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Identyfikacja czynników genetycznych, biochemicznych i komórkowych wpływających na przebieg mukopolisacharydoz ze szczególnym uwzględnieniem choroby Sanfilippo

Identification of genetic, biochemical and cellular factors influencing the course of mucopolysaccharidoses with particular reference to Sanfilippo disease

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Choć to moje nazwisko widnieje na stronie tytułowej, praca ta nie powstałaby, gdyby nie wsparcie, które otrzymywałam przez te wszystkie lata.

Chciałabym w tym miejscu podziękować wszystkim, którzy zachęcali mnie do rozwijania moich pomysłów i nie pozwolili mi zrezygnować, kiedy miałam naprawdę dość.

Jednak przede wszystkim, chciałabym podziękować tym, którzy zachęcili mnie, żeby w ogóle zacząć.

Dziękuję

Spis treści

Streszczenie rozprawy doktorskiej

Mukopolisacharydozy (MPS) to heterogenna grupa dziedzicznych zaburzeń metabolicznych, klasyfikowanych jako lizosomalne choroby spichrzeniowe. Główną przyczyną MPS są mutacje w genach kodujących enzymy lizosomalne, które odpowiadają za rozkład glikozoaminoglikanów (GAG). W wyniku niedoboru lub całkowitego braku aktywności tych enzymów dochodzi do akumulacji GAG w komórkach różnych tkanek i narządów, co z kolei prowadzi do ich stopniowego uszkodzenia i dysfunkcji. MPS charakteryzują się szerokim spektrum objawów klinicznych, takich jak dysmorfia twarzy, niski wzrost, deformacje kostne, przewlekłe bóle stawowe, hepatosplenomegalia, zmętnienie rogówki, niedosłuch, trudności oddechowe oraz powikłania sercowo-naczyniowe.

Do niedawna wyróżniano 11 typów i podtypów MPS, klasyfikowanych na podstawie rodzaju spichrzanego GAG oraz enzymu, którego dotyczy defekt. W ostatnich latach opisano nowo odkryte, ultrarzadkie jednostki o fenotypie MPS: MPS X oraz tzw. syndrom MPS-plus. MPS X jest spowodowana mutacją w genie *ARSK*, kodującym arylsulfatazę K, co prowadzi do akumulacji siarczanu dermatanu. Z kolei syndrom MPS-plus (MPS-PS) jest zaburzeniem o fenotypie zbliżonym do MPS, w którym obserwuje się podwyższony poziom GAG, jednak bez defektu któregokolwiek ze znanych enzymów lizosomalnych degradujących GAG. Patomechanizm choroby pozostaje nie do końca poznany, mimo zidentyfikowania mutacji w genie *VPS33A*, którego produkt uczestniczy w szlakach endocytozy i autofagii. Ze względu na brak klasycznego defektu enzymu degradującego GAG oraz pewne różnice w obrazie klinicznym (min. zaburzenia czynności nerek i nieprawidłowści układu hematopoetycznego), syndrom MPS-plus nie jest obecnie włączany do formalnej klasyfikacji MPS, lecz zaliczany do tzw. „chorób podobnych do MPS" (ang. *MPS-like disorders*). Aktualny podział obejmuje więc 12 "klasycznych" typów i podtypów MPS, które wraz z ich charakterystyką przedstawiono w **Tabeli 1**.

Dodatkowo MPS można podzielić na typy neuropatyczne (MPS I, II, III oraz VII), w których dominują objawy neurologiczne oraz formy nieneuropatyczne (IV, VI, IX oraz X), gdzie funkcje układu nerwowego pozostają nienaruszone. Objawy neuropatyczne pojawiające się w przypadku ciężkich postaci MPS I i II, wszystkich podtypach MPS III oraz w MPS VII obejmują zaburzenia poznawcze, agresję, hiperaktywność, zaburzenia rytmu dobowego, bezsenność, padaczkę, drgawki, trudności w mowie, utratę słuchu i zmiany osobowości.

Tabela 1. Charakterystyka opisanych dotychczas typów/podtypów MPS.

Typ/podtyp MPS	Nazwa zwyczajowa	Zmutowany gen	Spichrzany GAG	Komponenta neurodegeneracyjna
MPS I	Zespół Hurler, Scheie lub Hurler-Scheie	*IDUA*	Siarczan dermatanu, siarczan heparanu	Tak (ciężkie przypadki)
MPS II	Zespół Huntera	*IDS*	Siarczan dermatanu, siarczan heparanu	Tak (ciężkie przypadki)
MPS IIIA	Zespół Sanfilippo	*SGSH*	Siarczan heparanu	Tak
MPS IIIB		*NAGLU*		
MPS IIIC		*HGSNAT*		
MPS IIID		*GNS*		
MPS IVA	Zespół Morquio	*GALNS*	Siarczan keratanu	Może wystąpić w skutek drugo- i trzeciorzędowych zaburzeń
MPS IVB		*GLB1*	Siarczan keratanu, siarczan chondroityny	
MPS VI	Zespół Maroteaux-Lamy	*ARSB*	Siarczan dermatanu	Nie
MPS VII	Zespół Sly	*GUSB*	Siarczan dermatanu, siarczan heparanu, siarczan chondroitytny	Tak
MPS IX	Zespół Natowicza	*HYAL1*	Kwas hialuronowy	Nie
MPS X	-	*ARSK*	Siarczan dermatanu	Dane niejednoznaczne

