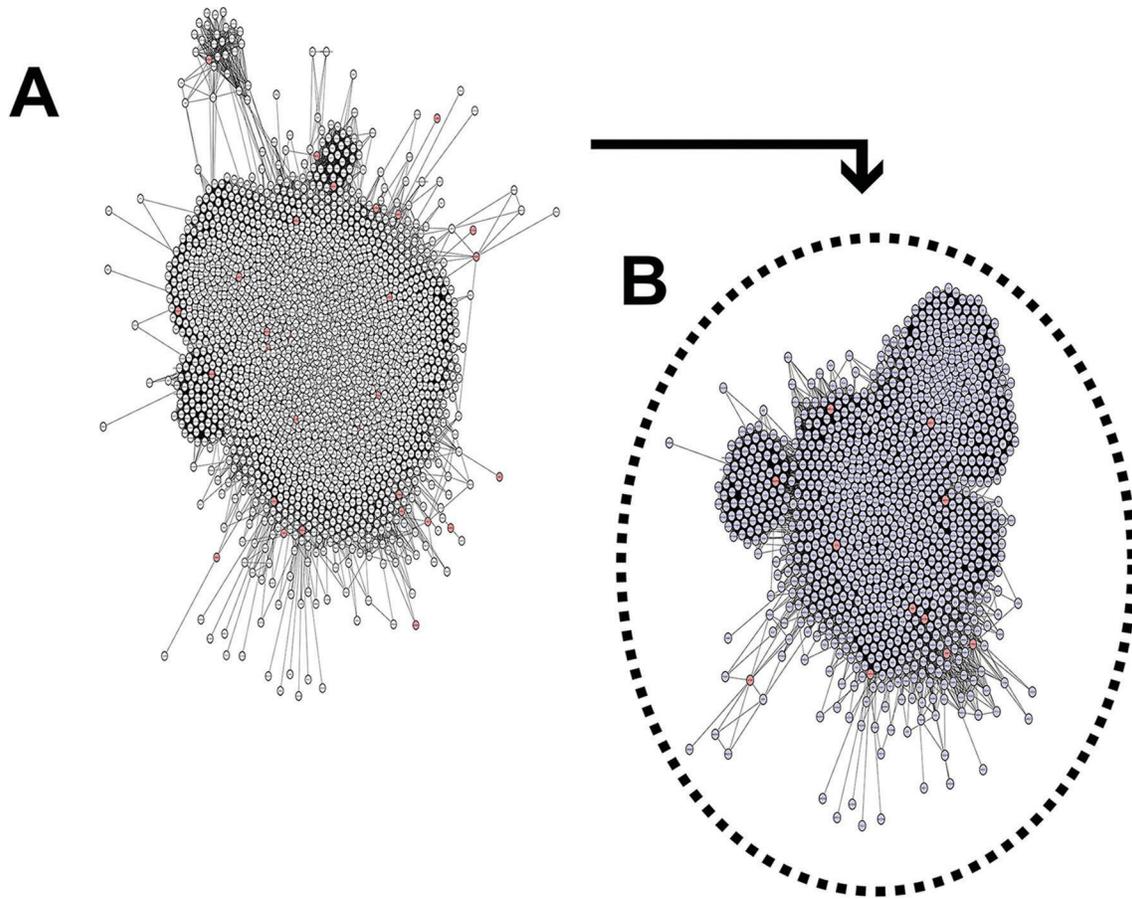
Figure 3 - The PPI network. The list of 44 genes was obtained from the human assembly of February 2009 (GRCh37/hg19). Interaction data from STRING were used to construct networks using Cytoscape software. (A) The primary network is composed of 2284 nodes and 83,340 edges. Red nodes are target proteins (SLC6A3, SRD5A1, CCT5, ADCY2, TAS2R1, MED10, MTRR, SLC12A7, CEP72, NDUFS6, MARCH6, LPCAT1, NKD2, CTNND2, TERT, CLPTM1L, MRPL18, MRPL36, UBE2QL1, PAPD7, and TPPP). (B) Secondary network composed of 1062 nodes and 41,309 edges. Red nodes are candidate proteins (CCT5, TPPP, MED10, ADCY2, MTRR, CEP72, NDUFS6, MRPL36, CTNND2, TERT, and SLC6A3) and immediate neighbors from SRO.

des. The network hubs (nodes with an above average number of connections) and betweenness (total number of non-redundant shortest paths going through a node or edge) indicate the most critical points in a biological network (Yu *et al.*, 2007). In our analysis, we observed 273 H-B nodes in the SRO network. Furthermore, we performed a cluster analysis that identified 16 major cluster regions above our cutoff score, and gene ontology (GO) analyses were performed in the identified modules.

Clusters taken into consideration for further analysis were those containing major proteins related to CdCs and deleted in all patients according to Espitiro Santo (2016), namely those containing combinations of SLC6A3, SRD5A1, CCT5, ADCY2, TAS2R1, MED10, MTRR, SLC12A7, CEP72, NDUFS6, MARCH6, LPCAT1, NKD2, CTNND2, TERT, CLPTM1L, MRPL18, MRPL36, UBE2QL1, PAPD7, and TPPP (Figure 4). In addition, the TERT protein was a commonly clusterized protein, and all clusters containing TERT were selected. Clusters that did not contain multiple combinations of the CdCs protein tar-

gets mentioned above, TERT, were excluded from the final analysis.

The most relevant GO terms are listed in Table S1. The main observed terms were: (i) nervous system-associated processes, such as development, synapsis, and learning; (ii) aging; (iii) double-strand break repair; (iv) regulation of apoptosis/cell death; (v) telomere maintenance; (vi) senescence; (vii) response to cytokine stimulus; (viii) regulation of interleukin (IL)-1; (ix) hormone biosynthetic processes, especially androgen biosynthesis; and (x) regulation of the NF-ĸB/IĸB pathway. The number of GO terms associated with each cluster can be found in Figure 5. Our analysis excluded GO terms that were not associated with significant biological processes related to the disease, or that were too general (e.g., regulation of biological process, signaling process, or response to endogenous stimulus).
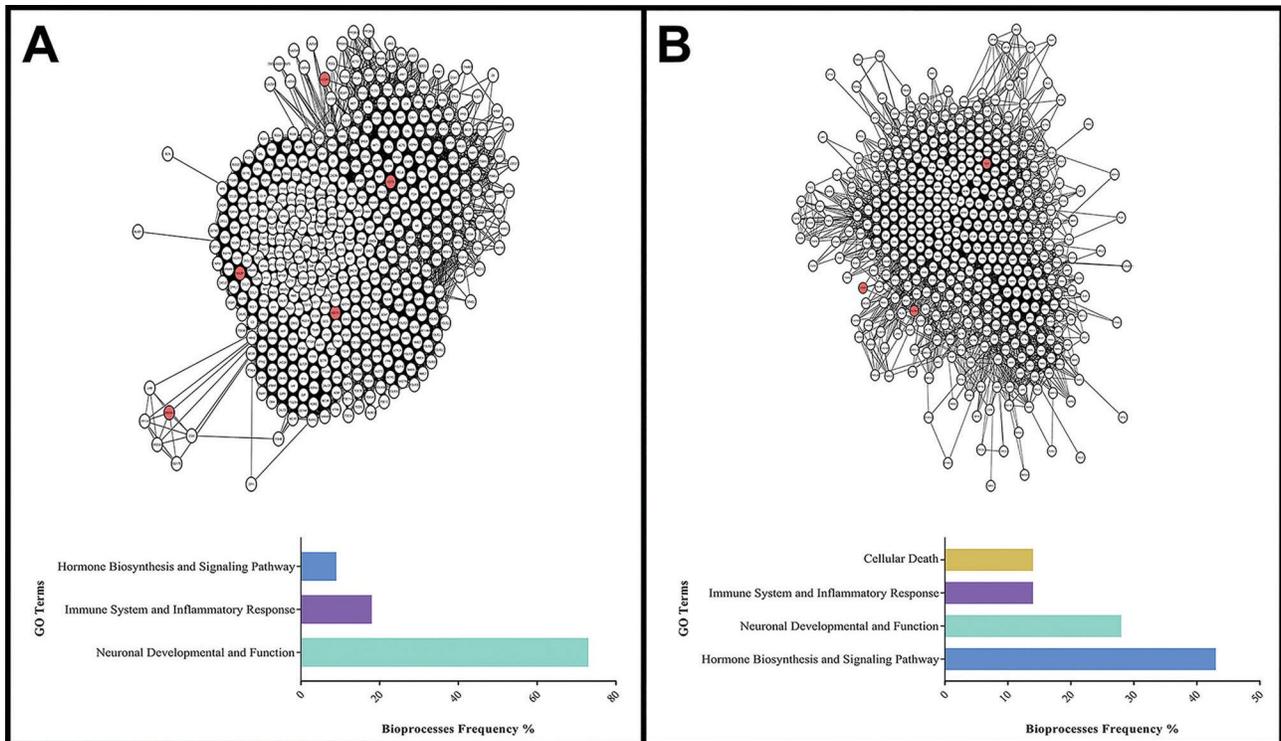
Figure 4 - Subnetworks derived from clustering analysis. Red nodes are target proteins. (A) Cluster 1, with Ci = 94,369, composed of 509 nodes and 24,064 edges. Target proteins: SLC6A3, SRD5A1, CCT5, ADCY2, and TAS2R1. Below, summary of the bioprocess frequency identified in the PPI network (B) Cluster 8, Ci = 23,208, contains 471 nodes and 5477 edges. Target proteins: SLC6A3, TERT, and TPPP. Below, summary of the Bioprocess frequency identified in the PPI network.
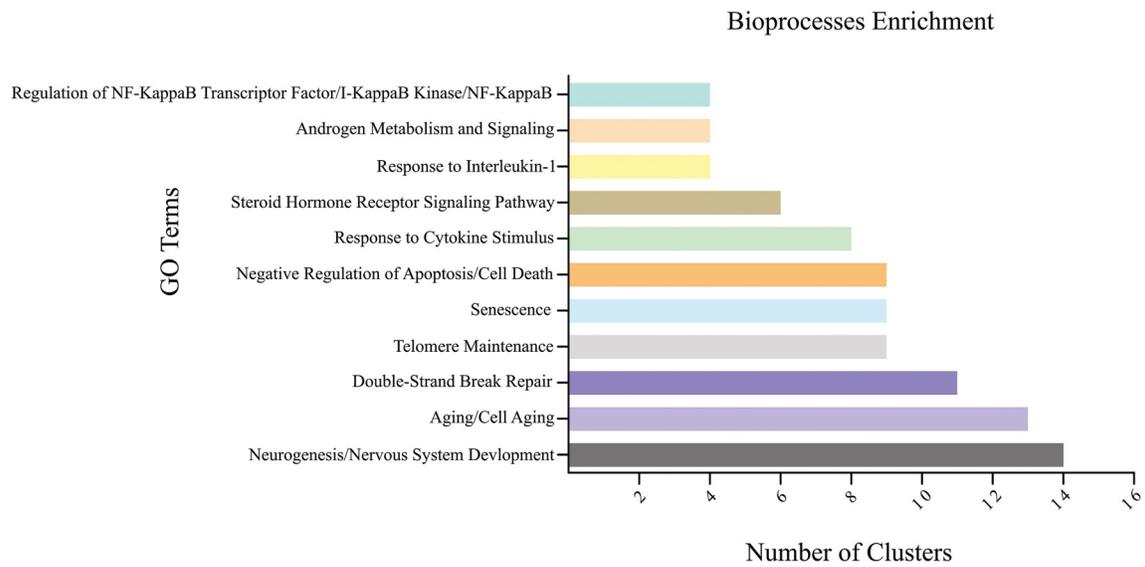

Figure 5 - Summary of the bioprocess enrichment identified in the PPI network. The colored horizontal bars show GO terms frequently present in the analyzed clusters.

Discussion

CdCs patients are traditionally diagnosed based on a detailed clinical evaluation and cytogenetic investigations. Furthermore, some studies have shown the importance of characterizing the genomic position of the critical chromo-

somal region associated with CdCs for a better understanding of genotype–phenotype correlations (Wu *et al.*, 2005; Zhang *et al.*, 2005; Damasceno *et al.*, 2016). Network-based approaches may contribute to the identification of specific genes distributions in a given disease and reveal common molecular mechanisms among genes affected by the condition. Furthermore, genes associated with the same

illness have been observed to interact with each other more frequently than expected by chance (Barabási *et al.*, 2011).

Interaction between SLC6A3 TPPP and CCT5 and Processes related to neuronal development and function in CdCs

In this study, the constructed networks and topological analysis, such as those in clusters 1 and 8 (Figures 2 and 4), showed interactions between SLC6A3, TPPP, and CCT5, genes which are located in the SRO, and interactions between processes related to neuronal development and function in CdCs. The GO analysis of clusters 1 and 8 indicated the presence of proteins deleted in hemizygous individuals in our study that are related to the regulation of glutamatergic and dopaminergic synaptic transmission, catecholamine uptake involved in synaptic transmission, and norepinephrine secretion and neurogenesis. Changes in patterns of neuronal activity modulated by dopamine and noradrenaline in the cortico-striatal region of the brain are able to influence the emergence of disturbances, such as attention deficit hyperactivity disorder (ADHD) (Del Campo *et al.*, 2011; Cummins *et al.*, 2012). Interestingly, ADHD is present in about 70% of children with CdCs (Nguyen *et al.*, 2015), and, in our study, hyperactivity was present in five out of the six subjects (Figure 1). SLC6A3, a dopamine transporter, regulates extracellular dopamine, is responsible for the reuptake of dopamine, and functions to balance levels of neuronal dopamine (Gizer *et al.*, 2009). Deficiency of this protein can lead to the accumulation of dopamine in the cytosol, with deleterious effects (Sotnikova *et al.*, 2005). These effects may be associated with hyper-locomotion, stereotyped behaviors, and hyperactivity, as in *Slc6a3* KO mice (Giros *et al.*, 1996; Pogorelov *et al.*, 2005; Lohr *et al.*, 2017), or decreased immobility, as in *Slc6a3*^{+/−} mice (Perona *et al.*, 2008). Therefore, SLC6A3 can be proposed as a good target on subsequent functional analyses that could increase the mechanistic knowledge related to those CdCs phenotypes. Interestingly, we observed that TPPP is among the direct neighbors of SLC6A3 in cluster 8 (Figure 4). TPPP functions in tubulin polymerization and microtubule stabilization (Vincze *et al.*, 2006). TPPP plays an important role in pathological conditions through the co-enrichment and co-localization of TPPP and α-synuclein in human brain inclusions, such as in Parkinson's disease (Oláh and Ovádi, 2014). Through the polymerization of the tubulin polymer, TPPP contributes to the extension of peripheral axons in sensory neurons (Aoki *et al.*, 2014). Changes in the expression of TPPP are associated with the phenotypes of depression and anxiety following early life stress in humans (Montalvo-Ortiz *et al.*, 2016). Therefore, these results identified by network analysis suggest an important perturbation between the proteins SLC6A3 and TPPP generating neural changes in CdCs individuals. SLC6A3 also interacts with the H-B CCT5 in cluster 1, in which processes related to cognition, memory, and learning

can be found (Figure 4, Table S2). The protein CCT5 is involved in cilia morphogenesis and neurodegenerative processes, and its deficiency may cause neurodegenerative diseases, such asspastic paraplegia (Bouhouche *et al.*, 2006; Posokhova *et al.*, 2011), supporting the GO results. Individuals with spastic paraplegia may present with atrophy of the spinal cord and defects in the upper limbs. These results indicate that SLC6A3*,* CCT5 and TPPP show important connection. Thus, we could consider that disruption of these interactions may change the processes related to neuronal development and function underlying in some patients with CdCs.

Interplay between of genes in the SRO and behavioral and cognitive impairment

The proteins encoded by *CTNND2*, *TERT*, and *MED10*, which are located in the SRO determined in this study (Figure 2), are commonly deleted in CdCs and interact in several modules associated with neuronal development/function and cellular death, specifically clusters 3, 5, 6, 8, 10, and 11 (Tables S4, S5, S6, S7, S9, S11 and S12). This suggests an interplay between genes in the SRO and behavioral and cognitive impairment*.* These genes are expressed during important periods of embryonic and neuronal development (Yui *et al.*, 1998; Kwon *et al.*, 1999; Ho *et al.*, 2000). *CTNND2*, considered a bottleneck in our analysis, encodes ο-catenin, a component of adherens junction complexes (Kosik *et al.*, 2005) that regulates spine morphogenesis and synapse function in hippocampal neural cells during development (Arikkath *et al.*, 2009). ο-Catenin is stabilized by N-cadherin, which binds to PDZ domain proteins in the post-synaptic compartment at synapse junctions and regulates spine architecture during hippocampal development and the differentiation of neurons via downstream effectors that bind to actin in the cytoskeleton (Kosik *et al.*, 2005; Yuan *et al.*, 2015). Among the bioprocesses investigated in the protein interaction network, we identified the negative regulation of the Wnt receptor signaling pathway. Through Wnt signaling, ο-catenin prevents Rho GTPase signaling, modulating the Ras superfamily in cytoskeletal reorganization (Lu *et al.*, 2016). Perturbations in this pathway, observed after depletion of ο-catenin, may contribute to functional neurological alterations (Arikkath *et al.*, 2009). In this sense, the loss of a copy of *CTNND2* in CdCs may be associated with intellectual disability, reading problems (Medina *et al.*, 2000; Belcaro *et al.*, 2015; Hofmeister *et al.*, 2015), learning difficulties, and autism spectrum disorder (ASD) (Asadollahi *et al.*, 2014) (Figure 1). The interplay of ο-catenin with cadherin suggests its influence on Wnt/ℽ-catenin signaling (Lu *et al.*, 2016), increases keloid cell proliferation and inhibits apoptosis through its interaction with telomerase (Yu *et al.*, 2016). This mechanism perhaps explains the enrichment of the negative regulation of apoptosis process in the GO analysis

(Figure 5). In addition, reduction in MED10 levels enhances Wnt signaling and is required for the expression of developmentally regulated genes (Kwon *et al.*, 1999; Lin *et al.*, 2007). The H-B MED10 is crucial for DNA-binding factors that activate transcription via RNA polymerase II (Sato *et al.*, 2003). Lastly, the telomerase reverse transcriptase, encoded by *TERT*, which behaved as an H-B, was the most clusterized protein (Tables S15 and S17). The hemizygosity of *TERT* has been associated with shorter telomeres in lymphocytes from CdCs patients and contributes to the phenotypic changes seen in the syndrome (Zhang *et al.*, 2003). However, another study with 52 individuals affected by CdCs showed that the telomere length in CdCs patients was within the normal range, though the average was shorter than that in normal controls (Du *et al.*, 2007). These data suggest that the contribution of *TERT* to CdCs may involve alterations in other biological processes or pathways. For instance, *TERT* can exert protective effects. Under dietary restriction conditions, TERT accumulates in the mouse brain, leading to reductions in free radicals in the mitochondria, DNA damage, and apoptosis through the inhibition of the mTOR cascade (Miwa *et al.*, 2016). These processes were present in all clusters except 1 and 13 (Tables S2 and S14).

Therefore, analyses of centrality suggest that the deficiency in *CTNND2*, *TERT*, and *MED10* genes expression during important stages of development may affect processes related to neurogenesis and the regulation of apoptosis and DNA repair, being inherent in the cognitive and behavioral impairments seen in CdC patients (Figure 1).

Control of NF-kB transcription factor/interleukin 1 and inflammatory response

In several clusters, GO analysis identified processes related to the immune system and inflammatory response. Considering this, we explored the control of the NF-κB transcription factor/IL-1 and the inflammatory response. The appearance of respiratory and intestinal infections during the first years of life is common in patients with CdCs, though it has been rarely discussed (Mainardi, 2006). Processes related to immune response-activating signal transduction, response to IL-1, leukocyte activation, and regulation of the IκB kinase/NF-κB cascade, which has an important role in inflammation (Deacon and Knox 2018), were observed in our study, especially in clusters 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, and 12 (Figure 5, Tables S2-S4 and S6-S13). With the use of telomerase inhibitors and telomerase-targeting small interfering RNAs, it has been found that H-B TERT reduces TNF-α-induced chemokine expression in airway smooth muscle cells (SMCs) (Deacon and Knox, 2018). Another protein involved in the immune response is adenylyl cyclase (ADCY2), which is also an H-B according to the centrality analysis. This protein catalyzes the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), involving va-

rious signal transduction pathways. ADCY2 regulates the production of IL-6 in inflammatory processes and enhances its expression in SMCs (Bogard *et al.*, 2014; Jajodia *et al.*, 2016). In addition, single-nucleotide polymorphisms in ADCY2 have been associated with severe chronic obstructive pulmonary disease (Hardin *et al.*, 2012).

These data suggest that the presence of specific pathways related to the immune response can be affected by genes commonly deleted in CdCs (Figure 5). These results bring new insights into the pathogenesis of the syndrome, in an attempt to explain the emergence of recurrent respiratory and intestinal infections during the first years of life in individuals with CdCs (Mainardi, 2006).

Association between genes in SRO and congenital malformations.

Regarding the association between genes in the SRO and the multiple congenital malformations observed in CdCs, the network analysis demonstrated interactions between MTRR, CEP72, NDUFS6, MRPL36, and MED10 in clusters 2 and 4, in which the GO analysis identified processes related to DNA repair, cell cycle control, cellular death, and mitochondrial ATP synthesis, and electron transport (Figure 5). *MTRR* encodes a methionine synthase reductase that is fundamental for the remethylation of homocysteine, which regenerates functional methionine synthase via reductive methylation. Individuals with neural tube defects (NTDs) exhibit elevated homocysteine concentrations (Steegers-Theunissen *et al.*, 1993; Zhu *et al.*, 2003; Cheng *et al.*, 2015). The protein MTRR emerged as a bottleneck in our protein interaction network. Heterozygous mutations that lead to MTRR deficiency have been implicated in homocysteine accumulation, resulting in adverse reproductive outcomes and congenital heart defects in mice (Zhu *et al.*, 2003; Li *et al.*, 2005). Therefore, defects in the activity of MTRR could be associated with frequent clinical manifestations of CdCs, such as cardiac abnormalities. Furthermore, neurodevelopmental disorders such as primary microcephaly are associated with mutations in proteins that interact with the centrosomes, such as the CEP72 (Kodani *et al.*, 2015), which was considered an H-B in our analysis. CEP72 regulates the localization of centrosomal proteins and bipolar spindle formation (Oshimori *et al.*, 2009). Therefore, CEP72 is involved in centriole duplication and biological processes such as control of the cell cycle, and deficiency of this protein may contribute to dysmorphic phenotypes in CdCs (Figure 1).

Another protein in cluster 2 was the H-B NDUFS6, an accessory subunit of the mitochondrial chain NADH dehydrogenase (Murray *et al.*, 2003). Deletion of NDUFS6 or mutation of its Zn-binding residues blocks a late step in complex I assembly (Kmita *et al.*, 2015). Mutations in this protein may also cause lethal neonatal mitochondrial complex I deficiency (Kirby *et al.*, 2004) and fatal neonatal lactic acidemia (Spiegel *et al.*, 2009). Besides these proteins,

MRPL36, a component of the ribosomal subunit (Williams *et al.*, 2004), emerged as a hub in our network of protein interactions. Decreases in MRPL36 prevent the correct folding and assembly of translation products, leading to rapid degradation of these molecules and defects in the biogenesis of respiratory chain complexes in the mitochondria (Prestele *et al.*, 2009). Therefore, the hub MRPL36 may contribute to oxidative stress-related processes found in cluster 2 (Table S3) and may be associated with excess apoptosis and NTDs (Yang *et al.*, 2008).

Excessive apoptosis in fetal central nervous tissues can cause NTDs by decreasing the number of cells in the neural folds or by physical disruption of the dorsal midline, consequently resulting in embryonic dysmorphogenesis (Chen *et al.*, 2017; Lin *et al.*, 2018). Furthermore, the H-B MED10, located in clusters 2 (Figure 4), 3, and 4, regulates heart valve formation in zebrafish (Just *et al.*, 2016). In addition, network analysis demonstrated an interaction between MED10 and the protein encoded by *MED24/TRAP100*, located on chromosome 17. MED24 is necessary for enteric nervous system development in zebrafish (Pietsch, 2006). Together, these findings contribute to our understanding of the emergence of congenital heart defects, microcephaly, and occasional abnormalities such as agenesis of the corpus callosum, cerebral atrophy, and cerebellar hypoplasia, which may be present in CdCs.

Conclusion

The possibility of using microarrays to characterize chromosomal rearrangements has led to several studies aimed at establishing genotype-phenotype correlations in several contiguous gene deletion syndromes, and some of them have proposed the regions of susceptibility to each specific condition. However, no consensus has been reached on the exact identity of the genes and cell signaling pathways involved in promoting these symptoms, as e.g. in the CdCs. This is the first study to explore the interaction network of the proteins encoded in the critical region associated with CdCs by combining cytogenomic data and systems biology tools. This study identified and demonstrated the biological processes involving genes previously found to be associated with CdCs, such as *TERT*, *SLC6A3,* and *CTDNND2*. Furthermore, through analysis of the protein interaction network, we identified other possible candidate proteins, including CCT5, TPPP, MED10, ADCY2, MTRR, CEP72, NDUFS6, and MRPL36, with potential contributions to the phenotypes observed in CdCs. Further functional analysis of these proteins is required to fully understand their involvement and interplay in CdCs. Additional research in this direction may confirm those that are directly involved in the development of the CdCs phenotype and improve genotype–phenotype correlations.

Conflict of interests

There are no conflicts of interest to declare.

Author contributions

All authors contributed to the analysis and interpretation of data; all authors participated in the writing of the manuscript and approved the version submitted for publication.