Diagnostyka w kierunku MPS po zauważeniu objawów mogących sugerować chorobę metaboliczną, rozpoczyna się od analizy biochemicznej, w której wykrywa się nadmiar GAG wydalanego z moczem. Potwierdzenie diagnozy wymaga oceny aktywności specyficznych enzymów lizosomalnych w leukocytach lub fibroblastach pacjenta. Współczesne techniki molekularne umożliwiają identyfikację mutacji w genach, których dysfunkcje warunkują każdy typ MPS, co pozwala na precyzyjne określenie typu/podtypu choroby i podjęcie odpowiedniego leczenia, jeśli takie jest dostępne. Proces diagnostyczny jest jednak niezwykle trudny i czasochłonny ze względu na rzadkie występowanie tych chorób, zróżnicowany obraz kliniczny (zarówno między poszczególnymi typami jak i w obrębie jednego typu), postępujący i wielonarządowy/układowy charakter choroby. Ze względu na rzadkość występowania, rozwój objawów w różnym czasie i ich podobieństwo do innych (częstszych) chorób, MPS są często błędnie diagnozowane. Najczęściej rozpoznawane są jako zaburzenia neurologiczne (tj.: zaburzenia ze spektrum autyzmu, nadpobudliwość psychoruchowa czy niepełnosprawność intelektualna), choroby reumatologiczne i ortopedyczne (tj.: młodzieńcze idiopatyczne zapalenie stawów, choroba Perthesa, krzywica czy dystrofia mięśniowa). Postawienie prawidłowej diagnozy może trwać nawet latami, a samo rozpoznanie MPS to wciąż jedynie połowa sukcesu. Ważną częścią procesu diagnostycznego jest prawidłowa identyfikacja typu/podtypu choroby w

znacznym stopniu rzutująca na planowanie/dostępność terapii. Problemy diagnostyczne pacjentów z MPS, choroby z którymi mogą być one mylone, a także sposoby zapobiegania temu zjawisku, opisane zostały przeze mnie i współautorów w artykule przeglądowym Wiśniewska K, Wolski J, Gaffke L, Cyske Z, Pierzynowska K, Węgrzyn G. (2022) Misdiagnosis in mucopolysaccharidoses. *Journal of Applied Genetics*; 63(3):475-495.

Zarejestrowane metody leczenia MPS obejmują przede wszystkim enzymatyczną terapię zastępczą (ERT, ang. *enzyme replacement therapy*) oraz przeszczep hematopoetycznych komórek macierzystych (HSCT, ang. *hematopoietic stem cell transplantation*). ERT polega na podawaniu brakującego enzymu w celu zmniejszenia spichrzania GAG, co łagodzi niektóre objawy somatyczne. Stosowana jest w praktyce klinicznej w MPS I, MPS II, MPS IVA, MPS VI i MPS VII. Nie wpływa ona jednak na objawy neurologiczne (z uwagi na brak możliwości przekraczania bariery krew-mózg przez cząsteczkę enzymu) oraz zaburzenia układu kostno-szkieletowego (brak możliwości dotarcia enzymu do kości i chrząstek). HSCT, polegająca na przeszczepieniu prawidłowych krwiotwórczych komórek macierzystych w celu odbudowy układu krwiotwórczego i odpornościowego pacjenta, stosowana jest najczęściej u najmłodszych pacjentów z MPS I i II. Prowadzone są badania nad wieloma innymi podejściami terapeutycznymi, które pozostają na razie w fazie badań klinicznych lub przedklinicznych. Obejmują one tarapię genową, terapię redukcji syntezy substratu (SRT, ang. *substrate reduction therapy*) lub terapię molekularnymi chaperonami. Należy zaznaczyć, że dostępne metody terapeutyczne są leczeniem objawowym (za wyjątkiem terapii genowej), w dodatku ich możliwości są mocno ograniczone, a efekty nie są w pełni zadowalające.

Długo uważano, że magazynowane GAG są główną, a nawet jedyną, przyczyną MPS. Jednak fakt, że żadna z terapii ukierunkowanych na obniżenie poziomu GAG (ERT, SRT, terapia genowa) nie prowadziła do całkowitej korekty objawów zaczął budzić wątpliwości co do mechanizmów zaburzeń obserwowanych w przebiegu MPS. Uwagę zwróciło również szerokie spektrum objawów występujące pomiędzy pacjentami różnych typów. Przykładem takiej zmienności klinicznej może być MPS III charakteryzująca się neurodegeneracją, ciężkimi objawami neuropsychiatrycznymi i stosunkowo łagodną komponentą somatyczną oraz MPS IV charakteryzująca się prawidłowym rozwojem ośrodkowego układu nerwowego (OUN) ale równocześnie ciężkimi schorzeniami kości i stawów. Nawet jeśli tak duża zmienność objawów może wynikać ze spichrzania różnego rodzaju GAG (co nie jest wykluczone) to w MPS I i MPS II, w których gromadzą się te same rodzaje GAG (siarczan dermatanu i heparanu), ich cechy biochemiczne oraz objawy

mogą się znacząco różnić. Dla przykładu, zmętnienie rogówki pojawia się u pacjentów z MPS I, ale nie z MPS II. Natomiast zmiany skórne (charakterystyczne grudki) są obecne w MPS II, a nieobecne w MPS I. Także nadpobudliwość i agresywne zachowania są jedną z cech charakterystycznych dla pacjentów z neuropatycznym typem MPS II, ale nie MPS I, gdzie jeśli nawet występuje znaczny niedobór poznawczy, to pacjenci są łagodni i spokojni.