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Supplementary material

The following online material is available for this article:

Table S1 - List of genes located in the smallest overlap region.

Table S2 - List of Go terms identified by BiNGO in the Cluster 1.

Table S3 - List of Go terms identified by BiNGO in the Cluster 2.

Table S4 - List of Go terms identified by BiNGO in the Cluster 3.

Table S5 - List of Go terms identified by BiNGO in the Cluster 4.

Table S6 - List of Go terms identified by BiNGO in the Cluster 5.

Table S7 - List of Go terms identified by BiNGO in the Cluster 6.

Table S8 - List of Go terms identified by BiNGO in the Cluster 7.

Table S9 - List of Go terms identified by BiNGO in the Cluster 8.

Table S10 - List of Go terms identified by BiNGO in the Cluster 9.

Table S11 - List of Go terms identified by BiNGO in the Cluster 10.

Table S12 - List of Go terms identified by BiNGO in the Cluster 11.

Table S13 - List of Go terms identified by BiNGO in the Cluster 12.

Table S14 - List of Go terms identified by BiNGO in the Cluster 13.

Table S15 - List of Go terms identified by BiNGO in the Cluster 14.

Table S16 - List of Go terms identified by BiNGO in the Cluster 15.

Table S17 - List of Go terms identified by BiNGO in the Cluster 16.

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7.2.   Artigo II. Corrêa T, Venâncio AC, Galera MF and Riegel M (2020) Candidate Genes Associated with Delayed Neuropsychomotor Development and Seizures in a Patient with Ring Chromosome 20. Case Rep Genet 2020:1–6. https://doi.org/10.1155/2020/5957415

Case Report
Candidate Genes Associated with Delayed Neuropsychomotor Development and Seizures in a Patient with Ring Chromosome 20

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Ring chromosome 20 (r20) is characterized by intellectual impairment, behavioral disorders, and refractory epilepsy. We report a patient presenting nonmosaic ring chromosome 20 followed by duplication and deletion in 20q13.33 with seizures, delayed neuropsychomotor development and language, mild hypotonia, low weight gain, and cognitive deficit. Chromosomal microarray analysis (CMA) enabled us to restrict a chromosomal segment and thus integrate clinical and molecular data with systems biology. With this approach, we were able to identify candidate genes that may help to explain the consequences of deletions in 20q13.33. In our analysis, we observed five hubs (ARFGAP1, HELZ2, COL9A3, PTK6, and EEF1A2), seven bottlenecks (CHRNA4, ARFRP1, GID8, COL9A3, PTK6, ZBTB46, and SRMS), and two H-B nodes (PTK6 and COL9A3). The candidate genes may play an important role in the developmental delay and seizures observed in r20 patients. Gene ontology included microtubule-based movement, nucleosome assembly, DNA repair, and cholinergic synaptic transmission. Defects in these bioprocesses are associated with the development of neurological diseases, intellectual disability, neuropathies, and seizures. Therefore, in this study, we can explore molecular cytogenetic data, identify proteins through network analysis of protein-protein interactions, and identify new candidate genes associated with the main clinical findings in patients with 20q13.33 deletions.

1. Introduction

Ring chromosomes are rare structural rearrangements in humans, exhibiting an estimated frequency of 1 in 25,000 recognized conceptions [1] and approximately 1 in 30,000–60,000 births [2]. The ring formations are generally *de novo*, being only 1% inheritable [3–5]. Ring chromosome 20 (r20), which was first described in 1972 [6], is a syndrome characterized by refractory epilepsy, intellectual impair- ment, and behavioral disorders. Facial dysmorphism or other congenital malformations are rarely reported. r20 patients may present normal development until seizure onset, with the cognitive-behavioral decline being observed later, which suggests that the syndrome can be considered an epileptic encephalopathy [7, 8]. Most likely, r20 formation is

the result of intrachromosomal fusions from the direct union of unstable telomeres or the occurrence of two breaks, one in each chromosomal arm, resolved by the junction of the telomere ends of both arms, short and long, forming a circular structure [9, 10]. In the latter case, deletions, du- plications, and/or inversions usually occur at the chromo- somal ends [11, 12]. The diagnosis of ring chromosome 20 syndrome requires identification of ring formation by conventional cytogenetic techniques with the complement of chromosomal microarray analysis to detect small losses and gains in genetic material.

There are fewer than 20 cases described in the literature of patients carrying subtelomeric deletions in 20q13.33 [13–18]. Common manifestations of these individuals in- clude skeletal and growth abnormalities, behavioral

problems, developmental delay, and seizures [19]. However,there are at least three factors that impair the clinical characterization of affected individuals and the identifica- tion of causal genes. First, there are notably few individuals molecularly characterized with high-resolution techniques. Second, there is no pattern regarding the presence of specificclinical manifestations in 20q13.33 deletions. Third, the significance of the ring morphology or chromosomal du- plications in clinical abnormalities is unknown. These fac- tors hinder efforts to explain the pathogenesis of 20q13.33 deletions and the probable molecular mechanisms involved in the phenotypic presentation of these individuals.

In this report, we describe an individual carrying a ring chromosome 20 with duplication and deletion in 20q13.33. The integration of cytogenetic, clinical, and protein-protein interactions data enabled us to identify genes that help to explain how the patient's phenotype is affected by the 20q13.33 deletion present on the ring chromosome.

2. Case Presentation

The proband is female, aged 2 years and 8 months, and is the only daughter of nonconsanguineous parents. The 35-year- old father and the 29-year-old mother reported gestation with high blood pressure and cesarean section with a ges- tational age of 41 weeks. The girl was born with a weight of 2860 g, length of 45 cm, and cephalic perimeter of 33 cm. The first convulsion of the child was manifested at 15 days of life as a generalized epileptic crisis, which was repeated 24 hourslater. At 21 days, new seizures were characterized as focal seizures with secondary generalization and treatment with phenobarbital. The first neuropediatric assessment occurred at 4 months, showing delayed neuropsychomotor devel- opment, mild hypotonia, low weight gain, and cephalic perimeter of 36 cm ($<$ 3 Z scores). The electroencephalo- gram, cranial resonance, and screening tests for inborn errors of metabolism were unchanged. The child was re- ferred for genetic evaluation. At the age of examination, the patient showed some facial dysmorphic features as enlarged nasal dorsum, bulbous nasal tip, short *columella*, and long nasolabial filter. The treatment with phenobarbital was ef- ficient with control of the epileptic seizures for approxi- mately 1 year, when it presented decompensation of the convulsive conditions, making it necessary to change the anticonvulsant to oxcarbazepine and levetiracetam. The child started walking independently at 26 months, and at 2 years and 6 months of age, the child presented delayed motor development and language and high cognitive deficit.

Karyotyping from the proband was performed on metaphase spreads prepared from peripheral blood samples.The chromosomal analysis was conducted after GTG banding at 550-band resolution, and at least 100 cells were analysed (Figure 1A). The karyotype showed results 46,XX,r(20). The parental karyotype was normal. At least 100 cells from each individual were analyzed. The DNA sample from the child was investigated using chromosomal microarray analysis (CMA) with a 60-mer oligonucleotide- based microarray with a theoretical resolution of 40 kb(8 × 60 K, Agilent Technologies Inc., Santa Clara, CA, USA).

The images were analyzed using Cytogenomics v 2.0 and 2.7with the statistical algorithm ADM-2 and a sensitivity threshold of 6.0 (Figure 1B). It is recommended to provide confirmation of CMA results with other methods such as FISH or real-time PCR, especially for the refinement of breakpoints of structural chromosomal abnormalities. The authors pursued to perform it; but unfortunately, there is no sample left of this patient anymore, and the family is not available to obtain a new sample. The protein-protein in- teraction (PPI) metasearch engine STRING 10.0 (http:// string-db.org/) was used to create PPI networks based on40 genes and gene predictions located in the deleted region from our patient (Figure 2A). The list of genes was obtained from CMA analyses and subsequent research into the hu- man assembly of February 2009 (GRCh37/hg19) [20, 21]. The parameters used in STRING were (i) degree of confi- dence, 0.400, with 1.0 being the highest level of confidence; (ii) 500 proteins in the 1st and 2nd shell; and (iii) all pre- diction methods enabled, except for textmining and gene fusion. The final PPI network obtained through STRING was analyzed using Cytoscape 3.5 [22]. The MCODE tool was used to identify densely connected regions in the final Cytoscape network. The PPI modules generated by MCODE were further studied by focusing on major biology-associ- ated processes using the Biological Network Gene Ontology (BiNGO) 3.0.3 Cytoscape plugin [23]. The degree of func- tional enrichment for a given cluster and category was quantitatively assessed (p value) using a hypergeometric distribution. Multiple test correction was also implemented by applying the false discovery rate (FDR) algorithm [24] at a significance level of $p <$ 0.05. Finally, two major parameters of network centralities (node degree and betweenness) were used to identify hub-bottleneck (H-B) nodes from the PPI network using the Cytoscape plugin CentiScaPe 3.2.1 [25].

3. Discussion

The underlying biological mechanism in individuals with ring 20 has not been determined. Hypotheses include (i) gene silencing by the influence of telomere position; (ii) uniparental disomy of chromosome 20; (iii) deleted genes inthe chromosomal segment, or (iv) effect of ring instability incellular functions [26]. In this study, we investigated ge- notype-phenotype correlations through deleted genes in 20q13.33 using systems biology approaches to explain the associated clinical spectrum in our patient. With this ap- proach, we identified candidate genes that may be involved in the pathophysiology of ring chromosome 20. To measure the importance of the protein-protein interaction network of genes located in the deleted region (Figure 1B), we examined the topological properties of the network using centrality analyses.

The proteins ARFGAP1, HELZ2, and EEF1A2 presented a high node degree in the network. These nodes are con- sidered hubs and have central functions in a biological network [25]. ARFGAP1 serves as a regulator of vesicular trafficking of proteins [27]. HELZ2 is a helicase that acts as a transcriptional coactivator [28]. EEF1A2 promotes the GTP- dependent binding of aminoacyl-tRNA during protein
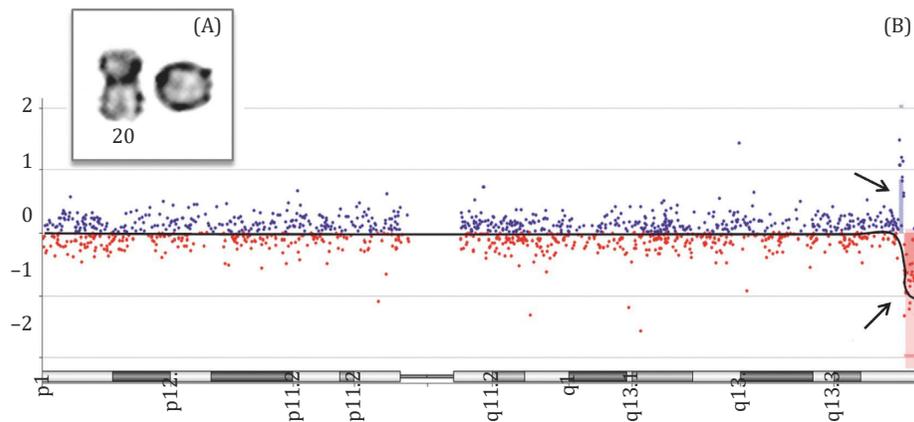
Figure 1: (A) Chromosome banding showing the chromosome 20 pair, with a normal chromosome and the other on the ring (20). (B) CMA profile of chromosome 20 showing the hybridization pattern. The genomic segment with a gain of ~302,774 kb (61,142,577–61,445,350) isshown (vertical blue band). The arrow indicates the shift of the median ratio log^2 to +1.0. The segment with loss of ~1.4 Mb (61,472,348–62,872,839) is demonstrated (vertical red band). The arrow indicates the shift of the median ratio log^2 to − 1.0.
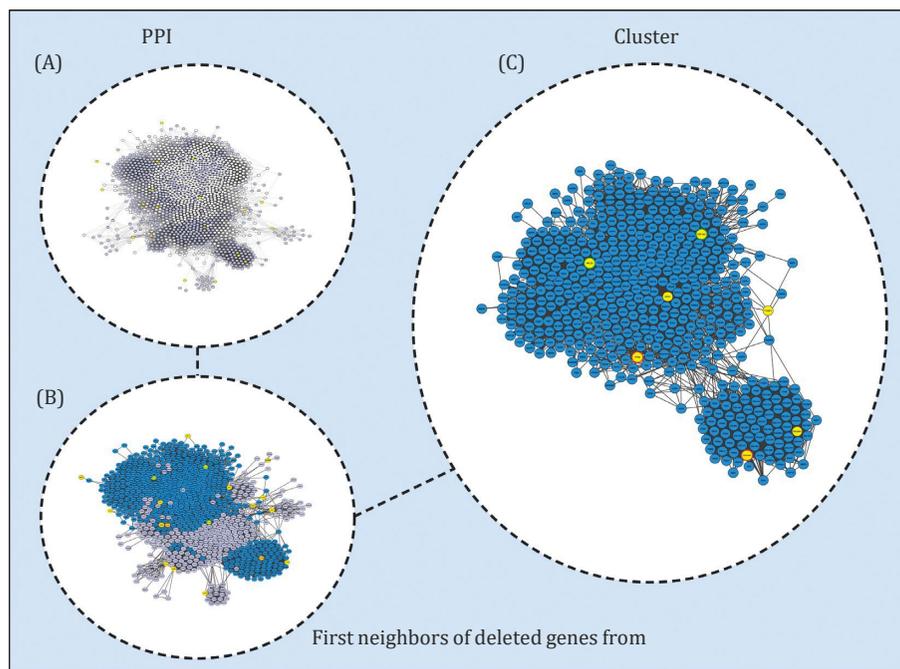
Figure 2: PPI network. A list of 40 genes and gene predictions was obtained from the human assembly of February 2009 (GRCh37/hg19) (Table S12). Interaction data from STRING were used to construct networks using cytoscape software. (A) The primary network is composed of 1,572 nodes (proteins) and 54,925 edges (interactions). (B) The secondary network is composed of 794 nodes and 16,805 edges; yellow nodes are candidate proteins with data available in string (ZGPAT, HELZ2, EEF1A2, TNFRSF6B, ZBTB46, DIDO1, ARFGAP1, RTEL1, SRMS, GMEB2, YTHDF1, PTK6, GID8, BIRC7, COL9A3, COL20A1, KCNQ2, ARFRP1, and CHRNA4). (C) Cluster 2, with C_i ◆0,863, is composed of 510 nodes and 10,388 edges. Nodes with green borders are considered hubs; orange are bottlenecks; and red arehub bottlenecks.

biosynthesis and plays a role in the regulation of actin function and cytoskeletal structure. *EEF1A2* knockout mice showed degeneration of neurons in the spinal cord and brain stem [29], and heterozygous mutations in the gene were associated with intellectual disability, developmental delay, autistic behaviors, and epilepsy [30, 31]. Therefore, the EEF1A2 protein may be a good candidate to explain some of the symptoms present in individuals with deletion 20q13.33.

CHRNA4, ARFRP1, GID8, and SRMS were the nodes identified as bottlenecks in the network. ARFRP1 plays a role in membrane trafficking between the trans-Golgi network and endosomes. GID8 is a nuclear retention factor for β-catenin during Wnt signaling, and SRMS nonreceptor- type tyrosine kinases are a BRK family of kinases (BFKs) involved in the proliferation or differentiation of kerati- nocytes [32–34]. *CHRNA4* encodes alpha-4 nicotinic
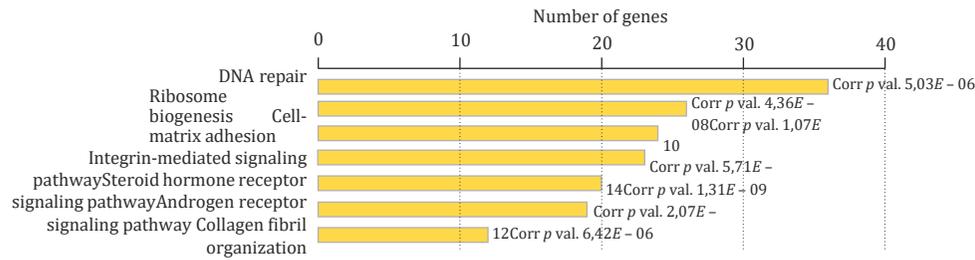
Figure 3: Summary of the bioprocess enrichment identified in cluster 2. The colored horizontal bars show GO terms frequently present.

acetylcholine receptor subunits, and different mutations in the gene cause autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [35, 36]. *CHRNA4* is a known gene associated with epilepsy in ring 20 patients [26]. Bottlenecks are related to the control of information between the in- teractions in the network [25, 37]; therefore, the identifi- cation of CHRNA4 already linked to the syndrome phenotype indicates that the haploinsufficiency of these bottlenecks could play a role in the development of our patient's phenotype.

Hub-bottlenecks (H-B) are nodes with a high degree and betweenness score. Among the nodes classified as H-B are two proteins, PTK6, another member of BFK, interacts directly with SRMS in the network. PTK6 functions as an intracellular signal transducer in epithelial tissues, con- tributing to the migration, adhesion, and progression of the cell cycle [38, 39]. COL9A3 is a structural component of hyaline cartilage, and mutations in the gene are associated with multiple epiphyseal dysplasia [40, 41].

Genes associated with the same illness have been ob- served to interact with each other more frequently than predicted by chance [42]. Therefore, we performed cluster analysis to examine densely connected regions in the final network and observe these novel candidate genes localized in 20q13.33 [43]. We analyzed a total of 11 clusters (data not shown). Interesting relationships were found; for example, the proteins HELZ2, EEF1A2, DIDO1, YTHDF1, PTK6, COL9A3, and COL20A1 interact with one another in cluster 2 (Figure 2C). Many of these genes are deleted in nonmosaic ring chromosome 20, and the clinical abnormalities iden- tified in these individuals include findings also seen in our patient as seizures, intellectual disability, and developmental delay [8]. Ring chromosome 20 is associated with refractory epilepsy, behavioral problems, and mild-to-severe cognitive impairment. *De novo* microdeletion of 20q13.33 is associ- ated with intellectual disabilities, developmental delay, speech and language delay, seizure, and behavioral problems such as autistic spectrum disorder. However, there is no pattern of abnormalities that would arouse clinical suspicion of a r(20) or *de novo* 20q13.33 microdeletion [19].

Functional enrichment analysis in the clusters revealed that the candidate genes were enriched in several biological processes, including microtubule-based movement, nucle- osome assembly, DNA repair, and cholinergic synaptic transmission (Tables S1–S11). Defects in these bioprocesses are associated with the development of neurological diseases, intellectual disability, neuropathies, and seizures [44–48]. In addition, such bioprocesses as cell-matrix adhesion and

integrin-mediated signaling were identified (Figure 3). These pathways are involved in neural stem cell differentiation, neuronal migration, neuroplasticity, maturation, and function of synapses in the peripheral and central nervous system and may have an important contribution to the emergence of intellectual disability and seizures in humans [49–51]. The biological processes involving candidate genes denote the heterogeneity of pathways disrupted by 20q13.33 deletions.

The ring chromosome associated with subtelomeric deletions and duplications can confound and limit geno-type-phenotype correlations. In fact, the circular structure of a ring chromosome, as described in this report, can change the ordinary 3D conformation of the chromatin in various ways and thus alter the expression of the genes present in the ring chromosome. The presence of an amplification iden- tified in our ring chromosome analysis hinders efforts to determine its impact on the patient's phenotype. The du- plication can be seen as a consequence of the mechanism of ring formation; in this case, an inverted duplication may be stabilized not only through telomere healing and telomere capture but also through circularization in the chromosomal ring [12]. Recently, chromothripsis and chromoanasynthesis have been proposed as two independent mechanisms that could explain the combination of deletions and duplications on the same chromosome. Indeed, various molecular ap- proaches, including whole genome sequencing, have shown that the concomitance of amplification, deletion, and ring chromosomes can be the result of a more complex rear-rangement with respect to a ring chromosome with only the loss of extremities [52, 53]. In this case, it is expected that the final phenotype of the proband is not only the result of the abnormal dosage of deleted genes but also of the altered expression of duplicate genes present in two copies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Table 1: list of GO terms identified by BiNGO in Cluster 1. Table 2: list of GO terms identified by BiNGO in Cluster 2. Table 3: list of GO terms identified by BiNGO in Cluster 3. Table 4: list of GO terms identified by BiNGO in Cluster 4. Table 5: list of GO terms identified by BiNGO in Cluster 5. Table 6: list of GO terms identified by BiNGO in Cluster 6. Table 7: list of GO terms identified by BiNGO in Cluster 7.

Table 8: list of GO terms identified by BiNGO in Cluster 8. Table 9: list of GO terms identified by BiNGO in Cluster 9. Table 10: list of GO terms identified by BiNGO in Cluster 10. Table 11: list of GO terms identified by BiNGO in Cluster 11. Table 12: list of genes located in the 0.9 Mb smallest overlap region encompassing band 20q13.33. (*Supplementary Materials*)

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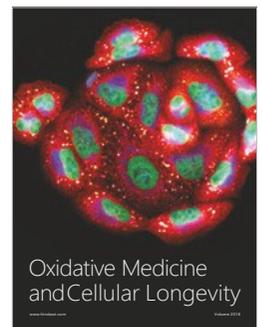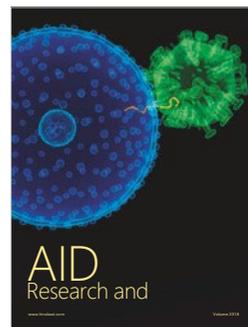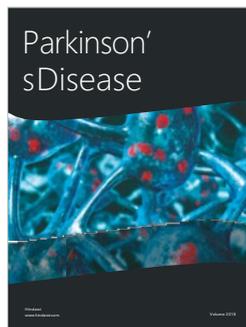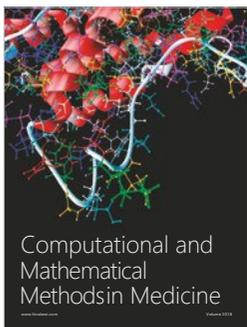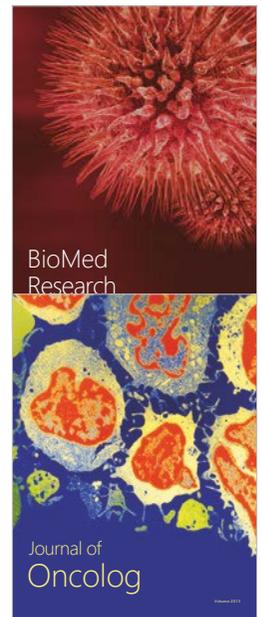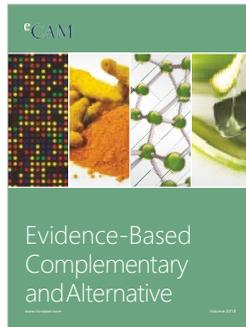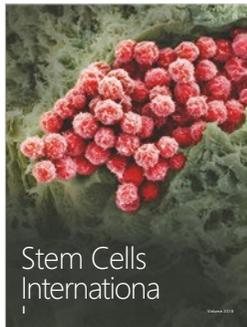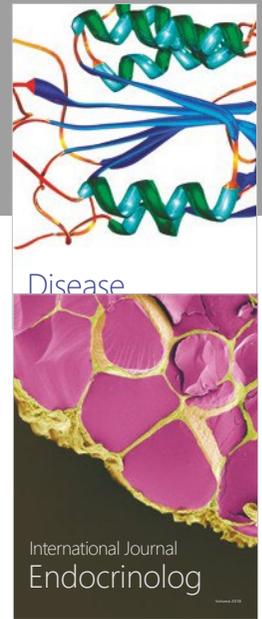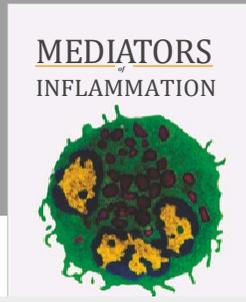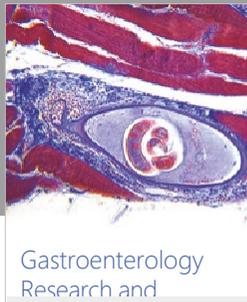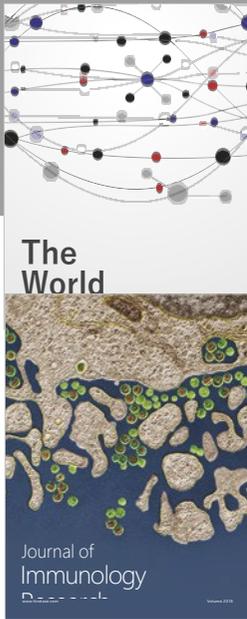
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Candidate Genes Associated With Neurological Findings in a Patient With Trisomy 4p16.3 and Monosomy 5p15.2

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In this report, we present a patient with brain alterations and dysmorphic features associated with chromosome duplication seen in 4p16.3 region and chromosomal deletion in a critical region responsible for Cri-du-chat syndrome (CdCS). Chromosomal microarray analysis (CMA) revealed a 41.1 Mb duplication encompassing the band region 4p16.3–p13, and a 14.7 Mb deletion located between the bands 5p15.33 and p15.1. The patient's clinical findings overlap with previously reported cases of chromosome 4p duplication syndrome and CdCS. The patient's symptoms are notably similar to those of CdCS patients as she presented with a weak, high-pitched voice and showed a similar pathogenicity observed in the brain MRI. These contiguous gene syndromes present with distinct clinical manifestations. However, the phenotypic and cytogenetic variability in affected individuals, such as the low frequency and the large genomic regions that can be altered, make it challenging to identify candidate genes that contribute to the pathogenesis of these syndromes. Therefore, systems biology and CMA techniques were used to investigate the extent of chromosome rearrangement on critical regions in our patient's phenotype. We identified the candidate genes *PPARGC1A*, *CTBP1*, *TRIO*, *TERT*, and *CCT5* that are associated with the neuropsychomotor delay, microcephaly, and neurological alterations found in our patient. Through investigating pathways that associate with essential nodes in the protein interaction network, we discovered proteins involved in cellular differentiation and proliferation, as well as proteins involved in the formation and disposition of the cytoskeleton. The combination of our cytogenomic and bioinformatic analysis provided these possible explanations for the unique clinical phenotype, which has not yet been described in scientific literature.