Uwagę należy zwrócić również na MPS typu III (choroba Sanfilippo), na którą położono szczególny nacisk w obrębie przedstawionej rozprawy doktorskiej. MPS III jako jedna z dwóch typów MPS (obok MPS IV) podzielona została na podtypy (MPS III A, B, C i D) z uwagi na podłoże genetyczne, deficyt innego enzymu lizosomalnego, jednak prowadzący do spichrzania tego samego GAG (w MPS III siarczanu heparanu). Podobnie jak w opisanym powyżej przypadku MPS I i II, pacjenci MPS III różnych podtypów potrafią znacząco się różnić klinicznie. Zaburzenia ze spektrum autyzmu występują u prawie 30% pacjentów z MPS IIIA i tylko u 8% pacjentów z MPS IIIC. Padaczka występuje u 17% pacjentów z MPS IIIA i 8% pacjentów z MPS IIIC. Podobnie, cechy dysmorfii twarzy i powiększenie wątroby, występują odpowiednio u 94% i 56% pacjentów z MPS IIIB oraz 85% i 39% pacjentów z MPS IIIC. Podtypy te ewidentnie różnią się także wiekiem pierwszego wystąpienia poszczególnych objawów. Na przykład średni wiek rozpoznania MPS IIIB wynosi od 2,5 do 5 lat, a MPS IIIC od 4,5 do 19 lat. Długość życia pacjentów również wykazuje znaczne zróżnicowanie i wynosi 15-18 lat dla MPS IIIA, 17-19 lat dla MPS IIIB i 19-34 lat dla MPS IIIC (brak dostępnych danych dla MPS IIID).

Wszystkie dotychczasowe obserwacje wskazują, że akumulacja glikozoaminoglikanów (GAG) jest procesem kluczowym dla patogenezy MPS, jednak nie może być jedynym czynnikiem determinującym obraz kliniczny. Również resztkowa aktywność enzymu lizosomalnego, choć istotna dla ogólnej ciężkości choroby – łagodniejsze postacie w przypadku zachowanej częściowej aktywności, cięższe przy jej całkowitym braku – nie wyjaśnia dużej zmienności fenotypowej pomiędzy pacjentami. Podobnie, różnice w rodzaju gromadzonych GAG są faktem biochemicznym, ale same w sobie nie tłumaczą, dlaczego poszczególne typy i podtypy MPS manifestują się tak odmiennym zakresem i dynamiką objawów, szczególnie w odniesieniu do układu nerwowego.

Coraz więcej badań wskazuje, że przebieg MPS jest modulowany przez dodatkowe procesy komórkowe i molekularne, które mogą działać niezależnie od akumulacji GAG lub rozwijać się wtórnie do niej. Należą do nich m.in. zaburzenia równowagi redoks, przewlekła aktywacja odpowiedzi zapalnej, stres oksydacyjny czy zakłócenia w regulacji

ekspresji genów. Mechanizmy te mogą w istotny sposób modyfikować przebieg choroby, wpływając na różnorodność objawów i tempo ich progresji.

W związku z tym **celem mojej pracy doktorskiej była identyfikacja czynników genetycznych, biochemicznych i komórkowych, które mogą modulować przebieg MPS, ze szczególnym uwzględnieniem choroby Sanfilippo.** Podejście to ma na celu lepsze zrozumienie, dlaczego pacjenci z teoretycznie tym samym defektem enzymatycznym i typem spichrzanego GAG mogą prezentować tak odmienny obraz kliniczny. Badania prowadziłam głównie na modelu fibroblastów skóry pobranych od pacjentów ze wszystkimi typami/podtypami MPS opisanymi do 2020 roku (kiedy rozpoczynałam badania). Charakterystykę wykorzystywanych linii komórkowych przedstawiłam w **Tabeli 2**.

Tabela 2. Charakterystyka wykorzystanych linii komórkowych

Typ MPS	**Defektywny enzym**	**Typ mutacji**	**Nr. katalogowy ****
MPS I	α-L-iduronidase	p.Trp402Ter/p.Trp402Te	GM00798
MPS II	2-iduronate sulfatase	p.His70ProfsTer29	GM13203
MPS IIIA	N-sulfoglucosamine sulfhydrolase	p.Glu447Lys/p.Arg245His	GM00879
MPS IIIB	α-N-acetylglucosaminidase	p.Arg626Ter/p.Arg626Ter	GM00156
MPS IIIC	Acetyl-CoA:α-glycosaminide acetyltransferas	p.Gly262Arg/pArg509Asp	GM05157
MPS IIID	N-acetylglucosamine 6-sulfatase	p.Arg355Ter/p.Arg355Ter	GM05093
MPS IVA	N-acetylglucosamine-6-sulfate sulfatase	p.Arg386Cys/p.Phe285Ter	GM00593
MPS IVB	β-galactosidase	p.Trp273Leu/p.Trp509Cys	GM03251
MPS VI	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	Not determined	GM03722
MPS VII	N-acetylgalactosamine 4-sulfatase	p.Trp627Cys/p.Arg356Ter	GM00121
MPS IX	Hyaluronidase	p.Glu268Lys/c.37bp-del;14bp-ins at nt 1361	GM17494
Linia kontrolna	N/A	N/A	N/A

*Numery katalogowe są zgodne z opisem linii komórkowej w Instytucie Coriell.

Chociaż fibroblasty mogą wydawać się nieoczywistym modelem do badania neurodegeneracji, mają one kilka istotnych cech wspólnych z neuronami. Oba typy komórek pochodzą z ektodermy, co sugeruje wspólne mechanizmy molekularne, w tym szlaki sygnałowe i procesy regulacji ekspresji genów. Cecha ta ma kluczowe znaczenie dla wyjaśnienia patogenezy chorób neurodegeneracyjnych. Ponadto fibroblasty wykazują zaburzenia w szlakach komórkowych, takich jak stres oksydacyjny, autofagia i metabolizm, które są również istotnymi czynnikami w neurodegeneracji. Fibroblasty mogą odzwierciedlać ogólnoustrojowe zmiany komórkowe, które wpływają na funkcjonowanie neuronów, zwłaszcza w zaburzeniach genetycznych, w których konsekwencje mutacji pozostają niejasne. Co więcej, fibroblasty znajdują się w pobliżu naczyń krwionośnych, w oponach mózgowych oraz w splocie naczyniówkowym mózgu i rdzenia kręgowego, gdzie odgrywają kluczową rolę w utrzymaniu funkcji OUN. Warto zauważyć też, że fibroblasty są łatwo dostępne dzięki minimalnie inwazyjnym procedurom, umożliwiającym regularne monitorowanie pacjentów, a ich stabilne hodowle ułatwiają długoterminowe badania, co czyni je cennym modelem do badań nad mechanizmami neurodegeneracyjnymi.

Część badań wykonałam również na tkankach pobranych z mysiego modelu MPS IIIB.

W badaniach nie uwzględniłam MPS X, typu MPS odkrytego dopiero po rozpoczęciu moich badań, a także syndromu MPS-plus-. Dostęp do komórek pacjentów cierpiących na te dwa typy MPS nawet po rozpoczęciu projektu był ograniczony z uwagi na bardzo małą liczbę pacjentów (nie przekraczającą 30 na całym świecie) oraz na fakt, że większość z nich pochodzi z objętej konfliktem zbrojnym Rosji.

Jak wspomniałam wcześniej, MPS dzieli się na 12 typów/podtypów ze względu na rodzaj spirzchanego GAG jak i defektywny enzym. Dodatkowo, MPS można podzielić na typy neuronopatyczne (MPS I, II, IIIA, IIIB, IIIC, IIID, VII) i nieneuronopatyczne (MPS IV, VI, IX, X) ze względu na obecność/brak cech neurodegeneracji. Jest to o tyle ciekawy podział, że przy z pozoru tej samej przyczynie choroby jaką jest spichrzanie GAG, widać wyraźne zróżnicowanie w występowaniu objawów somatycznych i tych związanych z neurodegenracją.

Obecnie nie ma możliwości jednoznacznego stwierdzenia, czy u pacjenta z MPS wystąpią objawy neurologiczne, a jeśli wystąpią trudno próbować prognozować tempo ich progresji czy stopień nasilenia. Możliwość przewidywania takiego komponentu miałaby

istotne znaczenie dla pacjentów i ich rodzin. Po pierwsze, stwarzałoby to możliwość wczesnego zastosowania terapii ukierunkowanych na ochronę funkcji neurologicznych, w tym dokanałowej lub dokomorowej ERT oraz terapii genowej podawanej dokanałowo, aby bezpośrednio oddziaływać na ośrodkowy układ nerwowy. Po drugie, umożliwiłoby to lepsze planowanie opieki specjalistycznej i rehabilitacyjnej, co mogłoby poprawić jakość życia i spowolnić postęp objawów. Wczesne rozpoznanie ryzyka neurodegeneracji pomogłoby również w dostosowaniu oczekiwań i przygotowaniu opiekunów na specyficzne wyzwania związane z postępującymi zmianami neurologicznymi. Dlatego tak ważne jest poszukiwanie markerów neurodegeneracji w MPS.

Wykorzystując bibliotekę RNA-seq, opracowaną we wcześniejszych latach przez zespół, w którym wykonywałam badania, przeprowadziłam analizę transkryptomiczną mającą na celu wyselekcjonowanie genów, których zmiany poziomu ekspresji w stosunku do komórek kontrolnych są specyficzne wyłącznie dla neuronopatycznych typów/podtypów MPS (I, II, IIIA, IIIB, IIIC, IIID i VII), przy jednoczesnym braku zmian poziomu ekspresji w typach nie-neuronopatycznych. Wyniki łącznie pokazały ponad 300 transkryptów, które mogą być związane z zaburzeniami układu nerwowego (dokładnie 322 unikalnych genów, których ekspresja jest znacząco zmieniona w co najmniej 2 neuronopatycznych typach/podtypach MPS, ale nie w typach nieneuronopatycznych). Do transkryptów ulegających zmianom w poziomie ekspresji w co najmniej 5 neuropatycznych typach/podtypach MPS (nie ulegając jednocześnie zaburzeniom poziomu ekspresji w typach nie-neuropatycznych) należą transkrypty o zwiększonej ekspresji np. *PDIA3* (kodujący izomerazę disiarczkową białek A3, PDIA3) jak i o obniżonej np. *ARL6IP6* (kodujący białko oddziałujące z GTPazą 6 podobne do ARF 6, ARL6IP6). Zaburzenia funkcji ARL6IP6 mogą prowadzić do nieprawidłowego różnicowania neuronów oraz zaburzeń homeostazy mitochondriów i retikulum endoplazmatycznego. Ponadto, białko to pełni rolę w przetwarzaniu APP w β-amyloid. Zmniejszenie jego poziomu może zatem wyjaśniać wzrost poziomu β-amyloidu i jego prekursorów, a także ich tendencję do agregacji. Dane literaturowe dotyczące badań z użyciem mysiego modelu *PDIA3*$^{-/-}$ wykazały, że brak *PDIA3* znacząco zmniejsza efektywność apoptozy, łagodzi stan zapalny i stres oksydacyjny, a ponadto poprawia funkcje poznawcze i zmniejsza objętość stłuczenia spowodowanego urazem w modelu urazowego uszkodzenia mózgu. Dodatkowo wykazano zależny od wieku wzrost poziomu PDIA3 w mózgowiu myszy z chorobą Alzheimera w przeciwieństwie do jego spadku wraz z wiekiem u myszy zdrowych. Dane te sugerują zdecydowanie, że wysoki poziom ekspresji *PDIA3* wykryty w przypadku

przeprowadzonej analizy w komórkach neuropatycznych typów/podtypów MPS może więc także częściowo przyczyniać się do neurodegeneracji.