Keywords: Cri-du-chat, 4p16.3, *PPARGC1A*, *CTBP1*, *TRIO*, *TERT*, *CCT5*

BACKGROUND

Cri-du-chat syndrome (CdCS; OMIM #123450) is a genetic condition caused by a deletion in the short arm of chromosome 5. The phenotype is characterized by a cat-like cry, microcephaly, facial dysmorphism, psychomotor delays, and intellectual disability (Nguyen et al., 2015). Deletions, which occur at the end of the chromosome, as well as interstitial which result

after two breaks, compose 80–90% of CdCS cases (Cerruti Mainardi, 2006). Unbalanced parental translocation occurs in approximately 10–15% of patients (Perfumo et al., 2000; Cerruti Mainardi, 2006). In addition, complex rearrangements, such as mosaicism, *de novo* translocation, or ring chromosomes, account for less than 10% of the cases (Perfumo et al., 2000). Wolf-Hirschhorn syndrome (WHS; OMIM #194190) is a contiguous gene deletion syndrome on the short arm of chromosome 4. It is characterized by facial dysmorphia, growth retardation, intellectual incapacity, and seizures (Zollino et al., 2008). However, duplication of the WHS critical region is a rare chromosomal condition causing mild clinical phenotypes, such as speech delay, facial dysmorphia, seizures, and delayed neuro and psychomotor development (Patel et al., 1995; Hannes et al., 2010; Carmany and Bawle, 2011; Cyr et al., 2011). However, the phenotypic and cytogenetic variability in affected individuals, such as the low frequency and the large genomic regions that can be altered, make it challenging to identify the candidate genes that contribute to the pathogenesis of these syndromes.

Here, we present an individual with duplication in the 4p16.3 region and deletion in the 5p15.2 region. The altered chromosomal segments are located in the critical regions of WHS and CdCS, respectively. This study reports a case never highlighted before in the literature. Systems biology and CMA were used to investigate the impact of chromosome rearrangement on critical regions in our patient's phenotype.

CASE PRESENTATION

A 5-day-old female was referred for investigation of congenital abnormalities such as imperforate anus and rectovaginal fistula, as well as atrial septal defect. Family history is noteworthy as it highlights consanguineous parents, and a brother who died with similar clinical presentation of imperforate anus, congenital heart defect, and clubfeet (**Figure 1A**). The pregnancy of the patient was uneventful, and the girl was born at home at the gestational age of 36 weeks, weighing 2,160 g, and a total length of 39 cm. On her first physical examination in our center, she had a low weight (2,045 g), down slanting palpebral fissures, short palpebral fissures, ptosis, widely spaced eyes, thin upper lip, clubfeet, overlapping fingers, micrognathia, and a high-pitched cry. Neurological examination was extraordinary as there was hypertonia of extremities and an absence of the Moro reflex. At the age of 1 month, the patient suffered seizure episodes with eye deviation that were controlled with phenobarbital drugs. In the electroencephalogram, acute wave discharges with multifocal distribution were observed in both hemispheres with predominance over the left temporal region. The brainstem illustrated that there was auditory potential; however, the scan showed abnormalities within the visual region. A brain MRI performed at the age of 5 months showed a thin corpus callosum, white matter volume loss, pontine hypoplasia, and dysgenesis of the cerebellar vermis (**Figures 1B,C**).

Despite this, myelination was in accordance with her age. After being subjected to surgical procedures which had no complications, she was discharged at the age of 5 months and 25 days. Although the patient had a tracheostomy and a nasoenteral tube, she was, clinically, in a stable condition.

Karyotyping identified typical patterns of GTG bands in the mother (46,XX), and paternal reciprocal translocation with breakpoints in 4p16.3 and 5p15.2 regions [46,XY,t(4;5) (p16.3;p15.2)]. The proband was identified with 4p16.3–p13 trisomy and 5p15.33–p15.2 monosomy [46,XX,der(5) t(4,5) (p16.3;p15.2)pat]. Fluorescence *in-situ* hybridization (FISH) analysis confirmed three fluorescence signals for the 4p16.3 band, and only one fluorescence signal in the 5p15.2 proband. CMA revealed duplication in chromosome 4 (41.1 Mb) encompassing the bands 4p16.3–p13. The approximate genomic position was defined in chr4:71552–41263831 (GRCh38/hg38), comprising 198 genes (**Figure 2A**). Chromosome 5 was outlined with a deletion of 14.7 Mb located between the bands 5p15.33 and p15.1. The genomic position was estimated in chr5:269963–15032936 (GRCh38/hg38), comprising 50 genes (**Figure 2B**).
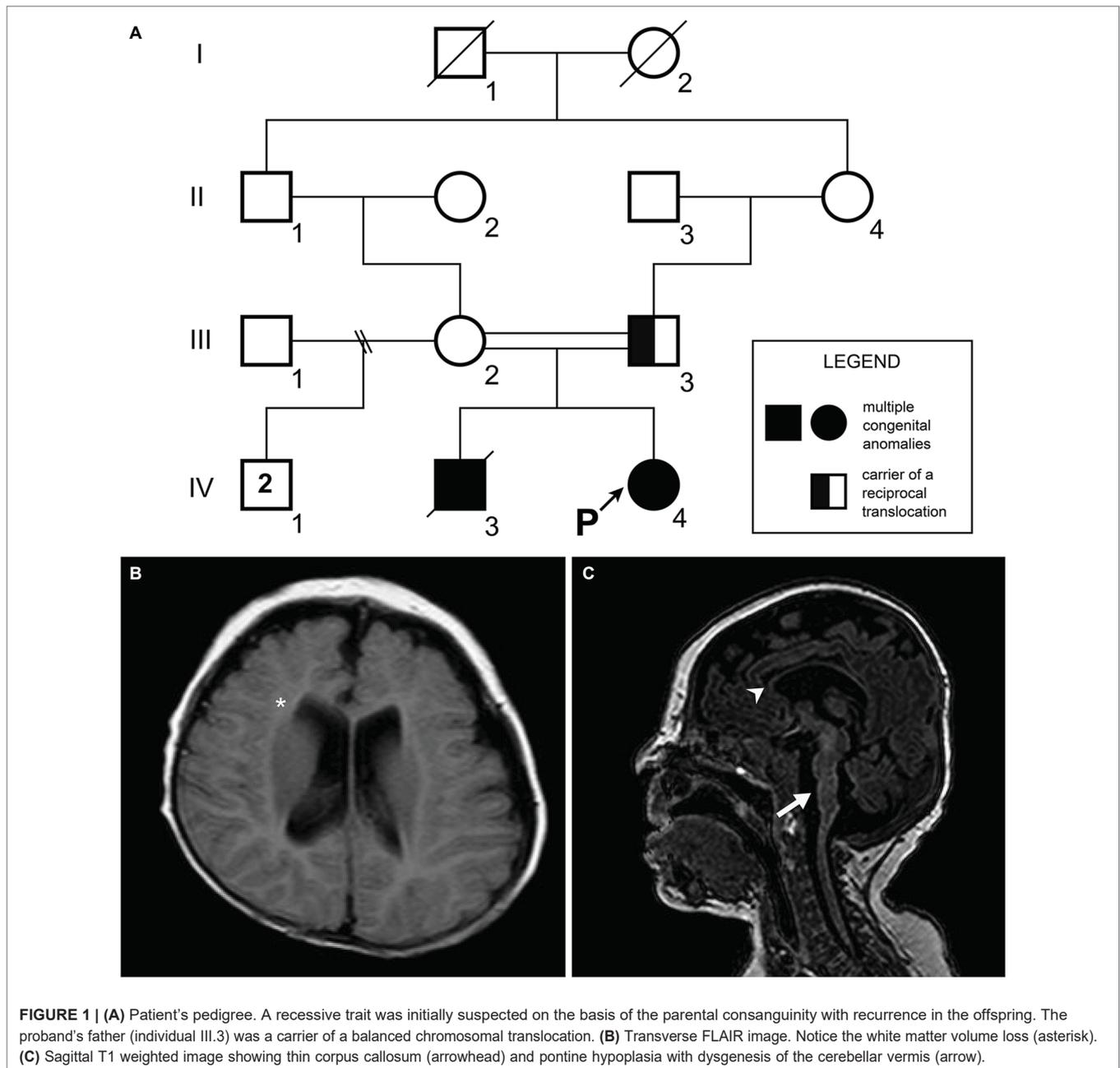
LABORATORY INVESTIGATIONS

Cytogenetic Studies

Karyotyping was performed on metaphase spreads prepared from peripheral blood samples. The chromosomal analysis was conducted through GTG banding at a 550-band resolution, and at least 100 cells were analyzed. FISH experiments were performed following standard techniques with commercially available locus-specific probes such as a dual-color commercial probe for the CdCS and WHSCR (Cytocell, UK). The *CTNND2* probe for 5p15.2 (red spectrum) contains a sequence homologous to the D5S2883 locus and covers approximately 159 kb of this locus. The probe for the 4p16.3 (red spectrum) contained a sequence that was homologous to the D4S166 locus and covered approximately 223 kb of this locus. At least 30 cells were analyzed per hybridization. The sample was mapped using CMA, using a 60-mer oligonucleotide-based microarray with a theoretical resolution of 40 kb (8 × 60 K, Agilent Technologies Inc., Santa Clara, CA, USA). The arrays were analyzed using a microarray scanner (G2600D) and feature extraction software (version 9.5.1, Agilent Technologies). The images were analyzed using Cytogenomics v2.0 and v2.7 with the statistical algorithm ADM-2 and a sensitivity threshold of 6.0.

Network Design

The protein-protein interaction (PPI) metasearch engine STRING 11.0 (http://string-db.org/) was used to create PPI networks based on deleted or duplicated genes located in the altered chromosomal regions. CMA, with a subsequent search in the UCSC genome browser of the human genome assembly (December 2013), retrieved 591 genes and predicted genes

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FIGURE 1 | (A) Patient's pedigree. A recessive trait was initially suspected on the basis of the parental consanguinity with recurrence in the offspring. The proband's father (individual III.3) was a carrier of a balanced chromosomal translocation. **(B)** Transverse FLAIR image. Notice the white matter volume loss (asterisk). **(C)** Sagittal T1 weighted image showing thin corpus callosum (arrowhead) and pontine hypoplasia with dysgenesis of the cerebellar vermis (arrow).

belonging to the duplicate area, as well as 246 from the deleted region (Kent et al., 1976; von Mering et al., 2005). The parameters used in STRING were: (i) degree of confidence, 0.400; (ii) 500 proteins in the first and second shell; and (iii) methods used were neighborhood, experiments, databases, and co-occurrence. The final PPI network was obtained through STRING and analyzed using Cytoscape 3.7.0 (Shannon et al., 2003).
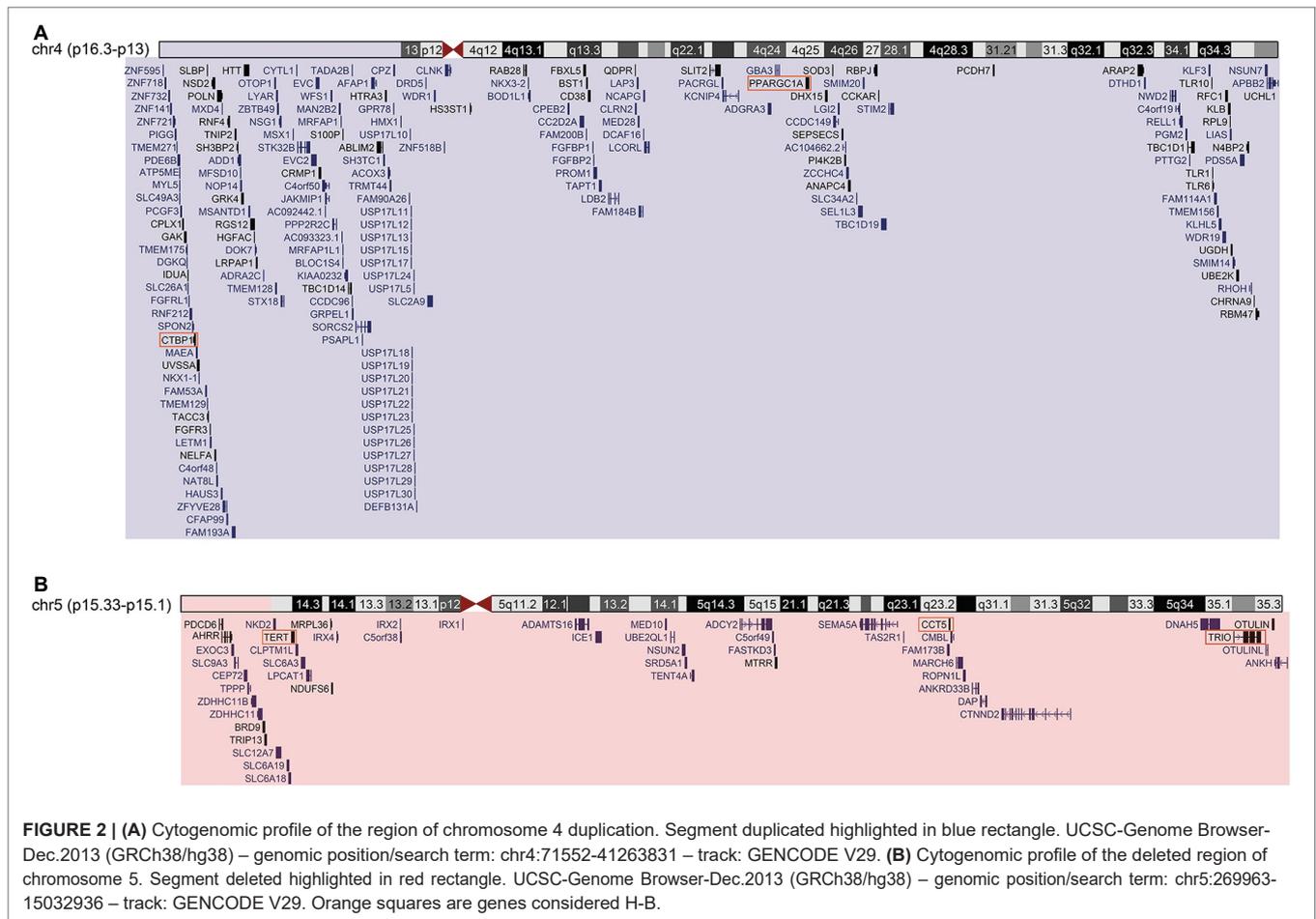
GO and Centralities Analysis

The Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome libraries were searched

using the ClueGO Cytoscape plugin (Bindea et al., 2009). Significant GO predictions were selected based on a $p \leq 0.05$, with the Bonferroni family-wise false discovery rate (FDR) test. Node degree and betweenness centralities were measured to identify hub-bottleneck (H-B) nodes from the PPI network using the Cytoscape plugin and CentiScaPe 3.2.1 (Scardoni et al., 2009).

Molecular Pathway Reconstruction

The PathLinker Cytoscape plugin was used to identify and reconstruct possible signaling pathways of interest from our PPI network (Murali et al., 2017). PathLinker computes the

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FIGURE 2 | (A) Cytogenomic profile of the region of chromosome 4 duplication. Segment duplicated highlighted in blue rectangle. UCSC-Genome Browser-Dec.2013 (GRCh38/hg38) – genomic position/search term: chr4:71552-41263831 – track: GENCODE V29. **(B)** Cytogenomic profile of the deleted region of chromosome 5. Segment deleted highlighted in red rectangle. UCSC-Genome Browser-Dec.2013 (GRCh38/hg38) – genomic position/search term: chr5:269963-15032936 – track: GENCODE V29. Orange squares are genes considered H-B.

k shortest paths that connect any source to any target in the network, and subsequently generates a subnetwork. It also creates a table with a rank of the shortest paths (Murali et al., 2018). The deleted gene network in the Cri-du-chat region (CdCR-Net) was used as a background, and the H-B CCT5, TERT, and TRIO were used as a source and targets for paths calculations. The parameters used in PathLinker were: (i) k: 50 (number of paths the user seeks); (ii) edge penalty: 1; and (iii) edge weight: weight probabilities, whereby it considers the edge weights as multiplicative, which result in the k highest cost paths (Murali et al., 2017).

DISCUSSION

Here, we have presented a patient with brain alterations and dysmorphic features resulting from chromosomal deletion in the critical region related to CdCS and duplication in the critical region related to WHS. The patient's clinical findings overlap with previously reported cases of both 4p duplication syndrome and CdCS (**Table 1**). Overall, the patients presentation is notably similar to CdCS patients as she presented with a weak, high-pitched voice and also

showed similar pathogenicity observed in the brain MRI. Furthermore, the patient's anorectal malformations are also similar to what can be observed in certain cases of CdCS (Marcelis et al., 2011). Nevertheless, she presents with some features that are common to both conditions discussed, or those more frequently described in patients with abnormalities of the critical region of WHS.

To identify possible candidates that could help explain this scenario, a centrality analysis was carried out to identify H-B. These proteins represent nodes with high degree and betweenness scores, which are frequently related to the control of information flow between groups of proteins with central functions in a biological network (Hahn and Kern, 2005; Scardoni et al., 2009).

Two H-B were identified in the WHR-Net (**Supplementary Figure S1A**). The H-B PPARGC1A is a transcriptional coactivator of a subset of genes related to oxidative phosphorylation, which regulate glucose and lipid metabolism, mitochondrial biogenesis, and muscle fiber development (Terada et al., 2002; Tunstall et al., 2002; Puigserver and Spiegelman, 2003; Finck et al., 2006). As expected, and through the enrichment analysis, PPARGC1A was found to be associated with the regulation of progesterone synthesized in the biosynthetic pathway (**Supplementary Figure S1B**).

TABLE 1 | Comparison of the clinical manifestations of this patient, and previously reported patients with Cri-du-chat syndrome and Trisomy 4p syndrome.

Clinical manifestations	This patient	Cri-du-chat patients (Honjo, 2018; Mainardi, 2001)*	Trisomy 4p patients (Patel et al., 1995; Dallapiccola, 1977)*,**
Imperforate anus	Present	−	−
Preterm birth	Present	+	+
Micrognathia	Present	++	+
Low birth weight	Present	+	+
Psychomotor retardation	Present	++	++
Downslanting palpebral fissures	Present	++	++
Widely spaced eyes	Present	++	+
Abnormalities of the fingers	Present	++	++
Prominent heels	Present	−	++
Weak, high-pitched voice	Present	++	−
Growth deficiency	Present	++	++
Seizures	Present	+	+
Microcephaly	Present	++	++
Pontine hypoplasia	Present	++	−

*++, presence of the manifestation in 50% or more of the patients; +, presence of the manifestation in more than 10%, but less than 50% of the patients; −, not frequently reported. *Based on overall reported frequencies in patients with variable chromosomal breakpoints. **Most previously reported trisomy 4p patients also have other chromosomal imbalances and variable breakpoints.*
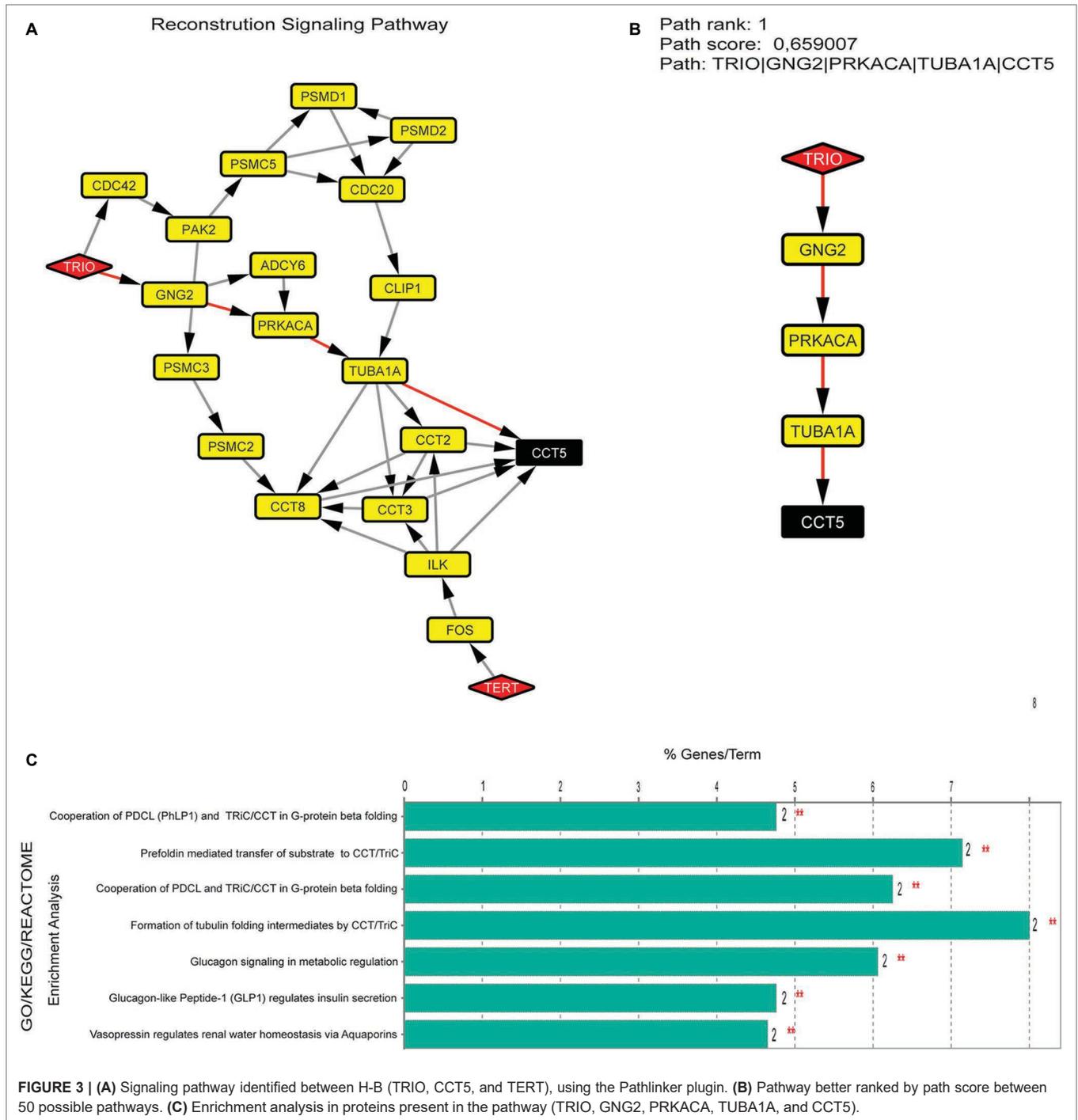
The deregulation of transcription and mitochondrial function caused by PPARGC1A is associated with conditions such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease (Weydt et al., 2006; Eschbach et al., 2013; Jesse et al., 2017). Additionally, the second H-B, CTBP1 plays a role in the regulation of gene expression during embryonic development, as well as participation in axial patterning and cellular proliferation and differentiation (Hildebrand and Soriano, 2002; Van Hateren et al., 2006). A *de novo* heterozygous missense mutation in the *CTBP1* (R331W) causes hypotonia, developmental delay, ataxia, and intellectual disability (Beck et al., 2016, 2019). As heterozygous null variants of *CTBP1* are commonly found in unaffected individuals, gain of function rather than loss of function mechanisms are more likely to be associated with these clinical findings (Beck et al., 2019). Moreover, *PPARGC1A* and *CTBP1* are duplicated in the 4p region in the patients with neuropsychomotor delay, intellectual disability, and speech delay (**Figure 2A**; Cotter et al., 2001; Paskulin et al., 2009; Carmany and Bawle, 2011). Consequently, topological analysis indicates that the increased dosage of the *PPARGC1A* and *CTBP1* genes may have contributed to the neuropsychomotor delay and neurological alterations found in our patient (**Table 1**).

TRIO, TERT, and CCT5 were identified as H-B in the CdCR-Net (**Supplementary Figure S2A**). TRIO has functions in cell migration and morphogenesis during cerebellum development, including neurite and axon outgrowth

(Briancon-Marjollet et al., 2008; Peng et al., 2010; Tao et al., 2019). *Trio* knockout causes reduction in the extension of granule neurons from the cerebellum and severe ataxia in mice (Peng et al., 2010). Furthermore, the *TRIO* haploinsufficiency in mice increases anxiety; impairs sociability and motor coordination, disrupts learning capacity and spatial memory, and decreases brain and neuron size (Zong et al., 2015; Katrancha et al., 2019). In this sense, the hemizygosity of *TRIO* may have contributed to the clinical findings in our patient at the age of 5 months, such as the thin corpus callosum, white matter volume loss, pontine hypoplasia, and dysgenesis of the cerebellar vermis (**Figures 1B,C**).

Moreover, damages in spatial memory are associated with TERT as its knockout in the hippocampus of adult mice impairs spatial memory processes during neural development (Zhou et al., 2017). The deficiency of *TERT* may also result in microvascular dysfunction in mice (Ait-Aissa et al., 2018). Furthermore, we found that TERT was associated with the negative regulation of apoptotic processes of endothelial cells in GO analysis (**Supplementary Figure S2B**). In addition, TERT shows interaction with CCT5 in the Y2H library screen (Wang et al., 2013). The H-B CCT5 is involved in cilia morphogenesis and survival of sensory neurons (Posokhova et al., 2011). Mutations in this gene may cause neurodegenerative diseases, such as spastic paraplegia and sensory neuropathy (Bouhouche et al., 2006; Pavel et al., 2016; Pereira et al., 2017). Additionally, *TERT* and *CCT5*, located in the critical region of CdCS, are associated with microcephaly and intellectual disability, reported in patients from several other studies (**Figure 2B**; Cerruti Mainardi, 2006). In this sense, deletion of *TERT* and *CCT5* genes could be involved with psychomotor retardation and microcephaly as presented in the present case (**Table 1**).

To investigate the importance of the H-B from CdCR-Net and their associated pathways (**Figure 3A**), we identified TRIO, GNG2, PRKACA, TUBA1A, and CCT5 as having the highest path score (**Figure 3B**). These proteins are involved in signaling mechanisms, including differentiation and proliferation, as well as roles in the formation and disposition of the cytoskeleton (Yajima et al., 2012; Tseng et al., 2017). In the latter case, TRIO, TUBA1A, and CCT5 play roles in the folding of actin and tubulin; reorganization; and assembly of the cytoskeleton during migration, growth, and differentiation of neurons (Seipel et al., 1999; Tian et al., 2010; Tracy et al., 2014). Genes that contribute to a common disorder tend to share core bioprocesses (**Figure 3C**; Goh et al., 2007). For instance, the chaperonin complex, CCT, which is also formed by the subunit CCT5, facilitates the formation of the heterodimeric form of the G-protein gamma subunits, similar to the GNG2 protein (Lukov et al., 2005). The formation of tubulin folding intermediates is also produced by CCT, in which unfolded actins and tubulins, such as TUBA1A are transferred to cytosolic chaperonin CCT (Frydman et al., 1992; McCormack et al., 2001). Interestingly, mutations or loss function of *TRIO*, *TUBA1A*, and *CCT5* is associated with intellectual

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FIGURE 3 | (A) Signaling pathway identified between H-B (TRIO, CCT5, and TERT), using the Pathlinker plugin. (B) Pathway better ranked by path score between 50 possible pathways. (C) Enrichment analysis in proteins present in the pathway (TRIO, GNG2, PRKACA, TUBA1A, and CCT5).

disability, defects in dendritic branching, synapse function, sensory neuropathy, and microcephaly in humans (Bouhouche et al., 2006; Morris-Rosendahl et al., 2008; Kumar et al., 2010; Ba et al., 2016; Pavel et al., 2016; Pengelly et al., 2016; Belvindrah et al., 2017).

Essential human genes are expected to encode central proteins, such as the H-B genes, and be expressed in different tissues (Goh et al., 2007; Loscalzo and Barabasi, 2011).

The haploinsufficiency of the H-B genes observed in our PPI-network could affect pathways related to the cilia morphogenesis, dendritic branching, and synapse function, including neurite and axon outgrowth, which consequently could have led to the neurodevelopment delay and microcephaly observed in our patient. In addition, the identification of CTBP1, PPARGC1A, CCT5, TERT, and TRIO with different approaches brought new insights on

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the pathogenesis involved in these rare chromosomal rearrangements, such as those presented here, in a case never reported before.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The study includes a statement on ethics approval and consent. The study was approved by the Ethics in Research Committee of Hospital de Clínicas de Porto Alegre (HCPA), under the reference number 10-560. Written informed consent form was obtained from the guardians of the participant for the publication of this paper.

AUTHOR CONTRIBUTIONS

TC, BF and MR conceived, designed the study and analyzed all the data. FP analyzed the clinical data. All authors contributed to the writing manuscript. MR revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00561/full#supplementary-material.

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Network-based analysis using chromosomal microdeletionsyndromes as a model

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Abstract

Microdeletion syndromes (MSs) are a heterogeneous group of genetic diseases that can virtually affect all functions and organs in humans. Although systems biology approaches integrating multiomics and database information into biological networks have expanded our knowledge of genetic disorders, cytogenomic network-based analysis has rarely been applied to study MSs. In this study, we analyzed data of 28 MSs, using network-based approaches, to investigate the associations between the critical chromosome regions and the respective underlying biological network systems. We identified MSs-associated proteins that were organized in a network of linked modules within the human interactome. Certain MSs formed highly interlinked self-contained disease modules. Furthermore, we observed disease modules involving proteins from other disease groups in the MSs interactome. Moreover, analysis of integrated data from 564 genes located in known chromosomal critical regions, including those contributing to topological parameters, shared pathways, and gene-disease associations, indicated that complex biological systems and cellular networks may underlie many genotype to phenotype associations in MSs. In conclusion, we used a network-based analysis to provide resources that may contribute to better understanding of the molecular pathways involved in MSs.

KEYWORDS
chromosomal critical regions, cytogenomics, interactome, microdeletion syndromes, network analysis

1 | INTRODUCTION

Biological functions in humans rely on diseases that can be viewed as local perturbations of the complex interactions between proteins, DNA, RNA, and small molecules within the cell, which impact associated pathobiological processes in cellular networks (Barabási, Gulbahce, & Loscalzo, 2011; Barabási & Oltvai, 2004). Most proteins interact with other proteins while performing their cellular functions, and are thus organized in biological networks. The relationships among multiple molecular processes are encoded in the interactome, a network that integrates all physical interactions within a cell, from protein-protein to regulatory protein-DNA and metabolic interactions (Menche et al., 2015).

Microdeletion syndromes (MSs) are disorders characterized by deletions of dosage-sensitive genes in a chromosomal segment with recognizable phenotypes (Harel & Lupski, 2018; Nevado et al., 2014; Schmickel, 1986). Clinical characteristics of MSs can encompass a wide variety of distinct or overlapping phenotypes such as multiple congenital anomalies, intellectual disabilities, developmental delay, and epilepsy (Stankiewicz & Beaudet, 2007). In the last decade, the "genotype-first" approach for assessing individuals with the same or overlapping chromosomal alterations has enabled delineation of each syndrome associated with a critical region containing breakpoints that encompass several genes (Mefford, 2009; Theisen & Shaffer, 2010). However, the individual effects exerted by most of these genes in the pathophysiology of these syndromes are unclear. Chromosomal imbalances in MSs may alter the

expression of multiple genes, leading to stoichiometric imbalances and perturbations in macromolecular complexes and their interactions in biological networks associated with dominant phenotypes (Barabási et al., 2011; Schadt, 2009; Theisen & Shaffer, 2010).

In network-sciences, biological networks comprise nodes representing units such as genes, proteins, or diseases, and edges representing interactions between the units such as physical interactions, transcription activation, or correlations of gene expression levels with diseases. For instance, cellular networks are not random but characterized by a power-law degree distribution, where most nodes have few edges but few nodes have many edges (Loscalzo, Barabási, & Silverman, 2017). The scale-free property represents an essential feature in molecular networks and allows greater robustness against the loss of random nodes, providing resilience against molecular disturbances (Barabási & Oltvai, 2004; Pavlopoulos et al., 2011). However, cellular networks are vulnerable to perturbations, such as infection and tumorigenesis, on any of the nodes with several connections (hubs) (Loscalzo et al., 2017). Besides, in network medicine, disease-related proteins are not randomly scattered in the networks but display significant functional clustering, creating specific regions or disease modules associated with diseases in the respective interactomes (Feldman, Rzhetsky, & Vitkup, 2008; Menche et al., 2015). According to the disease-module hypothesis, proteins associated with the same disease are usually found to interact with each other in a local neighborhood more frequently than expected by chance (Barabási et al., 2011; Goh et al., 2007; Oti, Snel, Huynen, & Brunner, 2006). The disease module facilitates an effective way of identifying new gene candidates as factors causing diseases to explore the associations of these genes with common characteristics, such as biological processes and disease phenotypes located in a local neighborhood of the human interactome (Loscalzo et al., 2017; Menche et al., 2015). Therefore, specific network perturbations, such as those in protein-protein interactions (PPI), can be identified to elucidate the etiologies of diseases (Loscalzo et al., 2017). PPI can be used to exploit the topologies of specific subregions in interactomes and analyze given spectra of biological processes to infer new disease-causing elements (Loscalzo et al., 2017; Tranchevent et al., 2011; Vidal, Cusick, & Barabási, 2011).

Nevertheless, per our knowledge, there are no reported studies employing network-based approaches to investigate the complex associations between chromosomal abnormalities and biological networks in multiple MSs. In this study, we obtained data of the most described MSs from the database of genomic variation and phenotype in humans using Ensembl resources (DECIPHER) (decipher.sanger.ac.uk/disorders#syndromes/karyotype), and applied a network-based approach to explore the MS-network (MSN) in the human interactome. We integrated the data of 28 syndromes and 564 genes located in chromosomal critical regions in the MSN, including topological parameters, clinical symptoms, pathways, and gene-disease associations. We observed that proteins in the MSN were usually located in specific regions of the interactome and emerged in the modules of specific syndromes. In addition to demonstrating the shared interactions and different phenotypes in MSs, we showed that topological parameters together with genetic metrics can contribute to predicting the impact of gene deletions.

2 | MATERIALS AND METHODS

2.1 | Curation of genes and phenotypes associated with MSs

The list of protein-coding genes located in the critical regions of chromosomes and the main phenotypes of the associated syndromes was obtained from the interactive web-based database DECIPHER (decipher.sanger.ac.uk/disorders#syndromes/overview). Disorders with susceptible locus or those that are not characterized as MS were not included. The list included 28 MSs and 811 protein-coding genes (Tables S1 and S2).

2.2 | Co-expression data

The GeneMANIA prediction server (Warde-Farley et al., 2010) was used to collect expression data and compare these with randomized networks having the same numbers of nodes. GeneMANIA Cytoscape plugin V.3.5.2 generated networks using automatically weighted evidence of co-expression. Parameters available in the plugin, such as related genes and top genes, were not used to create the networks.

2.3 | Extraction of haploinsufficiency, disease specificity index, and disease pleiotropy index data

We used the predictions of haploinsufficiency described by Huang, Lee, Marcotte, and Hurles (2010) for all 564 protein-coding genes. The file containing the haploinsufficiency scores is available at https://decipher.sanger.ac.uk/files/downloads/HI_Predictions_Version 3.bed.gz. Moreover, we used two metrics to extract gene-disease associations from MEDLINE using the BeFree system (Bravo, Piñero, Queralt-Rosinach, Rautschka, & Furlong, 2015). Disease specificity index (DSI) values represent the number of diseases associated with a particular gene, and disease pleiotropy index (DPI) of a gene indicate the number of different classes of diseases involving the gene (Piñero et al., 2017). DSI and DPI values of all 564 protein-coding genes were extracted from https://www.disgenet.org/downloads and are shown in Tables S3 and S4.

2.4 | MSN and expanded MSN network (eMSN)

To generate the MSN, we employed PPI data from a curated human interactome that encompassed 13,460 proteins and 141,296 interactions (Menche et al., 2015). The list of 811 protein-coding genes in critical regions of chromosomes was used to extract interactions among MSN-proteins. Through selecting first neighbors of MSN-proteins, to the MSN was expanded, generating the eMSN. Cytoscape V.3.7.0. software (Shannon et al., 2003) was used to analyze, visualize, and calculate the topological parameters of MSN and eMSN.

2.5 | Enrichment analysis

We accessed the major biological processes in the MSN using the Cytoscape plug-in Biological Network Gene Ontology (BiNGO) 3.0.4 (Maere, Heymans, & Kuiper, 2005). The hypergeometric distribution and false discovery rate (FDR) in different categories were quantitatively assessed (corr p-value) using an algorithm (Benjamini & Hochberg, 1995) with a significance level of $p < .05$. Our analysis excluded gene ontology terms that were not significantly associated with biological processes related to MSs or that were too general (e.g., regulation of biological process, signaling process, or receptor signaling protein activity).

2.6 | Prioritization of genotype-phenotype relationships

DisGeNET (http://www.disgenet.org/) is a comprehensive platform that integrates data on human gene-disease associations (Piñero et al., 2020). DisGeNET encompasses 30,170 diseases, including the Mendelian, complex, environmental diseases, and human clinical phenotypes, providing 1,134,942 gene-disease associations data from different repositories. The Cytoscape plug-in of DisGeNET version 7.0 (Piñero et al., 2017) was used to generate a gene-disease network with curated data from UniProt, PsyGeNET, Orphanet, CTD (human data), ClinGen, and the Genomics England with a score ≥ 0.4.

2.7 | Statistical analysis

The largest connected component size, betweenness, closeness, and average neighborhood connectivity (co-expression data) in MSN were used to calculate the Z-scores, according to Equation (1):

$$Z-\text{score} = \frac{V - \text{mean}ðV \text{ Random}Þ}{\sigma ðV \text{ Random}Þ}; \quad ð1Þ$$

The equation indicates the mean value and standard deviation of the random expectation. Spearman's correlation coefficient was used to measure the statistical relationship between genetic characteristics (HI; DSI; DPI) and centrality parameters.

3 | RESULTS

3.1 | The MS human interactome

The human interactome curated by Menche et al. (2015) (Figure 1(a)) provides high-quality PPI regulatory data. In this study, we identified 28 MSs in DECIPHER. Among the 811 protein-coding genes located in critical regions of chromosomes, 564 encoded proteins were detected in the interactome. The extraction of these proteins resulted in an MSN with 564 nodes/proteins and 479 edges/interactions (Figure 1(b)).

Topological features of the human interactome, random network, and MSN were compared. Node degree is defined by the number of connections among proteins in the networks. Topological analysis revealed an average degree of 21 in the human interactome, whereas that in the MSN was only 1.7. Both networks follow a power-law degree distribution, in which most proteins have few connections and few have many connections, as expected in biological networks (Barabási et al., 2011). Clustering coefficient (cl) indicates the tendency of a graph to be divided into clusters, allowing the identification of extensively connected regions (Pavlopoulos et al., 2011). The average clustering coefficient (cl) in the human interactome was 0.17, which was larger than that of the MSN (cl: 0.04). The diameter, which is the shortest distance between the two most distant proteins in a network, was 13 for both the human interactome and the MSN. The random network showed lower average for degree (k: 1.2), larger diameter (dmax: 17), and more fragmentation (372 isolated nodes) compared with the MSN (303 isolated nodes) (Figure 1(c)). These results indicate that the MSN-proteins may be located in the same network neighborhood in the human interactome, with some proximity.

3.2 | Modules

The use of topological and expression data may be useful for predicting proteins located in critical regions of chromosomes that may be involved in the pathogenesis of MS and tend to compose a disease module in the interactome (Loscalzo et al., 2017). Therefore, we analyzed the largest connected component and co-expression data of the 28 syndromes in the MSN to identify possible disease modules. In our analysis, we included all the 564 MS-associated proteins in the human interactome. Only 2q33.1 deletion, 16.p11.2-p12.2 microdeletion, 1p36 microdeletion, ATR-16, and Sotos syndromes exhibited enough interactions to allow further analysis. Each syndrome was compared with the same set of randomly extracted nodes from the interactome. z-scores >1.6 (p-value <.05) were considered significant.

The largest connected component in the MSN (214) was larger than in the random expectation (134), with a z-score of 31.4. Similarly, MSN-proteins had significantly higher expression (z-score: 3.2) than those in the random expectation. Besides, the largest connected component and co-expression data of these syndromes exhibited z-scores >1.6 (Figure 2(a)), indicating that the interactions among these proteins could not have emerged by chance and were associated with specific modules in the human interactome.

3.3 | Shared interactions and phenotypes

We investigated the overlap of interactions among MSN-proteins in the interactome (Figure 2(b)) and identified 265 interactions that were
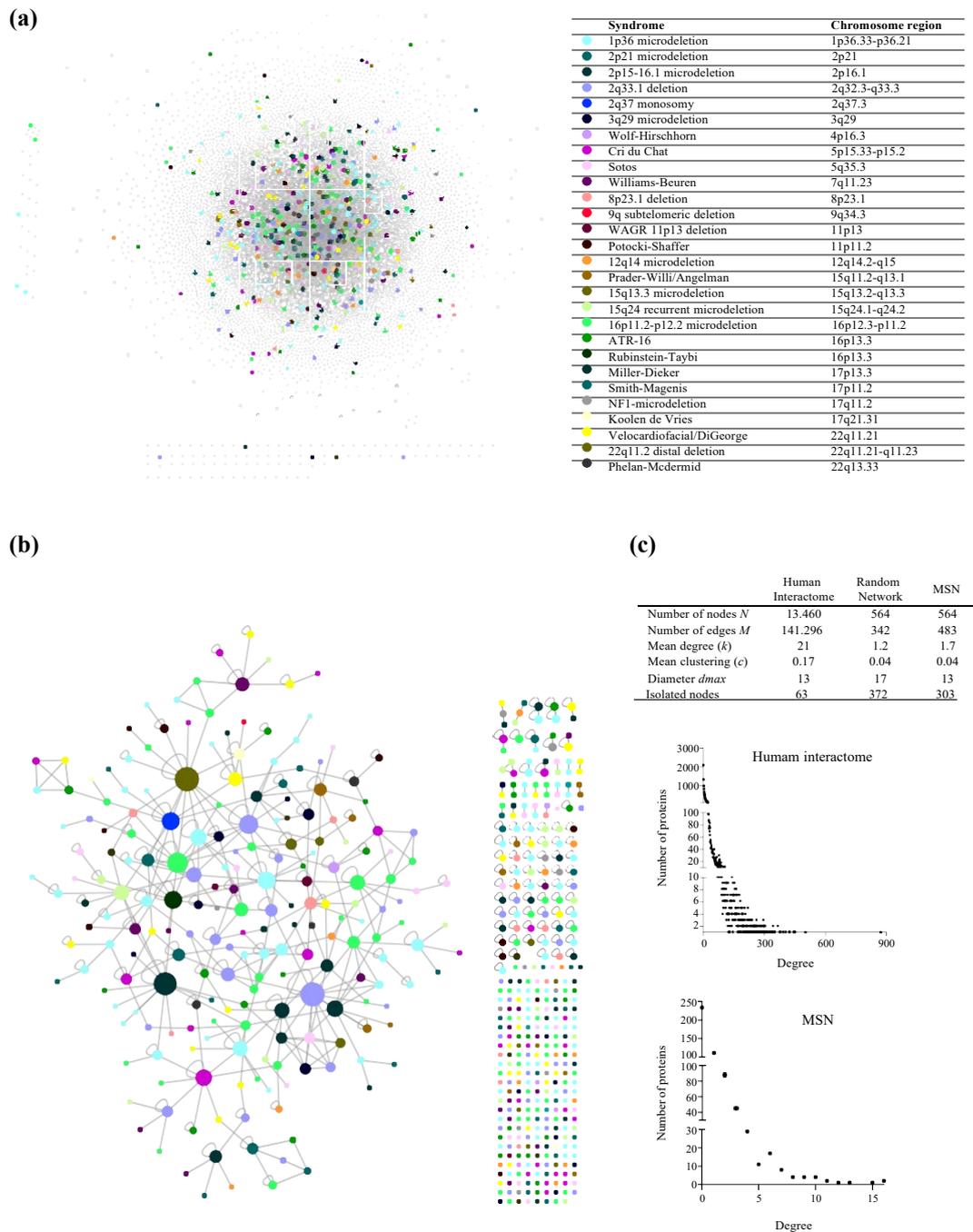
(a)

Syndrome	Chromosome region
1p36 microdeletion	1p36.33-p36.21
2p21 microdeletion	2p21
2p15-16.1 microdeletion	2p16.1
2q33.1 deletion	2q32.3-q33.3
2q37 monosomy	2q37.3
3q29 microdeletion	3q29
Wolf-Hirschhorn	4p16.3
Cri du Chat	5p15.33-p15.2
Sotos	5q35.3
Williams-Beuren	7q11.23
8p23.1 deletion	8p23.1
9q subtelomeric deletion	9q34.3
WAGR 11p13 deletion	11p13
Potocki-Shaffer	11p11.2
12q14 microdeletion	12q14.2-q15
Prader-Willi/Angelman	15q11.2-q13.1
15q13.3 microdeletion	15q13.2-q13.3
15q24 recurrent microdeletion	15q24.1-q24.2
16p11.2-p12.2 microdeletion	16p12.3-p11.2
ATR-16	16p13.3
Rubinstein-Taybi	16p13.3
Miller-Dieker	17p13.3
Smith-Magenis	17p11.2
NF1-microdeletion	17q11.2
Koolen de Vries	17q21.31
Velocardiofacial/DiGeorge	22q11.21
22q11.2 distal deletion	22q11.21-q11.23
Phelan-Mcdermid	22q13.33

(b)

(c)

	Human Interactome	Random Network	MSN
Number of nodes N	13.460	564	564
Number of edges M	141.296	342	483
Mean degree (k)	21	1.2	1.7
Mean clustering (c)	0.17	0.04	0.04
Diameter dmax	13	17	13
Isolated nodes	63	372	303

FIGURE 1 A platform for analysis of protein-protein interactions. In the network, proteins are nodes connected by links or interactions. Node colors indicate the origin syndromes. (a) Curated human interactome, composed of 13,460 proteins and 141,296 interactions. (b) MS network (MSN) with 564 proteins and 479 interactions. Node size is related to the number of connections (degree). (c) Topological parameters with degree distribution for all nodes of the interactome and MSN

common to different syndromes. The results showed that 16p11.2-p12.2 microdeletion and 1p36 MSs had the highest numbers of shared interactions (4.5%). Furthermore, 16p11.2-p12.2 microdeletion and 2q33.1 deletion syndromes shared 4.1% of interactions. Notably, 1p36 microdeletion syndrome showed significant overlap with other syndromes, accounting for 24% of all shared interactions, which is expected because this syndrome has the largest number of associated proteins identified in the interactome. The overlap of interactions among 16p11.2-p12.2 microdeletion, 2q33.1 deletion, and 1p36 MSs reached 11% of all interactions in the network.

Next, we quantified the overlap among the main phenotypes of MS (Table S2). Intellectual disability, microcephaly, muscular
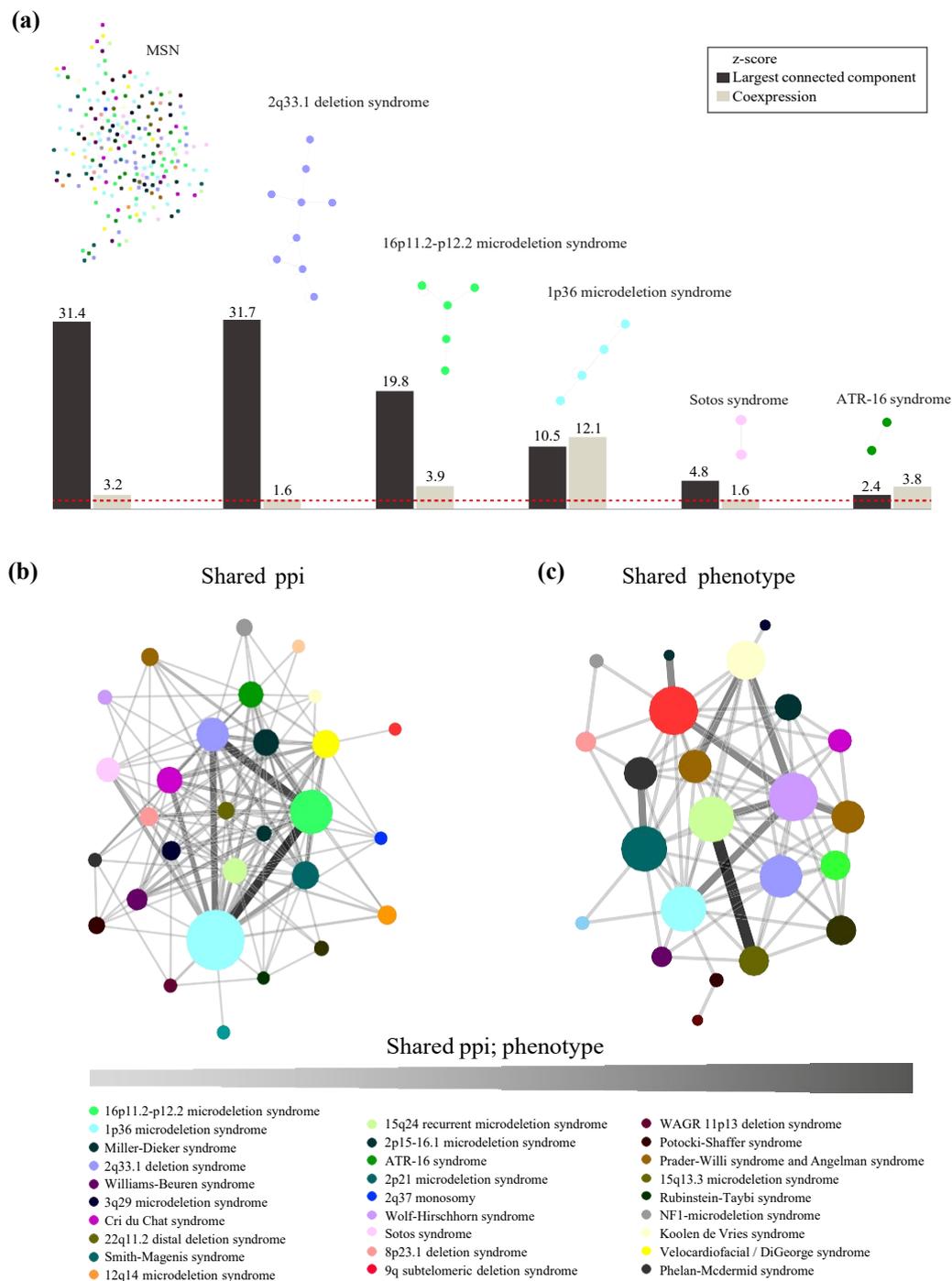
FIGURE 2 Characterization of subregions in the interactome (a) Largest connected component and co-expression data compared with the same random set of proteins. Dashed lines in red limit z-score > 1.6. (b) Protein-protein interactions (PPI) shared between MSs. The node size is proportional to the total number of interactions. The thickness of the edge represents the number of shared interactions between two specific syndromes. (c) Representation of the main shared phenotypes in MSs. Larger nodes share more phenotypes with other syndromes. The edge thickness is proportional to the number of common phenotypes expressed between two syndromes

hypotonia, feeding difficulties, and heart abnormalities were the most common clinical manifestations identified in the analysis of all syndromes in the MSN. Interestingly, 9q sub-telomeric deletion and Wolf-Hirschhorn syndromes are more connected in the network, sharing phenotypes with 13 other syndromes. Smith-Magenis, 15q24

recurrent microdeletion, and 1p36 MSs presented phenotypes that were common to 12 other MS (Figure 2(c)). The syndromes 15q24 recurrent microdeletion and 22q11.2 distal deletion shared four phenotypes (facial abnormality, intellectual disability, short stature, and small size for gestational age). Wolf-Hirschhorn syndrome is directly

connected with 15q24 recurrent microdeletion, 2p15-16.1 microdeletion, Cri du Chat, 9q subtelomeric deletion, and Angelman syndromes in the MSN; all of these syndromes share intellectual disability and microcephaly as common phenotypes.