Opisane powyżej wyniki badań nad *ARL6IP6* i *PDIA3* oraz innymi genami, sugerujące możliwe zastosowanie ich jako biomarkerów neurodegeneracji w MPS, opisane zostały w artykule: Wiśniewska K, Żabińska M, Szulc A, Gaffke L, Węgrzyn G, Pierzynowska K. (2024) The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease. *International Journal of Molecular Sciences*; 25(24):13447.

Jako, że interesowały mnie badania z zakresu funkcjonowania całej komórki, podobnej analizy dotyczącej mechanizmów neurodegeneracji dokonałam z podziałem na funkcjonowanie poszczególnych organelli komórkowych. Przy użyciu metod transkryptomicznych zidentyfikowałam geny związane z funkcją i budową poszczególnych organelli komórkowych (na podstawie bazy danych Ensembl), których poziomy ekspresji są zmienione w neuropatycznych typach MPS w porównaniu z komórkami kontrolnymi, pozostając niezmienionymi w nieneuropatycznych typach MPS. Ta analiza wskazała, że geny, których ekspresja jest zaburzona w neuropatycznym typie MPS, często są związane ze strukturami lub funkcjami jądra komórkowego, retikulum endoplazmatycznego lub aparatu Golgiego. Doświadczenia z użyciem mikroskopii fluorescencyjnej oraz elektronowej potwierdziły zaburzenia głównie w obrębie aparatu Golgiego, które pojawiały się znacznie częściej w komórkach pobranych od pacjentów neuropatycznych. Wyniki tych analiz przedyskutowano oraz porównano z tymi występującymi w innych chorobach neurodegeneracyjnych i zaburzeniach neurologicznych w artykule: Wiśniewska K, Gaffke L, Żabińska M, Węgrzyn G, Pierzynowska K. (2024) Cellular Organelle-Related Transcriptomic Profile Abnormalities in Neuronopathic Types of Mucopolysaccharidosis: A Comparison with Other Neurodegenerative Diseases. *Current Issues in Molecular Biology*; 46(3):2678-2700.

W kolejnym etapie badań skupiłam się na dwóch typach MPS – MPS III i MPS IV. Wybór ten był podyktowany kilkoma czynnikami. Są to jedyne postacie MPS, w których podział na podtypy wynika z różnic w defekcie molekularnym, a nie jak w przypadku MPS I – z odmienności przebiegu klinicznego.

Szczególnie intrygujący stał się dla mnie typ MPS III, w którym dominują objawy ze strony OUN. W tym typie gromadzony jest jedynie siarczan heparanu (HS) – związek,

którego akumulacja jest wiązana z neurodegeneracją również w innych chorobach, takich jak choroba Alzheimera. Neurodegeracja jest również tym, co m.in. różni typ IVA i IVB. Zaburzenia układu nerwowego mogą pojawiać się w przebiegu MPS IVB chociaż dominują w nim zaburzenia układu szkieletowego. Z tych powodów MPS III i IV są wyjątkowo wartościowym punktem odniesienia w badaniach nad neurodegeneracją w MPS.

W pierwszej kolejności, wykorzystując dane transkryptomiczne uzyskane z modelu komórkowego, dokonałam selekcji tych genów, które ulegały znacząco różnej ekspresji pomiędzy podtypami MPS III. Liczba transkryptów ulegających zróżnicowanej ekspresji była największa pomiędzy podtypami IIIA vs. IIIB, IIIA vs. IIID, IIIB vs. IIID i IIIC vs. IIID i wynosiła aż ponad 400. Podobnej analizy dokonałam pomiędzy podtypami MPS IV (IVA i IVB), w których liczba transkryptów wykazujących zróżnicowaną ekspresję wynosiła poniżej 100. Procesy komórkowe, w których uczestniczą produkty wspomnianych transkryptów, w dużej mierze obejmują metabolizm komórkowy i jego regulację, ale także odpowiedź komórki na stymulację lub komunikację komórkową (zgodnie z bazą danych Ensembl). Część tych transkryptów wykazało bardzo duże, bo ponad 16-krotne, różnice w poziomie ekspresji ($\log_2$fold change [FC, krotność zmiany] >4 lub <-4), szczególnie pomiędzy MPS IIIA i IIID, MPS IIIB i IIIC oraz MPS IIIB i IIID. Do transkryptów tych należą cząsteczki mRNA pochodzące od genów, których produkty związane są z funkcjami rybosomów (*RPLP2, RPL23, RPL10*), utrzymaniem prawidłowej struktury tkanki łącznej (*COL4A1, COL4A2, COMP*), zaangażowane w sygnalizację wewnątrzkomórkową (*NME2, WISP2, SRFP1*) oraz receptory i czynniki komórkowe (*RARRES2, CRLF1, IGFBP5, TFPI2*).

Podczas gdy duża zmienność ekspresji genów warunkujących prawidłową funkcję tkanki łącznej nie jest zaskakująca (została już opisana w kontekście patogenezy MPS), znaczna liczba takich genów zaangażowanych w funkcje rybosomów jest nowym odkryciem. Rybosomy są kluczowymi organellami zaangażowanymi w ekspresję genów, a uzyskane wyniki sugerują znaczne zróżnicowanie w wydajności syntezy białek w ogóle, co może być przyczyną różnego przebiegu choroby u pacjentów z czterema podtypami MPS III. Co więcej, zaburzenia ekspresji genu *RPL10* wskazano także w innych schorzeniach neurologicznych np. zaburzeniach ze spektrum autyzmu, mikrocefalii sprzężonej z chromosomem X, synodromicznej niepełnosprawności intelektualnej sprzężonej z chromosomem X lub padaczce. Zatem obniżony poziom ekspresji tych genów, wykryty w MPS IIIA w porównaniu do MPS IIIC i IIID, w pewnym stopniu mógłby wyjaśniać częstsze występowanie zachowań autystycznych i cięższe epilepsje u dzieci z