3.4 | Relationship between MSN-proteins and topological parameters

To extract topological features of the 28 syndromes, we expanded the MSN by selecting nodes that were immediate neighbors of the proteins in the network and created an eMSN of 5.353 proteins connected via 97.133 interactions (Table S1). The average degree in the eMSN was 36, which was higher than that in the human interactome (21) and the MSN (1.7). Mean clustering in the eMSN (hci: 0.24) was higher than in the human interactome (hci: 0.17) and MSN (hci: 0.04). However, the diameter (dmax: 8) and the mean distance of all node pairs in the eMSN (hdi: 3) were smaller than those in the human interactome (dmax: 13; hdi: 3.6) and MSN (dmax: 13; hdi: 5.1).

Hubs are defined as topologically relevant nodes with significant functions in cellular networks (Vidal et al., 2011), and hub proteins have degrees >100. Although only 5.5% of MSN-proteins had degrees >100 in the eMSN, these proteins were connected directly with 15.6% of other MSN-proteins. The results of all centrality analyses of the 564 MSN-proteins are shown in Table S2. To investigate whether centrality parameters can differentiate MSN-associated proteins from other proteins in the human interactome, we compared the averages of betweenness and closeness of the former in random networks. The centrality parameter betweenness represents the number of times a node occurs in the shortest path between other nodes, which corresponds to the number of nonredundant shortest paths passing through a node of interest (Girvan & Newman, 2002; Yu, Kim, Sprecher, Trifonov, & Gerstein, 2007). A bottleneck is a node with above average betweenness that may indicate the ability of a protein to promote a bridge and control communication between distant nodes; thus, bottlenecks are more likely to be essential proteins connecting different biological processes (Yu et al., 2007). Closeness is defined by the sum of the shortest path between a given node and all
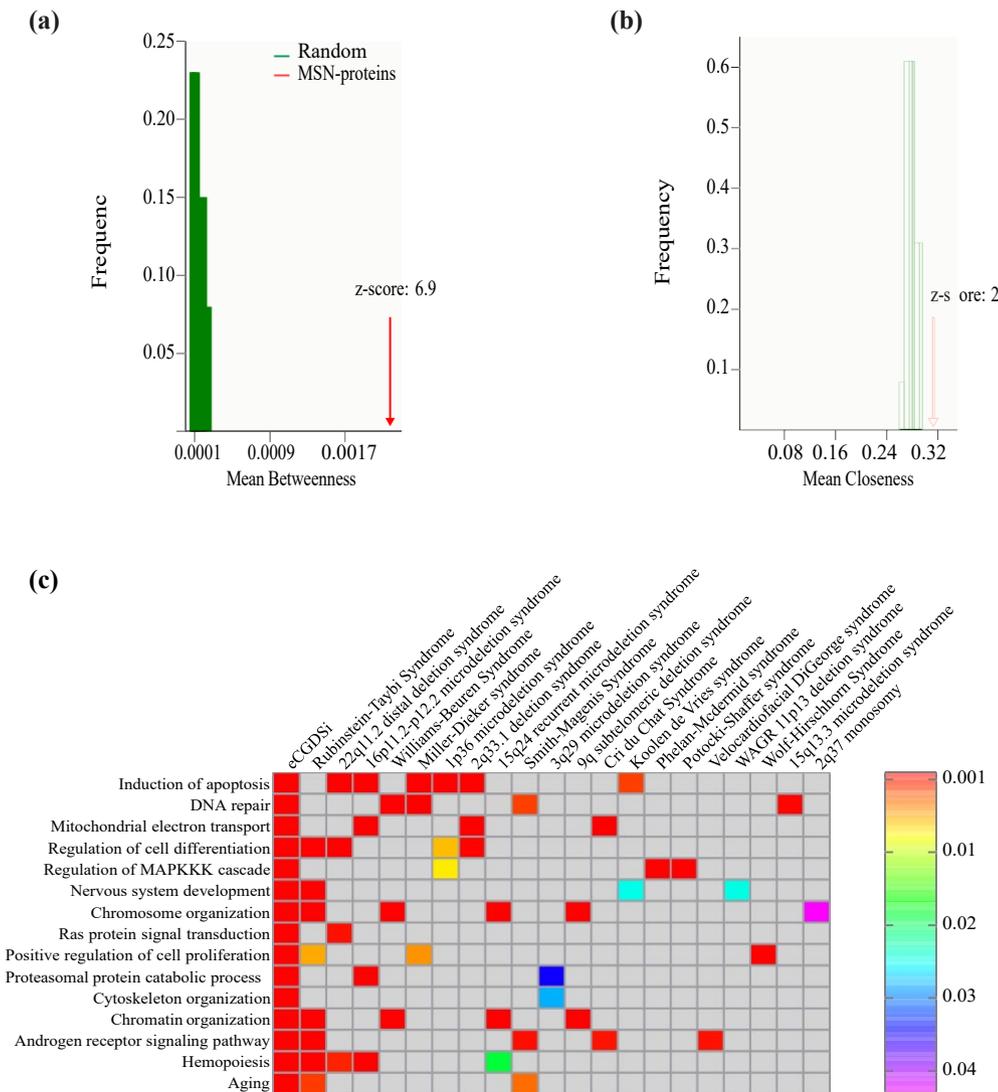

FIGURE 3 Analysis of the expanded MS network (eMSN) obtained from the human interactome. (a) Averages of betweenness and (b) closeness compared with random networks are significantly different from random expectation. Enrichment analyses with significance level of $p < .05$ to (c) biological processes

other nodes on the network, indicating how close a node is to all others (Scardoni & Laudanna, 2012). In the context of network information, nodes with high closeness maybe key to the regulation of many proteins, although few impact other proteins in the network (Scardoni & Laudanna, 2012). We found higher average values of closeness and betweenness in MSN-associated proteins compared to those in the random expectation group, with z-scores of 2 and 6.9, respectively (Figure 3(a),(b)). Altogether, these results indicate that some of the protein-coding genes located in critical regions of chromosomes may encode more central proteins with significant biological roles, which are responsible for multiple cellular functions or are essential for communication between distant proteins.

3.5 | A wide spectrum of biological processes is associated with MS

Enrichment analysis of biological processes in each syndrome and their respective first neighbors in the eMSN revealed that biological processes involved in DNA repair (corr p-value: 7.9^{E-16}), regulation of cell differentiation (corr p-value: 3.7^{E-15}), chromatin organization (corr p-value: 8.4^{E-07}), nervous system development (corr p-value: 8.6^{E-10}), and cytoskeleton organization (corr p-value: 4.7^{E-07}) were likely to be dysregulated in MS. Notably, significant enrichments were more frequently found in induction of apoptosis (corr p-value: 1.19^{E-17}), chromosome organization (corr p-value: 4.4^{E-09}), and androgen receptor signaling pathway (corr p-value: 3.4^{E-06}). Rubinstein-Taybi syndrome shared the largest number of biological processes in common with the eMSN. Moreover, mitochondrial electron transport was associated with cri du chat, 16p11.2-p12.2 microdeletion, and 2q33.1 deletion syndromes (Figure 3(c)). Statistically significant biological processes were not found in 2p21 microdeletion, ATR-16, and NF1-MSs. The diversity of biological processes associated with the protein-coding genes in MS reflect the variability of the phenotypes observed in affected individuals. The complete results are provided in Table S3.

3.6 | Impact of gene-deletions

To evaluate the impact of the deletion of each of the 564 genes, we selected three genetic metrics. First, haploinsufficiency (HI) score allows prediction of the probability of haploinsufficiency for a given gene (Huang et al., 2010); genes with high HI scores exhibit extreme sensitivity to decreased gene dosage, and therefore, the loss of half of the active proteins is sufficient to impair or inhibit the wild phenotype or cause disease (Morrill & Amon, 2019). Second, DSI is a measure indicating whether a specific gene is associated with several or few diseases. Third, DPI indicates whether a gene is associated with multiple diseases belonging to the same or different MeSH disease class or classes, respectively, resulting in a pleiotropy index (Piñero et al., 2017). The complete list of metrics with ranks is shown in Tables S3 and S4.

Applying Spearman's correlation coefficient to identify possible associations between HI, DSI, and DPI with degree centrality, we found a moderate negative correlation ($\rho = -0.42$; $p < .005$) between HI and degree distribution. Thus, syndromes with hubs may exhibit a higher average HI score (Figure 4(a)). In addition, we identified a moderate negative correlation between degree values and DSI scores, with $\rho = -0.43$ and $p < .005$, indicating syndromes containing hubs involved with multiple diseases (Figure 4(b)). Finally, we found that DPI score positively correlated with degree centrality ($\rho = 0.38$; $p < .005$), suggesting that syndromes with high mean degree values may involve proteins associated with diseases of different classes (Figure 4(c)). Interestingly, Rubinstein-Taybi and 2q 37 monosomy syndromes had the highest scores for all metrics that were evaluated (Figure 4). Genes with high scores for these metrics are likely to be essential genes (Chavali, Barrenas, Kanduri, & Benson, 2010; Ohnuki & Ohya, 2018; Zotenko, Mestre, O'Leary, & Przytycka, 2008). Therefore, the deletion of these genes could generate a more severe phenotype in individuals.

3.7 | Association of MSN-genes with other diseases

Similar phenotypes are shared between MS and other genetic diseases caused by functionally related genes in a specific disease module in the human interactome. Thus, the identification of genotype-phenotype relationships allows the prioritization of candidate disease-associated genes to better understand the pathophysiology involved in these syndromes. For this purpose, we used curated data of gene-disease associations (Piñero et al., 2020). First, we generated a network with associations between MSs and disease classes (Figure 5(a)). Nervous system diseases (11.17%), neoplasms (11.17%), musculoskeletal diseases (11.17%), neonatal diseases and abnormalities (8.12%), and mental disorders (8.12%) were the disease classes most frequently associated with MS. In the human interactome 1p36 microdeletion and Koolen de Vries syndromes share genes related to 35 and 20 different diseases, respectively. Wolf-Hirschhorn Syndrome has the highest number of connections with the class of musculoskeletal diseases, which includes a total of 15 different diseases. Velocardiofacial and Cri-du-Chat syndromes interact with 12 and 5 different mental disorders, respectively. The syndromes connected with the highest numbers of disease classes were Smith-Magenis, 2q33.1 deletion, and 12q14 MSs, all of which were associated with 14 disease classes in the human interactome.

Next, we investigated the relationship between the nervous system disease class and MS-genes. The gene-disease associations in the MSN comprised 66 MS-genes and 126 diseases of the nervous system (data not shown). Intellectual disabilities were found to be connected with 15 MS-genes in the network. The genes having the most associations with different diseases were *MAPT* ($n = 17$), *MTHFR* ($n = 12$), and *NF1* ($n = 8$). The largest connected component seen in Figure 5(b) shows diverse common clinical manifestations present in MS. This specific subregion includes two clusters interconnected by
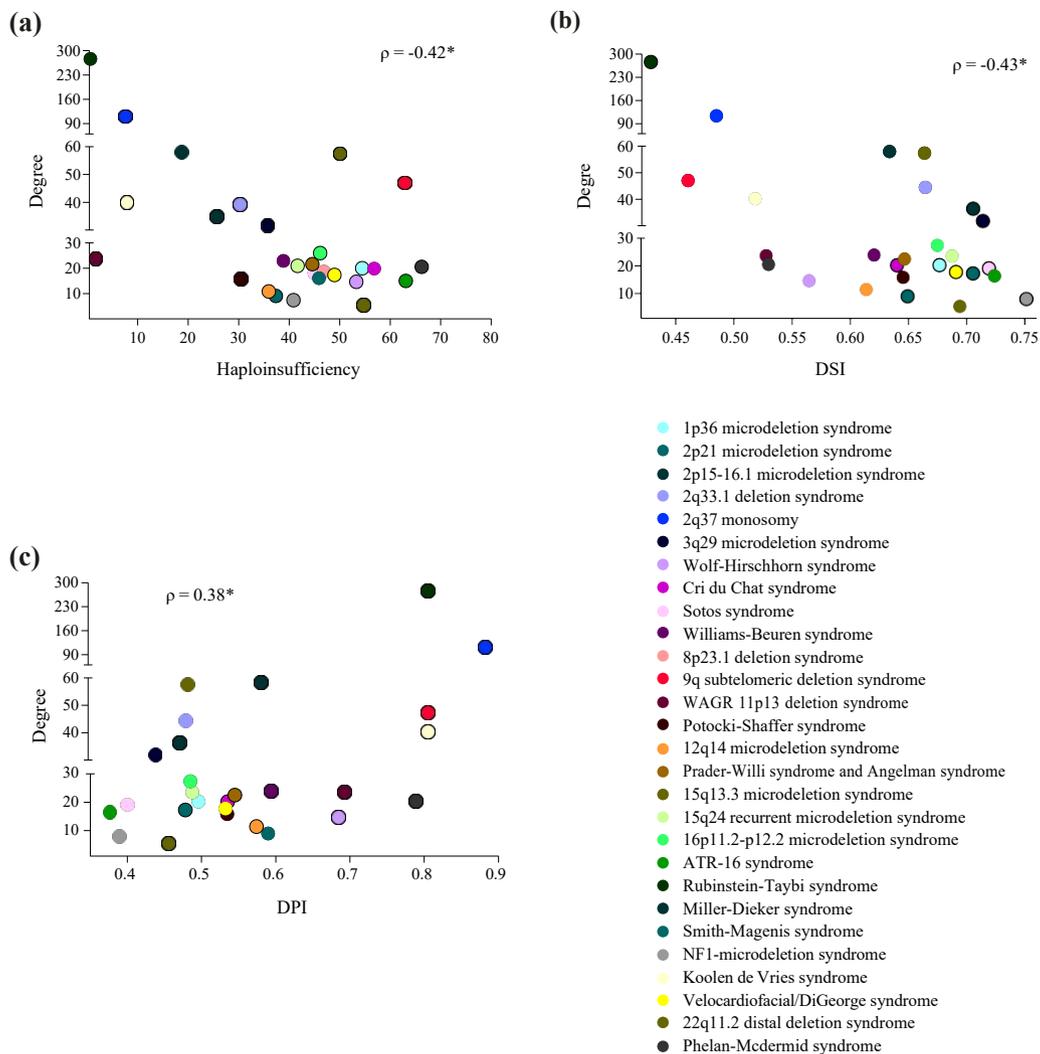
FIGURE 4 Association between genetic and centrality measures of the eMSN-proteins in each syndrome. (a) Correlation to HI (haploinsufficiency); High ranks (e.g., 0-10%) indicate a gene with a high probability of presenting HI, low ranks (e.g., 90-100%) indicate a gene most likely not to exhibit HI. (b) DSI (disease specificity index for the gene); the DSI ranges from 0.25 (many diseases associated) to 1 (one disease-associated). (c) DPI (disease pleiotropy index for the gene). DPI ranges from 0 to 1 (many associations with different disease-class). Spearman's correlation was used with *$p < .05$

genes associated with seizures, a phenotype present in several MS. Therefore, interactions of functionally related genes in the bipartite network can support the identification of candidate genes in critical regions of chromosomes, as well as help explain the overlap of the phenotypes found in MSs.

4 | DISCUSSION

MS presents severe manifestations in affected individuals, with a broad clinical spectrum. Although the "genotype-first" approach is widely used in studies of genotype-phenotype correlations, we are not aware of published studies investigating MSs systematically. However, microarray techniques and the availability of interaction data

generated by large-scale experiments allowed us to approach MSs with a holistic view in this study.

The investigation of protein-coding genes located in the critical regions of chromosomes using a platform of PPI indicated that MSN-proteins were likely to be located in specific regions of the interactome (Corrêa et al., 2018; Corrêa, Feltes, Poswar, & Riegel, 2020; Corrêa, Feltes, & Riegel, 2019; Corrêa, Venâncio, Galera, & Riegel, 2020). Disease-associated proteins usually interact with each other in a local neighborhood of the human interactome more frequently than expected by chance (Goh et al., 2007; Oti et al., 2006; Vidal et al., 2011). In this sense, 2q33.1 deletion, 16.p11.2-p12.2 microdeletion, 1p36 microdeletion, ATR-16, and Sotos syndromes encompass proteins located in the same module of the interactome. However, the data generated so far covers less than 20% of all
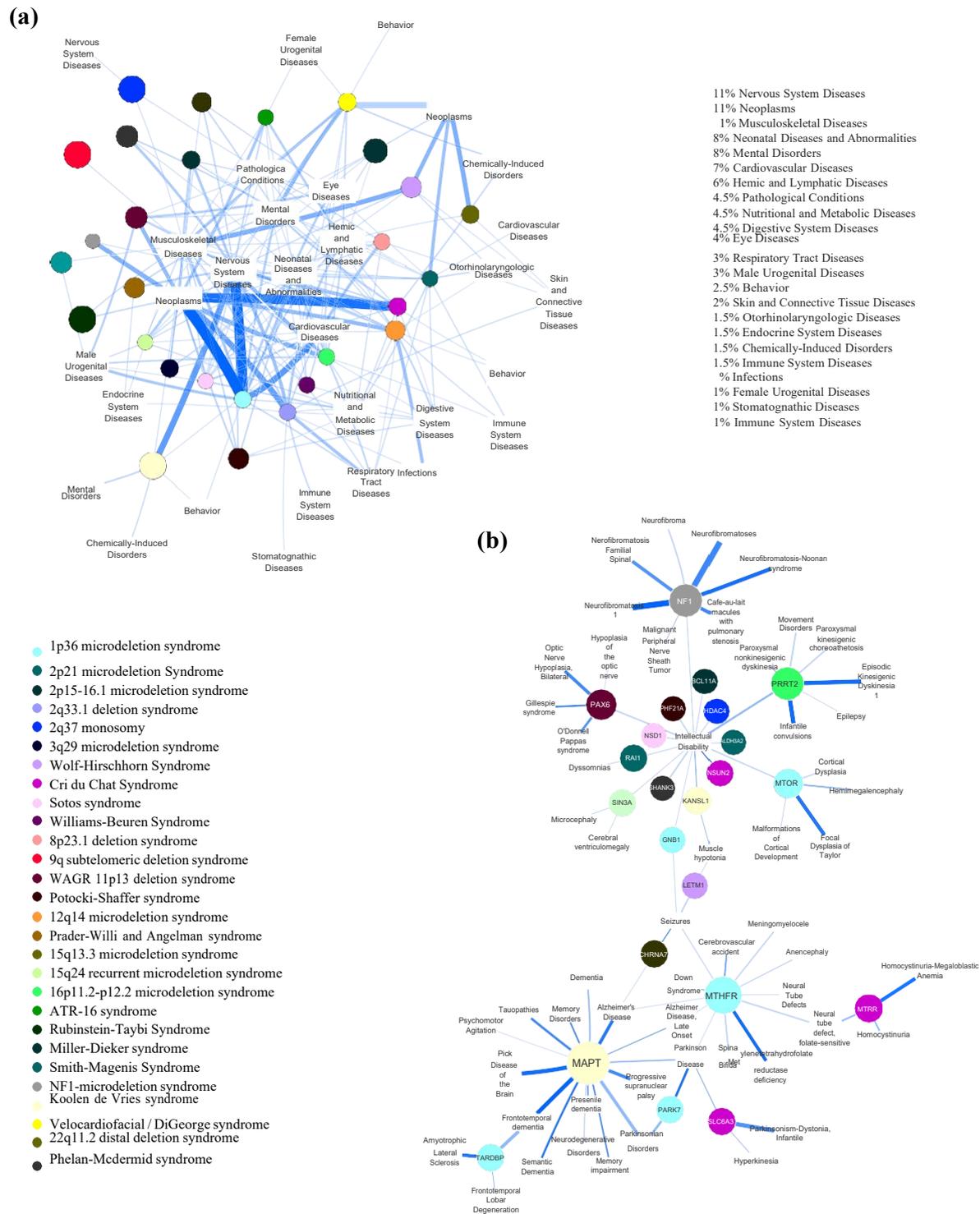
FIGURE 5 Overview of MS with respect to other diseases (a) Representation of associations in the MS-network with syndromes (circular nodes) and disease classes (rectangular nodes). The size of the circle represents the proportions of genes associated with the disease classes; edge thickness refers to the number of diseases within a class. (b) The largest connected component of human gene-disease associations network with genes (circular nodes) and nervous system diseases (rectangular nodes). The size of the node corresponds to the number of diseases associated with a given gene. Scores values are shown proportionally by the thickness of the edges

potential interactions among proteins in human cells (Caldera, Buphamalai, Müller, & Menche, 2017; Rolland et al., 2014); thus, a likely lack of interaction data may have limited the identification of modules for other syndromes. We generated MSNs and identified shared interactions and phenotypes among different syndromes, which are characteristics that may be reflected clinically.

MSN-proteins may be central in the human interactome and influence the communication of distant proteins (bottlenecks nodes) or the flow of information from neighboring proteins (node with high closeness) in the network. Interestingly, we identified a central position in the network for two hub-bottlenecks with MS-associated with a single gene deleted in the critical chromosomal regions: the histone lysine acetyltransferase (*CREBBP*) deleted in Rubinstein-Taybi syndrome presented a degree value of 276 and betweenness of 0.013, while the histone deacetylase 4 (*HDAC4*) deleted in 2q37 monosomy showed a degree of 110 and betweenness of 0.003. CREBBP protein is essential for embryonic development, growth control, and coactivation of different transcription factors (Das et al., 2014), while HDAC4 is involved in transcriptional regulation, cell cycle progression, and developmental events (Wang et al., 1999; Williams et al., 2010). Moreover, 2q37 monosomy, 9q subtelomeric deletion, and Rubinstein-Taybi syndromes showed that highly connected protein-coding genes in the network are more likely to be associated with many diseases, different classes of diseases, and high haploinsufficiency scores (exception of 9q subtelomeric deletion syndrome). Importantly, topological features in a network reflect multiple functions in the biological context. Therefore, it is expected that some MSN-proteins may have a greater impact on the topological features, that is, phenotypes caused by genomic deletions, together with genetic metrics such as HI, DSI, and DPI, contributing to the identification of proteins with greater influence on the pathophysiology in MSs.

However, disease-associated genes are frequently nonessential and do not encode central proteins but are rather normally localized in peripheral regions in the network (Goh et al., 2007; Zotenko et al., 2008). The evolutionary explanation is that mutations in genes with central topological features may affect regions that are extensively interconnected in the network, compromising embryonic development or important physiological functions in individuals (Goh et al., 2007; Yu et al., 2007). Therefore, it is noteworthy that most MSs involving many genes located in critical regions tend to present protein-coding genes with low degree values, haploinsufficiency, and pleiotropy, indicating that these proteins are localized in more peripheral regions in the network and are involved in few cellular functions.

The phenotype in MSs is not merely the result of a deficient protein, but also disturbances that spread in the network, affecting functionally related genes involved in different disorders in the specific disease module of the human interactome (Barabási et al., 2011; Schadt, 2009). Disturbances in neighboring proteins may occur in MSs, and the undesirable impact may be greater in cases where genes associated with several other diseases are deleted. The bipartite network of gene-disease associations identified different classes of diseases related to clinically observed MSs phenotypes such as nervous system, musculoskeletal, and neonatal diseases. Besides, we identified a broad spectrum of diseases of the nervous system, and showed that intellectual disabilities highly connected with different genes in the network, which may explain the high frequency of this phenotype in MSs.

The contribution of the gene network-based analysis is that, regardless of the origin of the pathogenesis of a microdeletion syndrome (epigenetic alteration, loss of function of the deleted proteins, effect of position, change of transcription factor sites or deregulation of miRNAs), it considers all these mechanisms when estimating how the proteins interact or fail to interact with each other in the interactome. Therefore, we propose that such approach may help to predict the impact of genomic imbalances within critical chromosomal regions.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

7.5. Artigo V. Corrêa T, Santos-Rebouças CB, Mayndra M, Schinzel A and Riegel M (2021) Shared neurodevelopmental perturbations can lead to intellectual disability in individuals with distinct rare chromosome duplications. Genes (Basel). https://doi.org/10.3390/genes12050632

Article

Shared Neurodevelopmental Perturbations Can Lead to Intellectual Disability in Individuals with Distinct RareChromosome Duplications

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Abstract: Chromosomal duplications are associated with a large group of human diseases that arise mainly from dosage imbalance of genes within the rearrangements. Phenotypes range widely but are often associated with global development delay, intellectual disability, autism spectrum disorders, and multiple congenital abnormalities. How different contiguous genes from a duplicated genomic region interact and dynamically affect the expression of each other remains unclear in most cases. Here, we report a genomic comparative delineation of genes located in duplicated chromosomal regions 8q24.13q24.3, 18p11.32p11.21, and Xq22.3q27.2 in three patients followed up at our genetics service who has the intellectual disability (ID) as a common phenotype. We integrated several genomic data levels by identification of gene content within the duplications, protein-protein interactions, and functional analysis on specific tissues. We found functional relationships among genes from three different duplicated chromosomal regions, reflecting interactions of protein-coding genes and their involvement in common cellular subnetworks. Furthermore, the sharing of common significant biological processes associated with ID has been demonstrated between proteins from the different chromosomal regions. Finally, we elaborated a shared model of pathways directly or indirectly related to the central nervous system (CNS), which could perturb cognitive function and lead to ID in the three duplication conditions.

Keywords: duplication syndromes; intellectual disability; axon guidance; PPI-network

1. Introduction

Chromosomal duplication syndromes are caused by intrachromosomal rearrangements (due to genomic instability) and may result in overexpression of dosage-sensitive genes within the rearrangement and gene interruption or gene fusion at the breakpoint junctions [1,2]. As a consequence, chromosomal duplications can affect multiple functional proteins that need to be effective in terms of quantity, location, and time of activity. The consequence of these alterations can lead to damage in brain development and/or cognitive functioning [3,4]. Moreover, imbalances of proteins that compose multiprotein complexes may be extremely deleterious, when stochiometric changes in subunits affect biological processes [2,5]. Finally, the perturbation of hub-genes may also alter the expression and function of other sets of proteins, or even, produce aggregation of proteins that lead to cellular toxicity [2,6].

Chromosomal duplications have a prevalence of ~0.7/10.000 births and are commonly associated with syndromic forms of Intellectual Disability (ID), a heterogeneous condition with a worldwide prevalence of 1% [4,7] that impairs intellectual functioning and adaptive.

behavior, manifesting before adulthood [8]. Usually, duplication syndromes are identified by Chromosomal Microarray Analysis (CMA), considered as the first-tier test that offers 15–20% of diagnostic rate for individuals with unexplained global developmental delay/ID and/or congenital anomalies [9].

Neurological processes are tightly regulated during the development and throughout the individual's life in a manner that any change can have deleterious effects on cognitive function [10]. Many cellular processes are affected in ID, including neurogenesis, neuronal migration to axon guidance, synaptic plasticity, dendritic arborization, and regulation of transcription and translation. These bioprocesses can converge on similar and connected pathways, involving common phenotypic manifestations [4,10,11]. Pathophysiology causing ID comprises proteins that emerge in pathways and cellular networks involving several biological functions that occur through interactions represented by the human interactome [4,12]. Moreover, chromosomal rearrangements may include regions significantly enriched for genes involved in brain development that can generate multiple pathogenic mechanisms [2].

Herein we determined whether genes located in duplicated regions in three patients followed up at our genetics service with rare but relevant regions (8q24.13q24.3, 18p11.32p11.21, and Xq22.3q27.2) are involved in shared central molecular pathways associated with genes related with ID. The 8q24.13q24.3 duplication identified is a rare chromosomal rearrangement associated with dysmorphic features, growth delay, and ID [13–16]. Moreover, variable levels of ID and cerebellum hypoplasia were described in patients with 18p11 duplications, however, few cases of pure duplications in this region have been reported with similar rearrangements so far [17–21]. Duplication at Xq22.3q27.2 is a condition with region enriched in genes related to neurological function involving many cases of ID, behavioral problems, holoprosencephaly, and cerebellar vermis hypoplasia [22–26]. Therefore, we integrated several levels of data by identification of gene content, protein-protein interactions, and functional analysis on specific tissues to suggest a model with common or related pathways to the central nervous system (CNS) functions in individuals affected by these duplication syndromes.

2. Materials and Methods

2.1. Chromosomal Microarray Analysis (CMA)

Three patients with ID were followed in the Medical Genetics Service—HCPA. The duplications were mapped by CMA using a 60-mer oligonucleotide-based microarray with a theoretical resolution of 40 kb (8 × 60 K, Agilent Technologies Inc., Santa Clara, CA, USA). The labeling and hybridization were performed following the protocols provided by Agilent, 2011. The arrays were analyzed using a microarray scanner (G2600D) and the Feature Extraction software (version 9.5.1, both from Agilent Technologies). UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) was employed to identify all protein-coding genes from duplicated regions. The complete list of protein-coding genes can be seen in Supplementary Table S1.

2.2. Interactome Construction and the Expanded Duplication Syndromes Interactome (eDSi)

The human interactome was generated using the Human Integrated Protein-Protein Interaction Reference (HIPPIE) database (version 2.2) [27]. We filtered in the interactions with confidence score > 0.4 and limited our analysis to the largest connected component, containing 16,108 nodes and 256,552 links/edges. Next, we extracted only protein-protein interactions from the three selected duplicated regions (Supplementary Table S2) and selected their first neighbor to expand and generate the eDSi. Cytoscape V.3.7.0. software [28] was used for visualization, and calculations of centrality parameters of the networks.

2.3. Functional Modules Detection and Enrichment Analysis

The HumanBase database integrates functional networks in tissues, gene expression, and disease associations. Evidence is provided by a massive set of experiments containing more

than 14,000 publications and 144 tissue- and cell lineage-specific functional contexts [29,30]. We extracted significant biological processes in the eDSi, by using the detection of functional modules tool in specific tissues available in the HumanBase [31]. This tool allows the detection of tissue-specific functional modules, comprising related genes located in clusters that share local network neighborhood. The method uses k-nearest-neighbor (SKNN) and the Louvain community-finding algorithm to cluster the genes list into distinct modules of tightly connected genes [31]. q value was calculated using one-sided Fisher's exact tests and Benjamini–Hochberg corrections to correct for multiple tests and only values < 0.05 were considered (Supplementary Table S3). Moreover, we used the webserver Enrichr [32] to identify significant pathways involved with neuronal functions in genes from the tissue-specific network. The gene-set libraries used were BioCarta, BioPlanet, Elsevier Pathway Collection, Kegg, Reactome, Panther, and WikiPathways. We considered only bioprocesses with a p-value < 0.05.

2.4. Prioritization of Candidate Genes

To prioritize candidate genes associated with ID, we used a list of known ID-genes (Supplementary Table S4), available at http://www.disgenet.org/ (accessed on 6 January 2020). DisGeNET is a platform that integrates data from UNIPROT, CGI, ClinGen, Genomics England, CTD (human subset), PsyGeNET, and Orphanet on human gene-disease associations [33]. We used a query list of ID-genes to expand the selection of nodes, using network propagation through the Diffusion algorithm (V. 1.6.1) [34]. Network propagation can estimate the distance between different sets of proteins, and identify a subnetwork with nodes closely related to each other [34]. The proximity among candidate genes and query ID-genes in the eDSi was measured using 302 as a maximum diffusion rank (highest allowed value). The complete list of prioritized genes is shown in Supplementary Table S5.

2.5. Functional Tissue-Specific Data

We used the list of the prioritized genes (Supplementary Table S5) to identify gene expression in tissues and construct a gene-disease association network in the Human-Base [29,30]. Moreover, a tissue-specific network with 18 genes highly expressed in the CNS was generated using data from co-expression, protein interaction, TF binding, microRNA targets, and perturbations. We prioritized the most expressed genes in the CNS, or genes previously reported in the literature involving ID in individuals with duplication regions. The parameters used to generate the network were a confidence >0.10 and a value of 15 for the maximum number of genes.

3. Results

3.1. Identification of Rare Chromosome Duplications

Chromosome duplications were mapped using the samples of three patients with ID using hg/19 reference: 8q24.13q24.3 (Chr8:126,397,316–143,577,971); dup18p11.32p11.21 (Chr18:14,316–14,773,575); and dupXq22.3q27.2 (Chrx:106,283,188–140,340,737). The summary of CMA and clinical findings from the three patients with chromosomal duplications can be seen in Supplementary S1 and Table 1, respectively.

3.2. DSi Proteins Tend to Have High Values of Betweenness

The human interactome provided a network-based framework to investigate protein-protein interactions between DSi-proteins (Figure 1a). The extraction of protein-coding genes from the duplicated regions and their first neighbor resulted in a DSi composed of 3016 nodes/proteins and 4330 links/interactions (Figure 1b). DSi included 89 proteins from duplicated regions and 65 ID-genes. Four DSi-proteins (LAMA1, STAG2, NKAP, and ALG13) were also found among the ID-genes list [35].

Table 1. Summary of the CMA and clinical findings from the 3 patient with chromosomal duplications.

Patient	1	2	3
Sex	F	M	F
Age (years) *	8	12	7
Band location (duplicated)	Chr8 (q24.21-q24.3)	Chr18 (p11.32-p11.21)	ChrX (q22.3-q27.1)
CMA Deletion size (pb)	17,180,656	14,759,260	34,057,550
Genomic position (GRCh38/hg38)	Chr8:126,397,316–143,577,971	Chr18:14,316–14,773,575	Chrx:106,283,188–140,340,737
Clinical findings	ID; microcephaly; seizures; speech delay; global developmental delay	ID; speech delay; anxiety; learning difficulty; psychomotor agitation	ID; NPMDD; short stature; clinodactyly; blepharophimosis

ID: Intellectual disability; NPMDD: neuropsychomotor development delay. * age in years at the time of the CMA investigation.
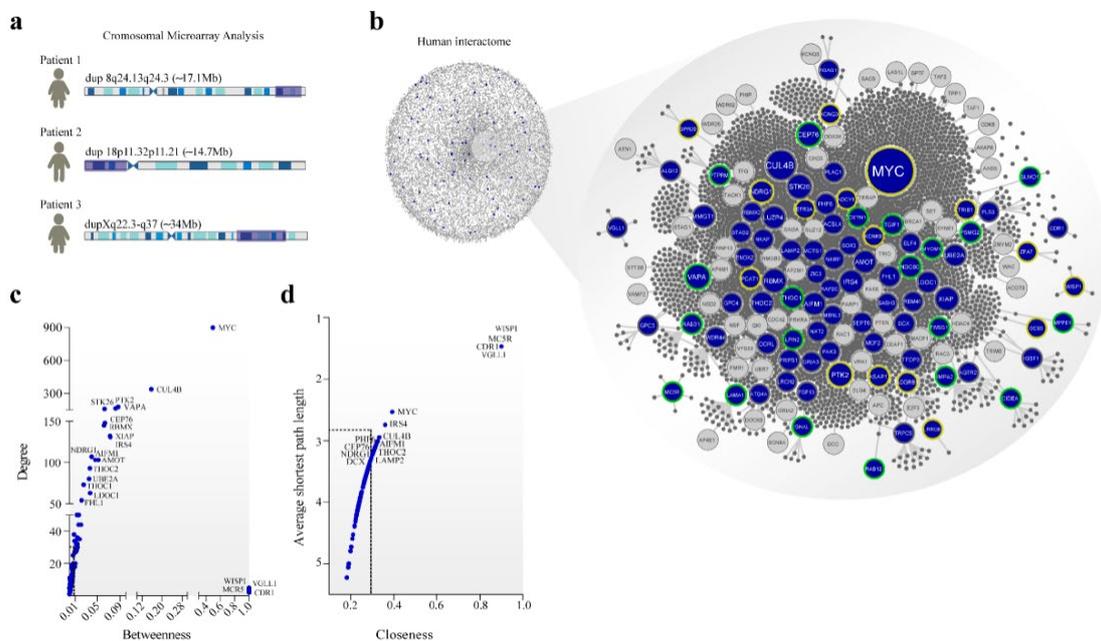
Figure 1. Cytogenetic to the interactome. (**a**) left: duplicated regions mapped by CMA in the patients; right: curated human interactome composed of 13,460 proteins and 141,296 interactions. In the network, proteins are nodes connected by interactions. (**b**) expanded duplication syndromes interactome (eDSi) with 3016 proteins and 4330 interactions. Blue nodes are protein-coding genes from duplicate regions; grey nodes are ID-genes. Node border colors represent the origin of duplication: dup 8q24.13q24.3 in yellow; dup 18p11.32p11.21 in green; dupXq22.3-q27 in grey. Node size is related to the number of connections (degree). (**c**) Topological parameters with degree and betweenness distribution and values of (**d**) average shortest path length and closeness for duplicated protein-coding genes in eDSi. Dashed lines in black indicate the average of these parameters for human interactome.

The average centrality measures in the human interactome were: degree (31.85), betweenness (0.00013), closeness (0.3249), and shortest path length (3.119). Degree centrality defines the number of connections of a specific node in the network, and in the biological context, nodes with a degree value > 100 links (hubs) may have multiple functions in cellular networks [36]. Betweenness corresponds to the number of nonredundant shortest paths that pass through a node of interest and may indicate the potential of a protein to create a bridge for communication between distant nodes [37,38]. The average shortest path length involves the summa of all shortest paths between nodes couples, divided by all pairs of nodes in the network, and the closeness indicates how close a node is to all other nodes in the network [39,40].

Many DSi-proteins showed higher values of centrality, compared to the mean of the human interactome, indicating topological relevance to specific DSi-proteins (Figure 1c). In this sense, 14% of DSi-proteins were considered hubs, including MYC, a transcription factor, and CUL4B, a central component of the ubiquitin-protein ligase complex, both acting in several biological processes. Moreover, other hubs, such as RBMX, PTK2, AIFM1, VAPA, and XIAP, are associated with ID [41–45].

Eighty percent of the DSi-proteins reached a betweenness centrality value higher than the average of the human interactome (Figure 1c). VGLL1 (coactivator for the mammalian TEFs), CDR1 (neuronal signal transduction protein), MC5R (melanocortin receptor coupled to the transmembrane G protein), and WISP1 (a member of the WNT1 inducible signaling pathway) showed high betweenness values. All of these proteins have significant roles in signal transduction or coactivation of transcription factors [46–49]. Furthermore, CDR1 is a putative neuronal protein identified in individuals with cerebellar degeneration [50].

Besides degree and betweenness, shortest path length and closeness were calculated. VGLL1, CDR1, WISP1, and MC5R also emerged in the network with high closeness and lower shortest path length values (Figure 1c). From a biological perspective, these nodes can have a major impact on proteins that are close to the node or serve as the shortest path among distant proteins in the network. About 22% of the proteins were identified with values of closeness and shortest path length above the average of the interactome. The main results of the topological characteristics of other DSi-proteins can be seen in Figure 1c,d.

3.3. Biological Processes Associated with Rare Duplications

We carried out enrichment analysis of the DSi-proteins to identify biological processes with a possible role in ID. Six clusters were detected grouping the main bioprocesses (Figure 2). Cluster 1 identified only proteins from dup18p11.32p11.21 with enriched bioprocesses related to chromosome segregation. DSi-proteins from 8q24.13q24.3, 18p11.32p11.21, and Xq22.3q27.2 were found in clusters 2 and 6, associated with telomere maintenance, DNA repair, epithelium developmental, and ion transport. Cell morphogenesis in clusters 3 and 4 was associated with proteins from duplicated regions on chromosomes 8, 18, and X. Cluster 5 is the only one to encompass proteins from the three duplicated regions, with enrichment for microtubule cytoskeleton organization, negative regulation of cell cycle, and neurogenesis. Cell pathways involving the ID pathophysiology can encompass changes in the cytoskeleton dynamics, neurogenesis, and morphology during synaptic plasticity or neuronal development [11,51].
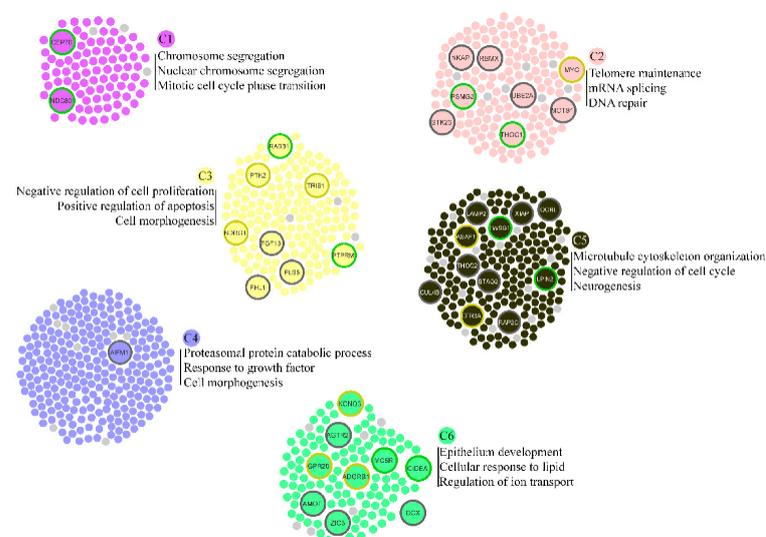

Figure 2. Functional cluster detection in eDSi. Node border colors represent the origin of duplication: dup 8q24.13q24.3 in yellow; dup 18p11.32p11.21 in green; dupXq22.3-q27 in grey. Small nodes in light grey represent ID-genes.

In addition, plasma membrane-bounded cell projection was enriched in the three duplicated regions. This process involves the formation of a prolongation bounded by the plasma membrane, such as an axon. Projection defects were reported in an ID mouse model [52]. Moreover, neuronal development and nuclear chromosome segregation were identified in functional enrichment analysis of ID-genes and DSi-genes from dupXq22.3q27.2 and dup18p11.32p11.21. The complete results are provided in Supplementary Table S3.

3.4. DSi-Genes Are Widely Expressed in the CNS

ID is caused by perturbations in the significant biological functions that impact cellular networks present in different regions of the CNS. We identified the influence of each of the 44 prioritized genes (Supplementary Table S5) in different tissues and found that these genes are mainly expressed in the CNS when compared with other tissues in humans (Figure 3) [14,16,53].
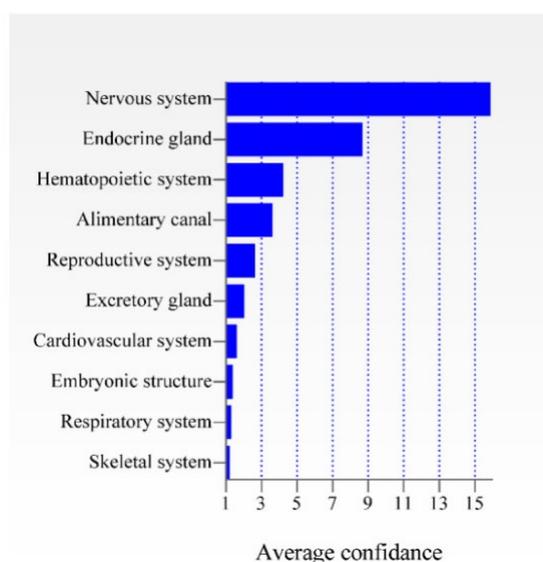
Figure 3. Expression of the 44 prioritized genes in ten different tissues. The average confidence value is shown for each tissue.

Therefore, we extracted expression data from multiple CNS regions to better understand the influence of each gene on this tissue (Figure 4). Many genes located at Xq22.3q27.2 are widely expressed in the CNS and were previously associated with syndromic/non-syndromic X-linked ID, such as ALG13, PAK3, THOC2, GRIA3, STAG2, OCRL1, AIFM1, PHF6, RMBX, SOX3, LAMP2, CUL4B, and UBE2A [54,55]. Moreover, patients with duplicated regions that encompass the X-linked genes SOX3, STAG2, AIFM1, GRIA3, PAK3, and OCRL exhibit ID [22–26,56–58]. Moreover, six genes from the duplicated region 18p11.32-p11.21 are highly expressed in several regions of the CNS, from which three of them (LAMA1, MYOM1, and TGIF1) were duplicated in individuals with ID [18–20]. Furthermore, patients with duplication of 8q24.13q24.3 region involving the KCNQ3, PTK2, ASAP1, and NDRG1 genes, which are widely expressed in CNS, presented ID [14,16,53].

3.5. Candidate Proteins from Different Chromosome Rearrangements Interact with Each Other in the CNS Network

To analyze the relevance of candidate proteins according to tissue specificity, we constructed a network with interactions from the CNS, in an attempt to identify clues about the likely contribution of each protein in the development of ID. The CNS network includes 32 nodes connected by 210 interactions, from which 18 are DSi proteins (Figure 5a). The most connected proteins are PTK2 (19), STAG2 (16), and TGIF1 (16). Interestingly, ID-genes WAC, QKI, and PPP1R12A emerge as interacting factors on the network by automatic addition of the database. It is worth mentioning that many links in the tissue-specific network result

from gene co-expression studies in the context of neurological conditions, such as recessive X-linked dystonia-parkinsonism, Rett syndrome, and Huntington's disease.
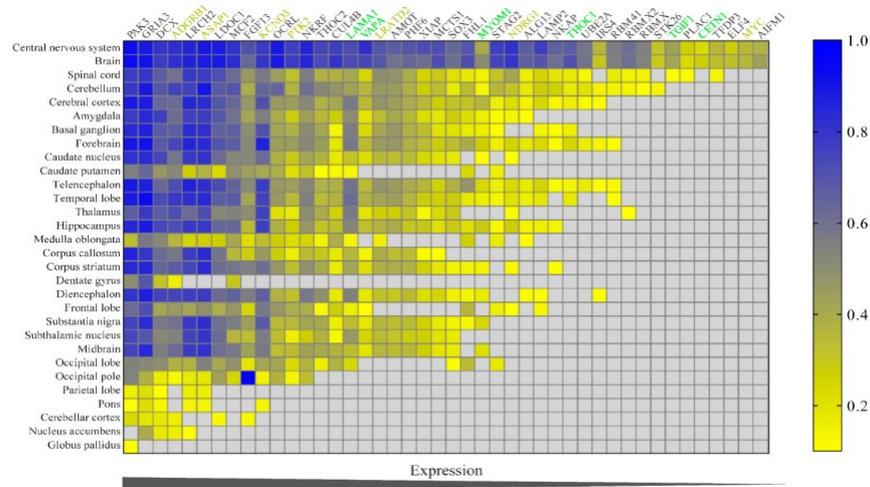

Figure 4. Heat map of expression of the 44 prioritized genes in CNS. Confidence value is calculated between 0–1.
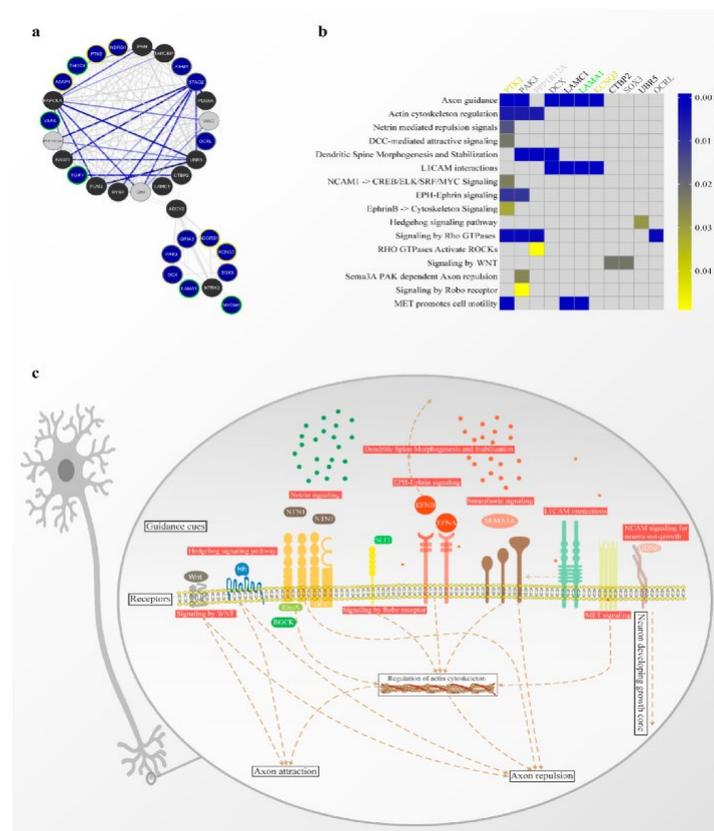

Figure 5. Tissue-specific gene network analysis. (**a**) functional network from CNS. Blue nodes are protein-coding genes from duplicate regions; grey nodes are ID-genes; black nodes were added by the database. Node border colors represent the origin of duplication: dup 8q24.13q24.3 in yellow; dup 18p11.32p11.21 in green; dupXq22.3-q27 in grey. Score values are shown proportionally by the thickness and intensity color of the edges. (**b**) heat map of genes from tissue-specific gene network with the most significant biological processes. (**c**) Scheme depicting the main pathways and molecules involved in axon guidance.

The functional enrichment analysis identified several pathways associated with axon guidance (Figure 5b). The genes directly involved with this biological process include PTK2 and KCNK3 (dup 8q24.13q24.3), LAMA1 (dup 18p11.32p11.21), and PAK3, DCX, SOX3, and OCRL (dupXq22.3-q27). As mentioned above, all these genes have already been identified in duplicated regions in individuals with ID. Moreover, LAMA1 was also present in our ID-list which used the candidate genes prioritization. Pathways related to functions necessary to axon guidance that encompasses these genes, include actin cytoskeleton regulation ($p = 0.0060$), L1CAM interactions ($p < 0.0001$), EPH-ephrin signaling ($p = 0.0107$), signaling by Rho GTPases ($p = 0.0029$) and MET cell motility promotion ($p = 0.0003$). However, other fundamental pathways in the axon guidance context can be seen in Figure 5b. The PPP1R12A gene, added to the database, is the only one not belonging to the duplicated region that appears in the ID-genes list and is involved in axon guidance.