tym pierwszym podtypem choroby Sanfilippo niż w innych podtypach. Innym przykładem genu związanego z padaczką jest *RARRES2*, kodujący chemerynę, której wysoki poziom jest wykrywany we krwi dzieci z padaczką idiopatyczną jako czynnik prognostyczny. Przeprowadzone przeze mnie analizy transkryptomiczne wykazały, że poziom ekspresji tego genu jest podwyższony w MPS IIIA w porównaniu z IIID, a epilepsja występuje częściej w pierwszym wymienionym podtypie niż w innych. Jak już wspomniano, tak dużych zmian w poziomie ekspresji poszczególnych genów nie zaobserwowano pomiędzy fibroblastami MPS IVA i IVB. W rzeczywistości różnice fenotypowe między pacjentami z różnymi podtypami MPS IV są znacznie mniejsze niż między różnymi podtypami MPS III. Sugeruję zatem, że zmiany we wzorach ekspresji genów mogą warunkować zróżnicowanie przebiegu choroby u pacjentów cierpiących na różne podtypy MPS III. Artykuł Wiśniewska K, Gaffke L, Krzelowska K, Węgrzyn G, Pierzynowska K. (2022) Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes. *Gene*; 812:146090 opisuje wyżej podsumowane rezultaty badań i wskazuje na możliwość korelacji między globalnymi wzorami ekspresji genów a rozwojem różnych objawów u pacjentów cierpiących na różne podtypy MPS III.

W drugim etapie badań zajęłam się identyfikacją tych transkryptów, które ulegają wspólnym zmianom poziomu ekspresji w stosunku do komórek kontrolnych (pobranych od osób zdrowych) we wszystkich podtypach MPS III (IIIA, IIIB, IIIC i IIID) oraz IV (IVA i IVB). Nigdy wcześniej nie przeprowadzono analizy zaburzeń ekspresji genów wspólnych dla wszystkich podtypów MPS III i MPS IV. Wyniki tej analizy wskazały na 45 transkryptów o podobnych zmianach poziomu ekspresji we wszystkich podtypach MPS III oraz aż 150 takich transkryptów w obu podtypach MPS IV w stosunku do komórek kontrolnych. Identyfikując te transkrypty, które cechują się najwyższymi wartościami krotności zmiany ekspresji ($log_2FC>3$ lub <-3) zauważyłam trzy, które wykazywały wysoki poziom zmian ekspresji we wszystkich podtypach zarówno MPS III jak i MPS IV w porównaniu do komórek kontrolnych. Są to ulegające zwiększonej ekspresji geny *PFN1* oraz *MFAP5*, a także ulegający obniżonej ekspresji *MMP12*. Geny te kodują kolejno profilinę (PFN1), białko 5 związane z mikrofibrylami (MFAP5) oraz metalopeptydazę macierzy 12 (MMP12). W komórkach wszystkich podtypów MPS III i IV przeprowadziłam immunodetekcję tych trzech białek technikami immunofluorescencyjnymi oraz western-blotting, która wskazała na wzrost poziomów PFN1 i MFAP5 w komórkach MPS III i IV oraz obniżenie poziomu MMP12 w komórkach MPS III. Ponieważ transkrypty te ulegały zmianom w poziomie ekspresji we wszystkich

typach/podtypach MPS III, interesującym wydawało się zagadnienie zależności ich poziomu od poziomu spichrzanych GAG. Poziom GAG w komórkach MPS został obniżony przy pomocy jednego z flawonoidów, genisteiny, która hamuje autofosforylację receptora naskórkowego czynnika wzrostu (EGFR), prowadząc ostatecznie do zahamowania ekspresji genów kodujących syntetazy GAG. W takich warunkach ponownie oceniono poziomy białek PFN1, MFAP5 i MMP12 a wyniki tej analizy wskazały, że wraz z obniżeniem poziomu GAG spada też poziom PFN1. Co więcej, wyciszenie ekspresji genu *PFN1* powodowało także spadek poziomu GAG, wskazując na możliwą nową ścieżkę terapeutyczną.

Profiliny kodowane przez gen *PFN1* to małe białka oddziałujące z cytoszkieletem komórkowym, gdzie wpływają na polimeryzację aktyny. Uczestniczą one również w transporcie błonowym, sygnalizacji komórkowej, transkrypcji i autofagii. Cytoszkielet odgrywa bardzo ważną rolę w synaptogenezie i neurotransmisji, a także bierze udział w rozwoju układu nerwowego i plastyczności dojrzałego mózgu. Jego zaburzenia często obserwuje się w chorobach psychicznych, takich jak schizofrenia, choroba afektywna dwubiegunowa, autyzm lub silna depresja. Jest to bardzo istotne, ponieważ problemy behawioralne są jednym z neuropatycznych objawów MPS. Co więcej, pacjenci z MPS III są czasami błędnie diagnozowani jako osoby z zaburzeniami ze spektrum autyzmu.

Mutacje w genie *PFN1* wykrywa się także u pacjentów z innymi chorobami neurodegeneracyjnymi, takimi jak stwardnienie zanikowe boczne, zespół łamliwego chromosomu X, rdzeniowy zanik mięśni, choroba Huntingtona i Parkinsona oraz adrenoleukodystrofia. Fakt ten wydaje się szczególnie interesujący, gdyż w wielu tych chorobach pojawiają się agregaty białkowe. Niektóre doniesienia literaturowe wskazują, że do ich powstania miałby przyczyniać się wzrost poziomu ekspresji właśnie *PFN1*. Wskazano bowiem, że niektóre zmutowane białka PFN1 wykazują właściwości podobne do prionów i działają jako czynniki, które wyzwalają konwersję białka TDP43 do toksycznych stanów konformacyjnych.