Taken together, these results indicate that genes from different duplicated regions may be related to each other and other genes previously associated with ID localized in cellular networks in the nervous tissue and involved in neurodevelopment processes (Figure 5c).

3.6. Candidate Genes Are Associated with the ID

Similar pathways are disrupted in ID and in other neurological diseases due to the functional relationships of genes located in the same module in the human interactome. Therefore, to identify DSi-genes implicated in other neurological diseases, and help to confirm our results, we generated a gene–disease association network (Figure 6).
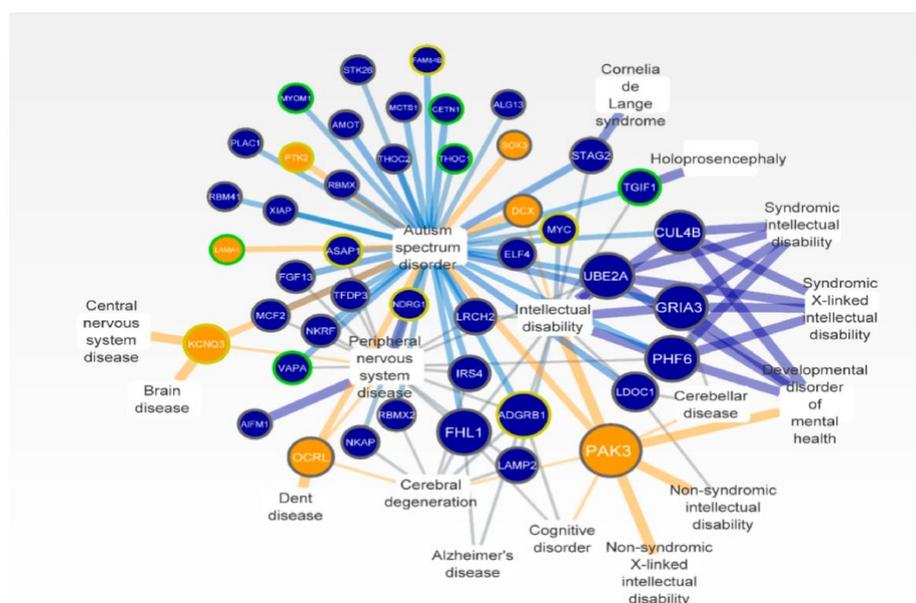

Figure 6. Gene–disease associations network. Duplicated genes (circular nodes) and disease of CNS (rectangular nodes). The size of the circle nodes corresponds to the number of diseases associated. Blue nodes are protein-coding genes from duplicate regions; orange nodes are candidate genes. Node border colors represent the origin of duplication: dup 8q24.13q24.3 in yellow; dup 18p11.32p11.21 in green; dupXq22.3-q27 in grey. Scores values are shown proportionally by the thickness and intensity color of the edges. Orange edges show interactions of candidate genes.

The most common diseases or phenotypes found on the network were autism spectrum disorder, peripheral CNS disease, and ID with 43, 17, and 15 associated genes, respectively. Maximum scores between disease and genes were seen in brain disease, holoprosencephaly, syndromic/non-syndromic ID, and syndromic/non-syndromic X-linked ID. The genes with the highest number of connections with other diseases were PAK3, GRIA3, and ADGRB1 associated with eight, six, and six neurological diseases, respectively. As expected, these genes were the most expressed in the CNS tissue (Figure 3). Previous

data support the known relationships of many genes on the network with neurological diseases, especially located in Xq22.3-q27.2 [55]. Moreover, the candidate genes PAK3, OCRL, DCX, PTK2, KCNQ3, SOX3, and LAMA1 were associated with autism, brain disease, Dent disease, and other conditions that present ID as a hallmark, corroborating our findings (Figure 6).

4. Discussion

Genomic disorders caused by duplications of chromosome segments confer potential risk of global developmental delay and ID, impacting the IQ, and educational achievement of individuals [59–61]. The imbalance in gene dosage caused by chromosomal duplications can destabilize several genes by spreading through interactions in cellular subnetworks during neurodevelopment. Moreover, the chromosome rearrangements identified in our patients are rare, with few cases reported so far. These duplicated regions have been reported as pathogenic and ID is a recurrent clinical finding in the affected individuals [16,21,26]. Therefore, we used network analysis in an attempt to identify the potential sharing of biological processes and genes responsible for the pathophysiology of ID in rare duplications. We found seven candidate genes: *PTK2* and *KCNK3* (dup 8q24.13q24.3), *LAMA1* (dup 18p11.32p11.21), and *PAK3*, *DCX*, *SOX3*, and *OCRL* from dupXq22.3q27, all duplicated in individuals with ID [15,16,19,23,25,55–57,61]. Furthermore, all candidate genes identified have been reported in duplicated regions of several ID patients in the web-based database—DECIPHER.

PTK2, protein tyrosine kinase 2, emerged with high degree and betweenness values (hub-bottleneck) through topological analysis in the eDSi (Figure 1c). This result correctly reflects the many biological functions performed by *PTK2* that involve the regulation of migration, adhesion, protrusion, and proliferation of the cell. Besides that, *PTK2* promotes axon growth and guidance and synapse formation during CNS development [62–65]. Therefore, changes in *PTK2* expression can impair brain development and lead to mental conditions [66]. Our topological analysis supports the identification of candidate disease genes that tend to be more central to the network, and not in peripheral regions as we expected [67,68]. Moreover, we identified many proteins from duplicated regions with high betweenness values considered bottlenecks, essential nodes in the information flow between distant proteins in cellular networks [38], indicating a potential impact in pathophysiology, when dysregulated.

We identified significant expression of duplicated genes in the CNS conversely to other tissues (Figure 3). Moreover, candidate genes present remarkable expression in regions of CNS associated with ID (Figure 4), such as the cortical region and the cerebellum [10]. Candidate genes from different chromosomes interact with each other in the tissue-specific network, demonstrating functional relationships among these genes in the CNS. For instance, *PTK2* (chr:8) interacts directly with *OCRL* (inositol polyphosphate-5-phosphatase—chr:X), *DCX* (doublecortin—chr:X) with *LAMA1* (laminin subunit α 1—chr:18), or yet, *KCNQ3* (potassium voltage-gated channel subfamily Q member 3—chr:8) and *LAMA1* are connected to each other by the neurotrophic tyrosine receptor kinase (*NTRK3*) (Figure 5a). Furthermore, *OCRL* and *PTK2* interacts directly with *WAC*, *QKI*, and *PPP1R12A*, genes previously associated with ID [69–72]. In the case of *PPP1R12A*, its loss-of-function causes holoprosencephaly and ID in individuals with stop gain variants and deletions/duplications, resulting in a frameshift effect [72]. PPP1R12A protein is present in pathways, such as RHO actin cytoskeleton regulation, ROCKs activation by GTPases, dendritic spine morphogenesis, and stabilization, all bioprocesses directly or indirectly involved with axon guidance mechanisms.

Axon guidance was the most enriched term in the tissue-specific network, besides the identification of various signaling pathways directly or indirectly involved in this biological process (Figure 5b). The axon guidance process plays an essential function in neuronal wiring in the developing spinal cord, where it is responsible for extending axons and reaching their targets to form synaptic junctions. These mechanisms allow the connection between the central and peripheral nervous system during neurodevelopment, through extracellular and transmembrane molecules and their cell surface receptors [73–75]. The main axon guidance

pathways and mechanisms involving our candidate genes were schematized in Figure 5c. The disruption or disintegration of neural circuit formation during CNS development affects cognitive function and can result in mental conditions such as ID [76–78]. The current model of the axon orientation mechanism reveals that the expression of guidance receptors occurs in the growth cone to indicate their targets and allow migration by controlling attractive and repulsive forces containing many guidance molecules present in their environment [75,77]. Therefore, the model of neural circuit formation supports the idea that changes in gene dosage caused by chromosomal duplications may impair the balance of this mechanism during the CNS development [79], where the gain or loss-of-function can impair the tight regulation of gene sets and cause disturbances in neighbor proteins in networks. However, expression data from patients with these chromosomal duplications should be used to confirm this model.

We observed interactions in the gene-disease association network between neurological conditions with ID and DSi-genes of three different chromosomes (Figure 6). These data suggest that duplicated regions could generate perturbations and propagate through modules in the interactome associated with many diseases linked to the CNS. For instance, the partial duplication of the gene that encodes the neuronal development transcription factor SOX3 can cause impairment in pituitary development and cognitive functions [80]. *PAK3* is expressed in the brain, playing a role in the control of cytoskeleton regulation, cell migration, axonal guidance, and synaptic plasticity, while its deregulation causes neurological abnormalities, such as ID [81,82]. *PAK3* pathogenic variants in affected males were associated with spatial cognitive abilities, defects in attention, and speech difficulties [83,84], and a hemizygous missense variant in this gene was found in two male siblings with ID [85]. *OCRL* regulates the traffic in the endosomal machinery and its depletion affects the recycling of various classes of receptors [86]. Dent disease patients with pathogenic variants in the *OCRL* can present mental impairment [87,88]. Already, *DCX* plays a crucial role in the CNS, enhancing the axonal outgrowth in postnatal cortical neurons [89]. Variants in *DCX* result in X-linked lissencephaly in males, and its overexpression leads to destabilization of microtubules and inhibition of neurite outgrowth [90]. Beyond the *PTK2* gene (a duplicated region on chromosome 8), *KCNQ3* encodes a protein with functions in the regulation of neuronal excitability and plasticity [91,92]. Pathogenic variants in this gene were identified in patients with early-onset epilepsy and neurocognitive deficits [93]. Moreover, a gain of function variants in *KCNQ3* causes neurodevelopmental delay and autistic features [94]. Lastly, *LAMA1* (duplicated region on chromosome 18), laminin involved in cell adhesion and axon outgrowth during embryonic development is associated with cerebellar dysplasia and ID in individuals with homozygous variants [95–98].

The phenotype in these conditions is not only the result of deficient protein, but also perturbations that spread in the cellular networks. Therefore, the network-based analysis, regardless of the origin of the pathogenesis of chromosomal duplications (epigenetic alteration, gain of function, effect of position, change of transcription factor sites, or deregulation of miRNAs), can help to predict the consequence of these mechanisms by analyzing functional protein relationships and their interactions in a network [99,100].

5. Conclusions

We found functional relationships among genes from three different duplicated chromosomal regions, reflecting interactions of protein-coding genes and their involvement in common cellular subnetworks. Furthermore, the sharing of common significant biological processes associated with ID has been demonstrated between proteins from the different chromosomal regions. According to our results, we indicate potential molecules and signaling pathways responsible for neuronal wiring that can be deregulated during neurodevelopment and cause ID. Further analysis of gene expression would be necessary to generate experimental data for these conditions in order to show more evidence regarding the association between gene expression and ID.

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Distinct Epileptogenic Mechanisms Associated with Seizuresin Wolf-Hirschhorn Syndrome

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Abstract

Seizures are one of the clinical hallmarks of Wolf-Hirschhorn syndrome (WHS), causing a significant impact on the life quality, still in the first years of life. Even that the knowledge about WHS–related seizure candidate genes has grown, cumulative evidence suggests synergic haploinsufficiency of distinct genes within cellular networks that should be better elucidated. Herein, we evaluated common mechanisms between candidate genes from WHS seizure-susceptibility regions (SSR) and genes globally associated with epilepsy. For this purpose, data from 94 WHS patients delineated by chromosomal microarray analysis were integrated into a tissue-specific gene network with gene expression, drugs, and biological processes. We found functional modules and signaling pathways involving candidate and new genes with potential involvement in the WHS–related seizure phenotype. The proximity among the previous reported haploinsufficient candidate genes (*PIGG*, *CPLX1*, *CTBP1*, *LETM1*) and disease genes associated with epilepsy suggests not just one, but different impaired mechanisms in cellular networks responsible for the balance of neuronal activity in WHS patients, from which neuron communication is the most impaired in WHS–related seizures. Furthermore, *CTBP1* obtained the largest number of drug associations, reinforcing its importance for adaptations of brain circuits and its putative use as a pharmacological target for treating seizures/epilepsy in patients with WHS.

Keywords Wolf-Hirschhorn syndrome · Seizures · *PIGG* · *CPLX1* · *CTBP1* · *LETM1*

Introduction

Wolf-Hirschhorn syndrome (WHS; OMIM #194,190) is a contiguous gene syndrome caused by a hemizygous deletion of the 4p16.3 chromosome region that occurs in 1:20,000 to 1:50,000 births [1]. Terminal deletions are the most frequent causes of WHS, but interstitial deletions, unbalanced translocations, ring chromosomes, and other chromosome rearrangements can also lead to the loss of the critical chromosome segment [2, 3]. Besides, WHS–related deletions differ in size and gene content, impacting clinical findings and severity of the disease [2, 4].

The main phenotype presentations in WHS include typical craniofacial features, prenatal and postnatal growth impairment, intellectual disability, severe delayed psychomotor development, hypotonia, and seizures [2]. Seizures are one of the clinical hallmarks in WHS, occurring in over 90% of the patients, with a common onset at the first years of life and a peak of incidence at 6–12 months of age [4]. Generally, seizures are often triggered by fever, and the most common types are classified as generalized tonic–clonic seizures, tonic spasms, complex partial seizures, and clonic seizures. About 50% of WHS patients present unilateral/generalized clonic or tonic–clonic forms [4]. Nonetheless, even that seizures cause a significant impact on the quality of children's lives, its etiopathology in WHS remains to be elucidated.

The "genotype-first" approach for assessing WHS patients with similar or overlapping chromosomal deletions has enabled the identification of WHS seizure-susceptibility regions (SSR), comprising breakpoints

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encompassing one or more genes [2, 5]. Based on genotype–phenotype studies and the connection between mitochondrial dysfunction and epilepsy, *LETM1* was the first candidate gene related to seizures in WHS [6]. The *LETM1* protein is localized in the inner mitochondrial membrane and functions as a proton-dependent calcium efflux from the mitochondrion, essential for the conservation of tubular complexes and ensemble structure of the respiratory chain [7]. *LETM1* knockdown in rodent models leads to the emergence of seizures [7, 8]. However, the findings in humans are conflicting since WHS patients without seizures harboring deletions involving *LETM1* and WHS patients presenting deletions, not including *LETM1* with seizures, have been described [1, 9–15]. Therefore, three additional candidate genes have been recognized for contributing to WHS–related seizures. *CPLX1* encodes a cytosolic protein with a role in synaptic vesicle exocytosis, essential for the regulation of neurotransmitters release [16], in a manner that *CPLX1* deletion in WHS patients was indicated as a good candidate for seizure phenotype [13, 17]. Furthermore, *CTBP1* haploinsufficiency in patients with unusual deletions and animal models has also been implicated in the pathogenesis of seizures in WHS [10, 17, 18]. *CTBP1* encodes a transcriptional repressor function able to modulate the expression of neuronal genes during development [19]. Moreover, the use of a ketogenic diet in a rat model showed a reduction of epilepsy by promoting CTBP1 activity [20]. Lastly, *PIGG*, a phosphatidylinositol glycan anchor biosynthesis with a crucial role in the membrane anchoring of GPI-anchored proteins [21], was also associated with seizures in WHS patients [11, 22].

Although genotype–phenotype studies identified new WHS–related seizure candidate genes, cumulative evidence suggests that these genes cannot explain the WHS–associated seizures individually. Otherwise, the candidate genes require synergic haploinsufficiency of different genes within cellular networks [5, 15, 22, 23]. Moreover, it is worth mentioning that clinical conditions in contiguous gene syndromes are not merely the result of the genes' physical loss, but also a consequence of global perturbations functionally affecting related genes in the specific disease module across the human interactome [24, 25]. Thus, proteins associated with the same condition are commonly recognized to interact with each other in a local neighborhood more frequently than expected by chance [24, 26].

In this study, we evaluated shared mechanisms between candidate genes in the SSR in WHS and disease genes previously associated with seizures and epilepsy in humans. For this purpose, data from 94 WHS patients delineated by chromosomal microarray analysis (CMA) were integrated with a tissue-specific gene network, and data concerning gene expression, drugs, and signaling pathways were explored.

Connections between candidate and disease genes have been revealed in modules closely related to putative involvement in the pathophysiology of WHS–related seizures.

Methods

Sample Selection and Score Generation

Initially, a literature systematic review in PubMed for WHS patients reported from January 2004 until March 2021 was performed. Only publications comprising patients, in which breakpoints were defined by CMA, as well as patients with clinical records containing information about the presence or absence of seizures were retrieved. A score, similar to that used by Pelleri and colleagues (2019), was applied to assess the probability of the emergence of the seizure phenotype for each 100 Kb region in the 4p16.3 region [27]. Thus, the score was generated according to the deletion size and information related to the presence/absence of seizures in each individual. The deleted genomic position in patients with seizures was considered as $(+ 1)$, but the deleted regions in patients without seizures as $(- 1)$. Furthermore, a two-tailed Fisher's exact test was applied for comparing individuals with a deletion including or not the candidate genes (*CPLX1*, *PIGG*, *CTBP1*, *LETM1*) and their seizure phenotypes. GraphPad prism® was used for analysis. Significance was defined as $p < 0.05$.

Interactome Construction and Subnetwork Generation

The Human Integrated Protein–Protein Interaction Reference (HIPPIE) database (version 2.2) [28] was used to generate the human interactome. Only interactions with a confidence score > 0.4 and the largest connected component were considered for further analysis. The human interactome containing 13,460 proteins and 141,296 interactions provided a network-based platform to search for protein–protein interactions. Protein-coding genes within the SSR genomic position (Chr4:100,000–2,400,000; GRCh38/hg38) with the highest score were retrieved from the UCSC Genome Browser [29]. Next, protein–protein interactions in the SSR were extracted and its first neighbors were selected to expand and generate a subnetwork.

Selection of Gene-Disease Associations

DisGeNET [30] and EpilepsyGene [31] were used to capture disease genes previously involved with epilepsy/seizures and to search for these selected genes in the human interactome subnetwork. DisGeNET is a platform that combines data from UNIPROT, CGI, ClinGen, Genomics England,

CTD (human subset), PsyGeNET, and Orphanet on human gene-disease associations. The EpilepsyGene is a massive database for genes associated with epilepsy with data collected from a set of 818 epilepsy studies. For DisGeNET, the "curated gene-disease associations" file was used, whereas for EpilepsyGene, the "all epilepsy-related genes" file was downloaded.

Construction of a Tissue-Specific Gene Network

The four previous candidate genes (*CPLX1*; *PIGG*; *CTBP1*; *LETM1*) and the seizure-genes present in the subnetwork from the interactome were employed to construct a tissue-specific gene network in the HumanBase [32]. The HumanBase database integrates functional networks in tissues with evidence provided by a set from thousands of experiments in different tissue- and cell lineage–specific functional contexts [32, 33]. Data from co-expression, protein–protein interactions, transcriptional factors binding, microRNA targets, and chemical and genetic perturbation were analyzed. The maximum number of genes in the network has been established for 33 genes with a confidence > 0.10. Cytoscape V.3.7.0. software [34] was used for visualization of the network.

Enrichment Analysis and Search of Drugs

The webserver Enrichr [35] was employed to identify significantly enriched pathways and drugs associated with brain functions in the context of seizures in the tissue-specific gene network. The gene-set libraries used for pathway enrichment analysis were BioPlanet, Elsevier Pathway Collection, Kegg, Reactome, and WikiPathways. For identification of enriched drugs, data from DSigDB, old CMAP up, and DrugMatrix were considered. Only terms with a *p*-value < 0.05 were retrieved.

Expression Detection and Functional Modules

The Genotype-Tissue Expression (GTEx) portal V.8 [36] was used to recognize the expression profiles for genes identified in the tissue-specific gene network in twelve brain regions. For each gene, the transcripts per million (TPM) values for each tissue were obtained. Functional modules were acquired with the Minkowsky algorithm for distance metric and the k-medoid algorithm, a clustering method related to k-means clustering that minimizes the distance between points by partitioning the data set into k groups or modules (Amato, 2019). The expression data from RNA sequencing were employed as node attributes in the network. Modules were generated by the Cytoscape plugin Cluster-Maker V.1.3.1 [37].

Signaling Pathway Reconstruction

We used the PathLinker Cytoscape plugin [38] to identify underlying signaling pathways between candidate and disease genes. PathLinker is a computational tool that automates the reconstruction of any human signaling pathway. The algorithm recognizes multiple short paths from the receptors to transcriptional regulators in a pathway within a protein interaction network [38]. The human interactome subnetwork was used as a background network and genes within the functional modules were selected as source and targets. The parameters used in Pathlinker were (i) k: 50 (number of paths the user seeks); (ii) edge penalty: 1; and (iii) edge unweighted. Lastly, a table with a rank of the shortest paths was generated.

Results and Discussion

The Seizure-Susceptibility Region with the Highest Score Is in the WHS Critical Region

The systematic review on Pubmed retrieved 94 patients with WHS, whose breakpoints were located at 4p16.3 (Fig. 1A). The size of the deletions ranged from 0.4 to 32 Mb. Sixty-six (71%) patients were reported as having seizures and twenty-seven (29%) without it. The description of the selected references [1, 3, 5, 9, 10, 12–14, 17, 22, 39–47], individuals, presence/absence of seizures, and extension of the deletions are detailed in Supplementary Table-1. We agree that absence seizures in some cases can be non-recognizable by parents or even the clinician. EEG can identify absence seizures, but clinical suspicion is often lacking. In some more complicated cases, the video-EEG monitoring could be useful. The scores were generated according to the location of the deletions at 4p16.3. The regions with a high score (> 70) for each 100 Kb in the genome positioned within the range of 0.1–2.4 Mb were identified as regions for susceptibility to seizures. All candidate genes (*PIGG*, *CPLX1*, *CTBP1*, *LETM1*) were included in this region with high susceptibility scores. The genomic region between 0.5 and 0.6 Mb exhibited the highest score (Fig. 1B). Moreover, analysis using two-tailed Fisher's exact test reinforced that the genes *PIGG* ($p < 0.000001$; Attributable Risk (AR) = 0.62), *CPLX1* ($p < 0.000001$; AR = 0.59), *CTBP1* ($p < 0.000001$; AR = 0.45), and *LETM1* ($p = 0.030517$; AR = 0.19) correlate with the presence of seizures in WHS. Therefore, among the candidate genes, *PIGG* showed the strongest correlation with seizures. *PIGG* encodes one member of the phosphatidylinositol glycan (GPI) pathway that anchors a group of over 150 proteins on the cell surface, where several cellular signaling processes occur [21]. Deficiencies in different proteins of the GPI pathway have been associated with
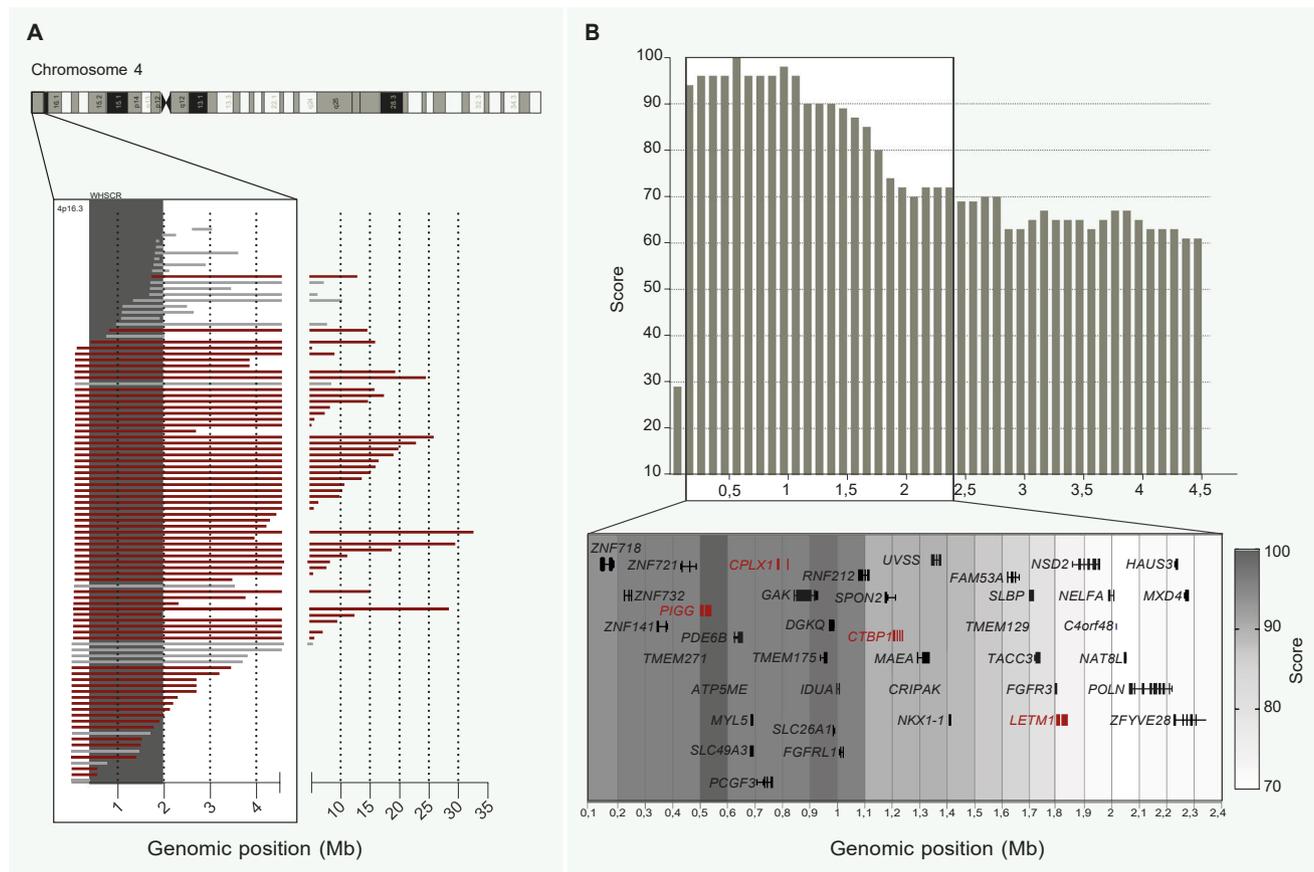
Fig. 1 Size and genomic locations of the 4p16.3 deletions in 94 individuals reported in the literature. **A** The rectangle with a black border highlights the 4p16.3 region. The vertical gray rectangle represents the WHS critical region (~0.4–2 Mb). The deletions in individuals with seizures are shown in red bars. The deletions in individuals without seizures are shown in gray bars. **B** The scores for each

100 Kb were generated according to the location of the deletions in 4p16.3. The vertical rectangle with a black border shows the regions with the highest scores (> 70). Below: region of susceptibility to seizures with scores in the range of 0.1–2.4 Mb and the gene content disposed by genomic position (Mb). Candidate genes are displayed in red

seizures in humans [48, 49]. In addition, homozygous or compound heterozygous pathogenic variants in *PIGG* cause seizures in individuals from consanguineous families [50]. Although our results are consistent with the previously refined (0.36–0.56 Mb) seizure susceptibility region in WHS [13, 22], there are still individuals with a deletion in this region without seizures. Therefore, it is strongly suggested that the combination of genes in the 4p16.3 region and other genes of the interactome cooperates in cellular networks to generate seizures in WHS. Therefore, approaches directed at isolated genes are unlikely to elucidate the mechanisms responsible for the WHS seizures.

Different Candidates and Disease Genes Can Be Responsible for WHS–Related Seizures

The extraction of protein-coding genes from the seizure-susceptibility region in the interactome (Fig. 1B) and their first neighbors resulted in a subnetwork composed of 658

nodes/proteins and 8,513 links/interactions (Supplementary Table-2). In the subnetwork, we identified 15 disease genes (*SMC1A*, *VAMP2*, *AP2M*, *MECP2*, *AKT1*, *AUTS2*, *NOS1*, *FLNA*, *AAK1*, *ARX*, *MKLN1*, *NCOR2*, *HDAC4*, *DDX50*, *HBS1L*) previously associated with epilepsy/seizures in the DisGeNET and EpilepsyGene databases [30, 31].

Next, we investigated possible relationships between candidate and disease genes. As expected, brain tissue represented the most significant data of interactions, so we generated a brain-specific network (BSN) composed of 33 genes and 290 interactions. The most connected gene among the candidates was *CTBP1*, interacting with the other 27 genes. The topological location of *CTBP1* in the network reinforces its function in controlling other genes through its role as a transcriptional repressor. In this sense, *LETM1* (11), *PIGG* (9), and *CPLX1* (8) are less central in the network with more peripheral functions in the cell. Interestingly, the four candidate genes are connected through *CTBP1*. Regarding the proximity to disease genes, *LETM1*, *PIGG*, and *CPLX1*

interact with two different disease genes (*LETM1*: *ARX* and *AAK1*; *PIGG*: *SMC1A* and *AUTS2*; *CPLX1*: *VAMP2* and *DDX50*) in their neighborhood of the BSN (Fig. 2A). Interestingly, *CTBP1* emerges linked to ten other disease genes in the BSN. CTBP1 can mediate synaptic-nuclear signaling mechanisms involved in functional changes in neurons during adaptations of brain circuits [51]. Furthermore, the repressor complex including *CTBP1* represses seizures induced by BDNF expression upon the use of anti-glycolytic compounds in an epileptogenic rat model [20].

Disease genes in the BSN have been linked to awakening epilepsy, myoclonic-astatic epilepsy, atonic absence seizures, generalized absence seizures, and myoclonic seizures, besides including the forms present in WHS, such as generalized tonic–clonic seizures, complex partial seizures, and

clonic seizures [4]. Therefore, the observation of functional relationships among genes involved in distinct types of epilepsy and candidate genes in the BSN suggests the involvement of different seizure-related mechanisms in WHS, other than those previously known. Thus, our data aid to explain why genotype–phenotype correlation studies found more than one candidate gene in the 4p16.3 region associated with the same phenotype.

Pathways Associated with Neuron Communication May Be the Most Impaired in WHS Seizures

To identify which specific processes are regulated by the BSN, we carried out functional enrichment analysis for genes with seizure-related biological processes (Fig. 2B).
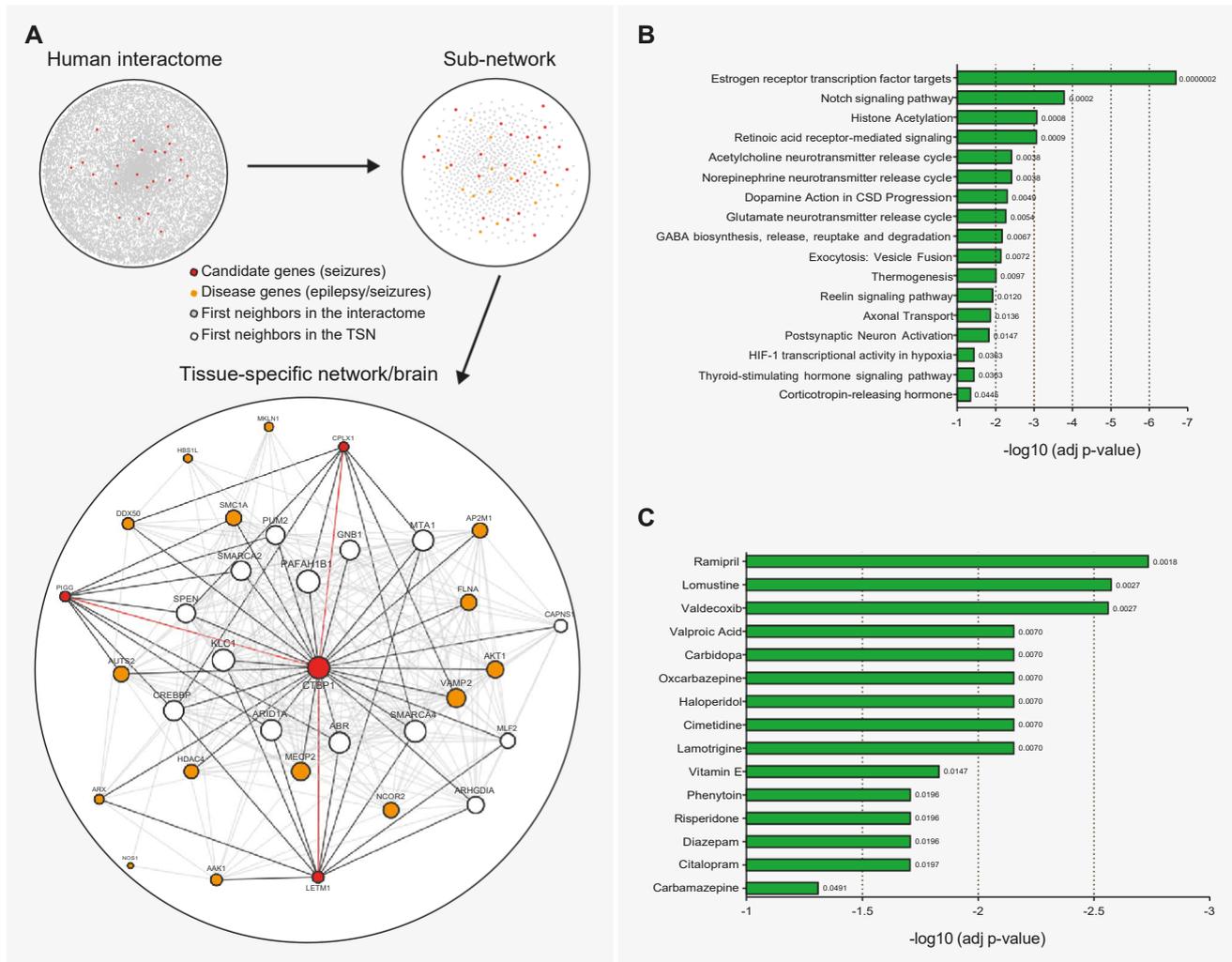

Fig. 2 From interactome to brain-specific network. **A** left: curated human interactome composed of 13,460 proteins and 141,296 interactions. In the network, proteins are nodes connected by interactions or edges. Right: expanded subnetwork from the interactome containing 658 proteins and 8513 interactions. Below: BSN with 33 genes/nodes and 290 interactions. Red nodes are candidate genes. Orange nodes are known disease genes. Red edges show interactions between candidate genes. **B** Functional enrichment analysis of the BSN with biological processes potentially involved in seizures with adj $p < 0.05$. **C** Most enriched drugs associated with BSN with adj $p < 0.05$

Estrogen receptor transcription factor targets (adj $p = 2.4^{-7}$), notch signaling pathway (adj $p = 1.6^{-4}$), and histone acetylation (adj $p = 8.4^{-4}$) were the most enriched terms in the BSN [52–54]. Moreover, many pathways related to neuron communication were seen in our analysis, being related to epileptogenic mechanisms such as the acetylcholine neurotransmitter release cycle, norepinephrine neurotransmitter release cycle, glutamate neurotransmitter release cycle, GABA biosynthesis, release, reuptake and degradation, dopamine action in cortical spreading depression progression, and exocytosis (vesicle fusion) [55]. The functional enrichment values for the most significant pathways involved with altered brain functions can be seen in Fig. 2B. Seizures originate from abnormal neuronal transmission in the brain due to an imbalance of excitatory and inhibitory neurotransmitters, such as glutamate and GABA. Furthermore, changes in the expression of receptors and ion channels activated by neurotransmitters are mechanisms involved in epilepsy pathogenesis [55]. In this sense, *CPLX1* was the most enriched candidate gene for the biological processes described above, since it encodes a presynaptic regulatory protein with an essential role in the modulation of neurotransmitter release [16].

The Repressor Complex Including CTBP1 May Be a Good Pharmacological Target

In an attempt to recognize targets and reinforce the importance of candidate and disease genes involved in WHS–related seizures, we searched for potential drugs that could act in the BSN. The significant values for the main enriched drugs are detailed in Fig. 2C. Many of the drugs found in our analyses are already used in the treatment of seizures/epilepsy, such as ramipril, lomustine, haloperidol, and risperidone [56], including first-prescribed drugs for seizures in WHS, such as valproic acid, diazepam, and oxcarbazepine [57, 58]. We also found drugs responsible to induce epilepsy if taken in overdoses, such as citalopram [59], and molecules with potential clinical intervention in seizures and epileptogenic processes involving excitotoxicity, neuroinflammation, and lipid peroxidation, such as vitamin E [60]. Interestingly, in a large cohort of individuals with WHS, lamotrigine showed an excellent result among the antiepileptic drugs, while carbamazepine, phenytoin, and oxcarbazepine were able to exacerbate seizures or achieve a poor seizure control performance [58]. Among the candidate genes, *CTBP1* obtained the largest number of drug associations (lamotrigine, phenytoin, lomustine, valproic acid, valdecoxib, and oxcarbazepine). These results reveal the importance of the repressor complex including CTBP1 for adaptations of brain circuits during development, suggesting that it may be a good pharmacological target for treating seizures/epilepsy in patients with WHS.

Genes in BSN Are Widely Expressed in the Regions Commonly Affected by Epilepsy

The correct functioning of the brain depends on the fine regulation of the expression of multiple genes, in which imbalances can lead to disturbances in the neuronal network associated with seizures. Therefore, we investigated the expression of genes present in the BSN in different regions of the human brain. The frontal cortex, cerebellum, and cortex showed the most significant expression levels (Fig. 3A). Abnormal neuronal connections in the frontal cortex, which covers the front part of the frontal lobe, and cerebellum are implicated in epilepsy in humans. Frontal cortex epilepsy is the second most common type of focal epilepsy due to the abnormal neuronal connections [61]. The cerebellum can already play a role in seizure networks, in which functional and structural changes in the cerebellum have been frequently observed in patients with epilepsy [62]. Different abnormalities in the EEG can be observed in patients with WHS, and the epileptic foci are commonly located in the posterior temporo-parieto-occipital regions [63, 64], represented by the cortex with prominent gene expression.

The ten top genes with the highest expression in the brain included the disease genes *VAMP2* and *AP2M1*, and one of the candidate genes for seizure-susceptibility in WHS (*CPLX1*) (Fig. 3B). *VAMP2*, involved with the fusion of synaptic vesicles during the release of neurotransmitters, is associated with epilepsy in humans in cases of non-synonymous variants [65]. *AP2M1* acts in the clathrin-dependent endocytosis and missense variants in this gene lead to epileptic encephalopathy [66]. Expression of *CPLX1* and *VAMP2* may be relevant in different regions of the brain, where they act in the same pathways responsible for the release cycle of neurotransmitters [67].

Moreover, we analyzed the expression in different regions of the brain separately. Candidate genes have higher expression in the cerebellum and cortex (except for *PIGG*). *CPLX1* and *CTBP1* are widely expressed in all surveyed regions of the brain, probably due to their multiple functions in the cell. In contrast, *LETM1* and *PIGG* show a more heterogeneous scenario with expression levels close to zero for some regions, indicating that their expressions are specific for particular brain regions. Besides, among the disease genes, there is considerable variability in brain expression levels (Fig. 3C), which could reflect the involvement of these genes in several types of epilepsy described above, affecting the activity of different regions of the brain.

Different Functional Modules and Signaling Pathways Involve Candidate and Disease Genes

We investigated how close the candidate genes are to each other or whether they are grouped in modules with disease
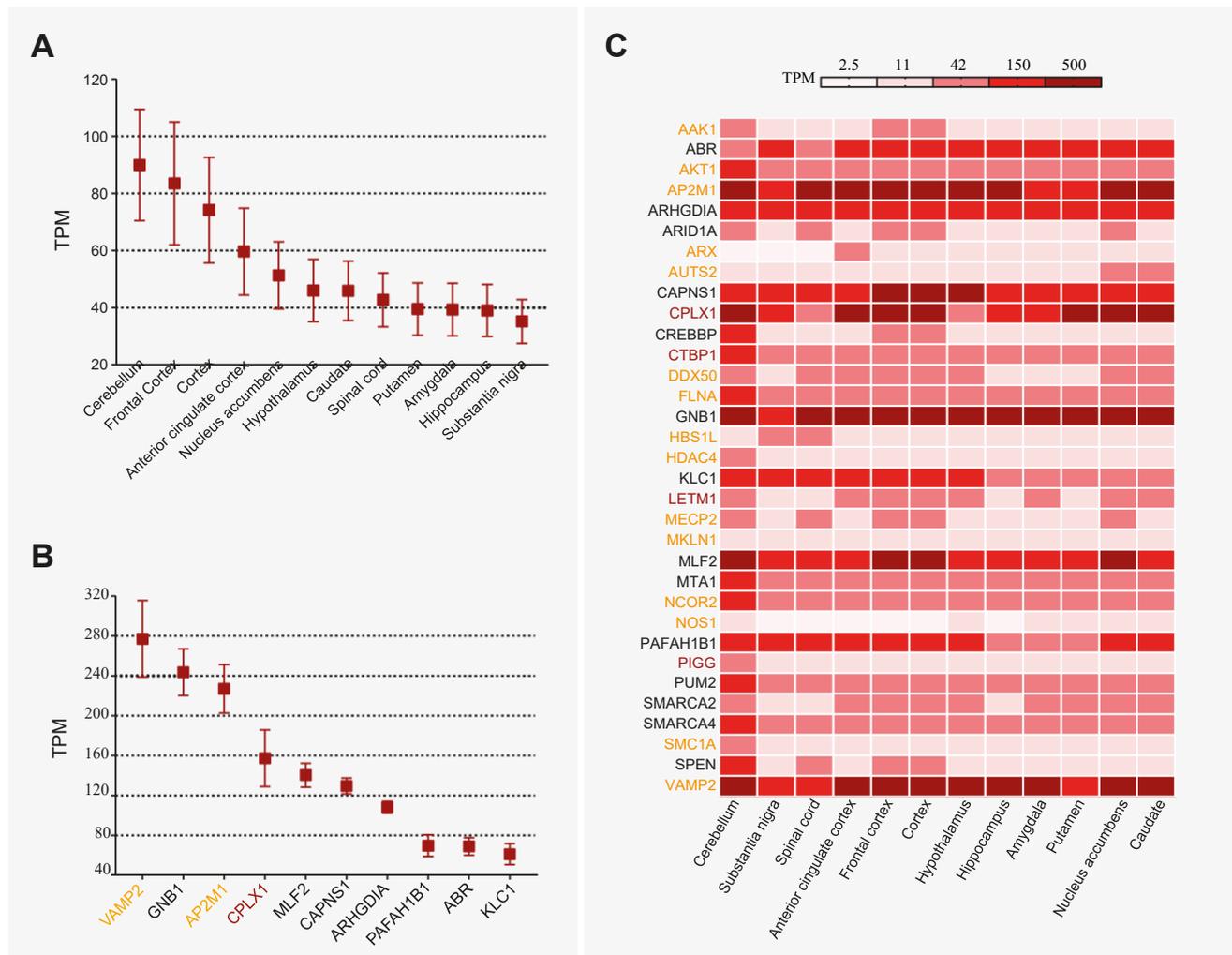
Fig. 3 Expression data for 33 genes in brain-specific network. **A** The average expression value is shown for each region of the brain. **B** The average expression value is shown for the ten most expressed genes in the brain. **C** Heat map of gene expression in different regions of the brain. Values are shown in transcripts per million (TPM). Candidate genes are in red. Disease genes from DisGeNET and EpilepsyGene are in orange

genes. Therefore, we used topological and expression data to generate functional modules capable of indicating clues about the pathogenic mechanisms related to seizures in WHS.

In the first module, *PIGG* is present with many other disease genes and interacts directly with *SMC1A* and *AUTS2* (Fig. 4A). The gene *SMC1A* encodes part of the cohesin complex involved in chromosome cohesion during the cell cycle and acts on the regulation of transcription [68]. The loss-of-function and heterozygous truncation variants in *SMC1A* cause therapy-resistant epilepsy and severe early-onset epilepsy with cluster seizures in females, respectively [69, 70]. *AUTS2* regulates neuronal gene expression in processes present in axon elongation and neuronal migration during brain development [71]. Intragenic deletions in *AUTS2* were identified in

individuals with juvenile myoclonic epilepsy or with features of atypical benign partial epilepsy [72]. However, regarding signaling pathways, we identified *APP* (amyloid-beta precursor protein) with potential impact in the signaling of the candidate gene *PIGG* and the disease genes *HDAC4* and *AAK1* (Fig. 4B). *APP* is a membrane receptor involved in physiological functions of neurons essential to neurite growth, neuronal adhesion, and synaptic function [73]. Moreover, *APP* is found with increased levels of protein expression in the temporal cortex and hippocampus of the refractory epilepsy patients [74]. Furthermore, *APP* is associated with the term fever-induced seizure (HP:0,032,894) together with other members of the phosphatidylinositol glycan pathway (*PIGP* and *PIGQ*), indicating a possible pathway altered in WHS–related seizures.
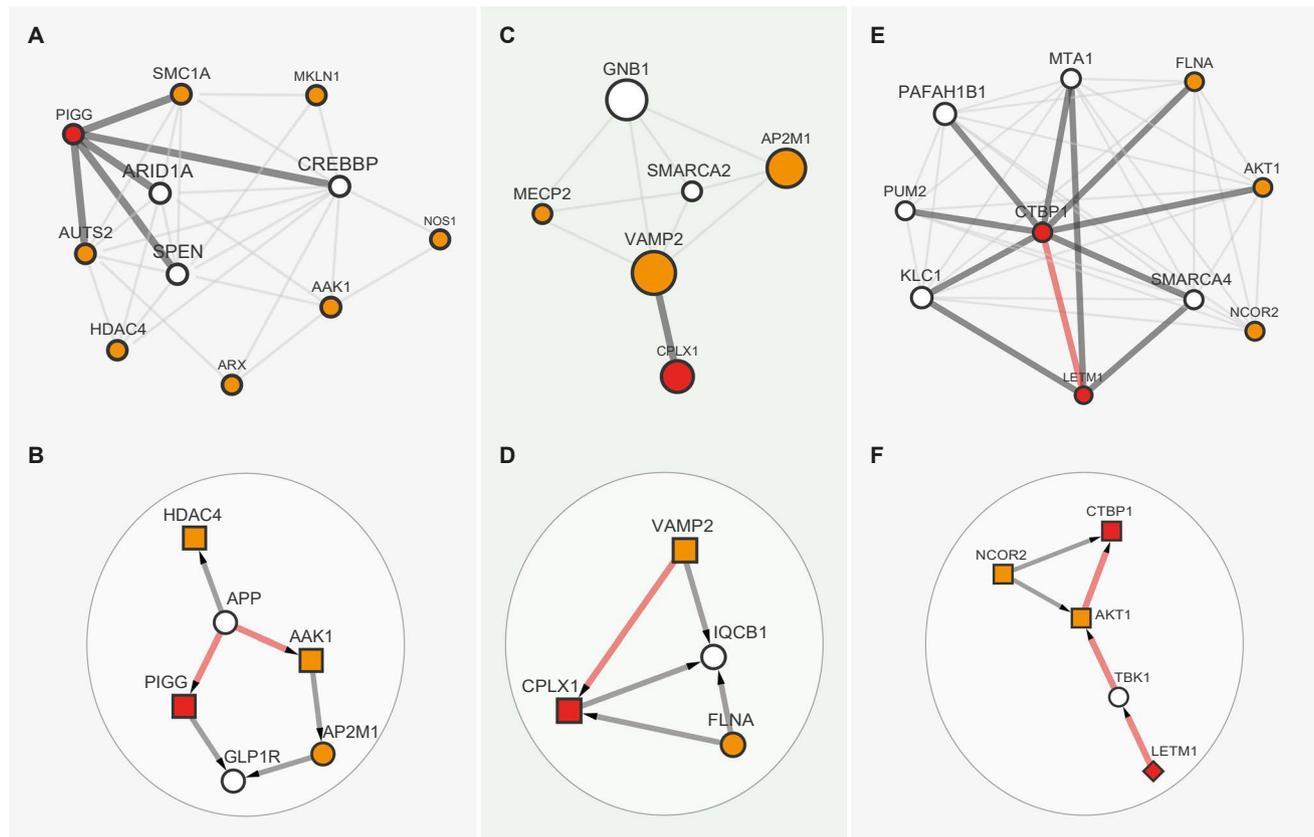
Fig. 4 Functional modules. Modules and signaling pathways show the candidate genes: **A/B** *PIGG*, **C/D** *CPLX1*, **E/F** *LETM1*, and *CTBP1*. Red nodes represent candidate genes, whereas orange nodes are known seizure-related genes. Transcriptional regulators are shown as squares and receptors as a rhombus. Red edges show interactions and short paths between candidate and disease genes

CPLX1 is a target of miR-135a-5p and is found dysregulated in humans and rats with temporal lobe epilepsy [75]. In the second module, *CPLX1* encompasses three disease genes (*MECP2*, *AP2M1*, *VAMP2*) (Fig. 4C), interacting directly with the presynaptic *VAMP2* gene, the most highly expressed gene in the brain and associated with cryptogenic epilepsy and electroencephalography abnormalities in humans [65, 76]. The involvement of *CPLX1* and *VAMP2* in signaling pathways with potential epileptogenic can be indicated by high scores of the shortest signaling pathway identified (Fig. 4D). Both *CPLX1* and *VAMP2* act in the SNARE complex and modulate synaptic vesicles involved with glutamate, dopamine, and acetylcholine neurotransmitter release cycle [16, 65, 67].

The candidate genes *LETM1* and *CTBP1* were found differentially expressed in blood cells of pediatric epileptic patients and a model of hyperthermic seizure in rats, respectively [77, 78]. In the third module, *LETM1* and *CTBP1* are grouped with three genes associated with seizures (*FLNA*, *AKT1*, *NCOR2*), but only *CTBP1* interacts directly with the disease genes *FLNA* and *AKT1* (Fig. 4E). *FLNA* encodes a protein with a role in anchoring several transmembrane proteins to the actin cytoskeleton, thus exhibiting important functions during contacts and adherent junctions between cells during brain development [79]. Loss-of-function variants in *FLNA* are involved in the X-linked inherited condition of periventricular nodular heterotopia with or without epilepsy [80]. *AKT1* is a serine/threonine kinase that modulates the AKT/mTOR pathway in the integration of newborn neurons during adult neurogenesis, covering the correct neuron positioning, dendritic development, and synapse formation [81]. Pathogenic variants in genes participating in the PI3K/AKT pathway can lead to epileptogenic brain malformations [82]. Besides that, our results indicate a possible involvement of this pathway in mediating signals between *LETM1* and *CTBP1* through *TBK1* (Fig. 4F), in which phosphorylates and activates AKT1 are dependent on PI3K signaling [83]. In this case, both candidate genes can contribute synergistically to WHS–related seizures.

Conclusions

Our study investigated previously candidate genes for epilepsy in WHS patients in the context of the human interactome and identified many new genes with potential involvement in the WHS–related seizure phenotype. The proximity among haploinsufficient genes in the WHS and disease genes globally associated with epilepsy suggests different impaired mechanisms in cellular networks responsible for the balance of neuronal activity in WHS patients. Therefore, the pathophysiology of seizures in WHS may be a consequence of distinct functional modules that encompass candidate genes from the critical seizure-susceptibility region, converging with other pathways and genes associated with epilepsy in humans. Further experimental analysis could validate these findings in iPSC–derived neurons.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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