Podsumowując, wskazane powyżej geny oraz ich produkty mogą być zaangażowane we wspólne ścieżki patogenezy różnych typów/podtypów MPS, z czego poziom PFN1 wydaje się być powiązany z poziomem GAG. Przedstawione powyżej wyniki badań dotyczących głównie zaburzeń poziomu PFN1 w komórkach MPS III i MPS IV opisane zostały w artykule: Wiśniewska K, Żabińska M, Gaffke L, Szulc A, Walter BM, Węgrzyn G, Pierzynowska K. (2024) Shared Gene Expression Dysregulation Across

Subtypes of Sanfilippo and Morquio Diseases: The Role of PFN1 in Regulating Glycosaminoglycan Levels. *Frontiers in Bioscience (Landmark Ed.)*; 29(12):415.

Wskazane powyżej wyniki dotyczące zaburzeń poziomu profiliny w komórkach MPS oraz jej udział w patogenezie innych chorób neurologicznych skłoniły mnie do wykonania szerokiej analizy poziomów białek, które znane są ze swojej tendencji do tworzenia agregatów w różnych chorobach neurodegeneracyjnych. W komórkach MPS III wszystkich podtypów dokonałam immunodetekcji technikami immunofluorescencyjnymi oraz western-blotting poziomów beta-amyloidu i jego prekursora (białka APP), białka tau i jego hiper-fosforylowanej formy (p-tau), a także alfa-synukleiny oraz białka TDP43 (białko TDP-43 nigdy wcześniej nie było badane w kontekście patogenezy MPS). Wyniki tych analiz wskazały na podwyższone poziomy białek APP, β-amyloidu, tau i TDP43 we wszystkich podtypach MPS III, a podwyższone poziomy p-tau i α-synukleiny we wszystkich oprócz MPS IIIC. Ponadto, widoczne pod mikroskopem fluorescencyjnym agregaty tworzone przez β-amyloid i tau obecne były we wszystkich podtypach MPS III, a agregaty tworzone przez p-tau, TDP43 i α-synukleinę we wszystkich podtypach MPS III z wyjątkiem IIIC. Podwyższony poziom wymienionych powyżej białek został zaobserwowany także w mózgowiu myszy stanowiących model MPS IIIB.

Jako że oznaczony w komórkach poziom GAG był najniższy ze wszystkich badanych linii właśnie w MPS IIIC (mimo, że w dalszym ciągu znacznie wyższy niż w komórkach kontrolnych), to nasunęło się pytanie o udział GAG w tworzeniu agregatów białkowych. Dlatego podobnie jak poprzednio, poziom GAG w komórkach MPS został obniżony przy pomocy genisteiny, a poziomy APP, β-amyloidu, tau, p-tau, TDP43 i α-synukleiny oraz ich agregatów zostały ponownie zbadane. Wyniki tych doświadczeń zaskakująco wskazały na obniżenie poziomu zarówno wszystkich wymienionych powyżej białek jak i tworzonych przez nich agregatów w komórkach poddanych działaniu genisteiny, z wyjątkiem α-synukleiny, której podwyższony poziom pozostawał niezależny od poziomu GAG. Sugeruje to ewidentny związek pomiędzy tworzeniem agregatów niektórych białek oraz poziomem GAG, co otwiera szereg pytań o rolę GAG w powstawaniu tego typu agregatów. Wyniki tych doświadczeń zostały opisane w artykule: Wiśniewska K, Rintz E, Żabińska M, Gaffke L, Podlacha M, Cyske Z, Węgrzyn G, Pierzynowska K. (2024) Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients. *Biochemical and Biophysical Research Communications*; 733:150718.

Ostatnim elementem mojej rozprawy doktorskiej było opracowanie przeglądu literatury poświęconego zaburzeniom behawioralnym i problemom ze snem w MPS III, czyli objawom, które są szczególnie charakterystyczne dla tego schorzenia i wyjątkowo obciążające zarówno dla pacjentów, jak i ich rodzin. Analizując dostępne dane, starałam się wyjaśnić podłoże zaburzeń, które obejmuje nakładające się procesy patologiczne w OUN. Potencjalne mechanizmy obejmują akumulację HS i wynikającą z niej neurodegenerację, przewlekły stan zapalny, stres oksydacyjny, dysfunkcję mitochondriów oraz zaburzenia w metabolizmie neurotransmiterów. Wszystkie te czynniki prowadzą do zaburzenia funkcji oraz struktur mózgu odpowiedzialnych za kontrolę zachowania, emocji i regulację rytmu snu i czuwania.

Najbardziej charakterystyczne objawy to nadpobudliwość psychoruchowa, impulsywność, napady agresji, stereotypie ruchowe, lęk oraz problemy ze snem, takie jak trudności w zasypianiu, częste wybudzenia, skrócenie całkowitego czasu snu czy odwrócenie rytmu dobowego. Co istotne, symptomy te bardzo często pojawiają się już we wczesnych stadiach choroby, zanim rozwiną się wyraźne objawy somatyczne. W przeglądzie omówiłam także możliwości łagodzenia tych zaburzeń – zarówno farmakologiczne (m.in. leki przeciwdepresyjne, przeciwpsychotyczne, melatonina), jak i niefarmakologiczne, takie jak modyfikacje środowiska, utrzymywanie stałych rytuałów snu, terapie behawioralne czy wsparcie psychologiczne. Zwróciłam jednak uwagę, że skuteczność tych metod jest zróżnicowana, a potrzeba dobrze zaprojektowanych badań klinicznych w tym obszarze pozostaje aktualna. W omawianej pracy chciałam przede wszystkim zwrócić uwagę na codzienne problemy związane z chorobą, z którymi muszą mierzyć się rodziny pacjentów, wskazać te najbardziej znaczące oraz omówić potencjalne możliwości radzenie sobie z nimi. Artykuł ten to praca: Wiśniewska K, Wolski J, Anikiej-Wiczenbach P, Żabińska M, Węgrzyn G, Pierzynowska K. (2025) Behavioural rdisorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications. *Eur Child Adolesc Psychiatry;* 34(6):1795-1816. Ta praca jest dla mnie szczególnie istotna, ponieważ zwraca uwagę na aspekt często pomijany w badaniach nad mechanizmami choroby i potencjalnymi terapiami przyczynowymi. Choć poszukiwanie skutecznych metod leczenia przyczynowego pozostaje priorytetem, nie można zapominać, że dopóki takie terapie nie będą dostępne, konieczne jest równoległe rozwijanie i udoskonalanie leczenia objawowego, które w chwili obecnej realnie poprawia jakość życia pacjentów i ich rodzin.

Podsumowując całość przedstawionych badań można wskazać, że odkrycia opisane w mojej rozprawie doktorskiej dostarczają nowego wglądu w mechanizmy molekularne leżące u podstaw patogenezy MPS oraz mechanizmy zróżnicowania między neuropatycznym i nie-neuropatycznym przebiegiem choroby. Ponad to moje badania pokazały jak zarówno jak wiele różnic i podobieństw jest między poszczególnymi podtypami MPS III, na każdym poziomie. Dodatkowo całość badań wskazuje na liczne podobieństwa pomiędzy neuropatycznymi typami MPS, a innymi chorobami neurodegeneracyjnymi, co może wskazywać nowe kierunki dla badań nad metodami leczenia.

Prace wchodzące w skład rozprawy doktorskiej

1. Wiśniewska, Karolina et al. “Misdiagnosis in mucopolysaccharidoses.” *Journal of applied genetics* vol. 63,3 (2022): 475-495. doi:10.1007/s13353-022-00703-1
2. Wiśniewska, Karolina et al. “The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease.” *International journal of molecular sciences* vol. 25,24 13447. 15 Dec. 2024, doi:10.3390/ijms252413447
3. Wiśniewska, Karolina et al. “Cellular Organelle-Related Transcriptomic Profile Abnormalities in Neuronopathic Types of Mucopolysaccharidosis: A Comparison with Other Neurodegenerative Diseases.” *Current issues in molecular biology* vol. 46,3 2678-2700. 21 Mar. 2024, doi:10.3390/cimb46030169
4. Wiśniewska, Karolina et al. “Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes.” *Gene* vol. 812 (2022): 146090. doi:10.1016/j.gene.2021.146090
5. Wiśniewska, Karolina et al. “Shared Gene Expression Dysregulation Across Subtypes of Sanfilippo and Morquio Diseases: The Role of PFN1 in Regulating Glycosaminoglycan Levels.” *Frontiers in bioscience (Landmark edition)* vol. 29,12 (2024): 415. doi:10.31083/j.fbl2912415
6. Wiśniewska, Karolina et al. “Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients.” *Biochemical and biophysical research communications* vol. 733 (2024): 150718. doi:10.1016/j.bbrc.2024.150718
7. Wiśniewska, Karolina et al. “Behavioural disorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications.” *European child & adolescent psychiatry* vol. 34,6 (2025): 1795-1816. doi:10.1007/s00787-025-02661-5

Abstract of doctoral dissertation

Mucopolysaccharidoses (MPS) are a heterogeneous group of hereditary metabolic disorders classified as lysosomal storage diseases. The leading causes of MPS are mutations in genes that encode lysosomal enzymes responsible for breaking down glycosaminoglycans (GAGs). As a result of the deficiency or complete lack of activity of these enzymes, GAGs accumulate in the cells of various tissues and organs, which in turn leads to their gradual damage and dysfunction. A wide range of clinical symptoms, such as facial dysmorphia, short stature, bone deformities, chronic joint pain, hepatosplenomegaly, corneal clouding, hearing loss, respiratory difficulties and cardiovascular complications, characterise MPS.

Until recently, 11 types and subtypes of MPS were distinguished, classified based on the type of stored GAG and the enzyme affected by the defect. In recent years, newly discovered, ultra-rare entities with an MPS phenotype have been described: MPS X and the so-called MPS-plus syndrome. MPS X is caused by a mutation in the *ARSK* gene, which encodes arylsulfatase K, leading to the accumulation of dermatan sulphate. MPS-plus syndrome (MPS-PS), on the other hand, is a disorder with a phenotype similar to MPS, in which elevated GAG levels are observed, but without a defect in any of the known lysosomal GAG-degrading enzymes. The pathomechanism of the disease remains unclear, despite the identification of a mutation in the *VPS33A* gene, whose product is involved in endocytosis and autophagy pathways. Due to the absence of a classic defect in a GAG-degrading enzyme and certain differences in the clinical picture (including renal dysfunction and haematopoietic abnormalities), MPS-plus syndrome is not currently included in the formal classification of MPS. Still, it is classified as *an "MPS-like disorder"*. Therefore, the current classification includes 12 "classic" types and subtypes of MPS, which are presented in **Table 1** along with their characteristics**.**

Additionally, MPS can be divided into neuropathic types (MPS I, II, III and VII), in which neurological symptoms predominate, and non-neuropathic forms (IV, VI, IX and X), in which the functions of the nervous system remain intact. Neuropathic symptoms occurring in severe forms of MPS I and II, all subtypes of MPS III and in MPS VII include cognitive impairment, aggression, hyperactivity, circadian rhythm disorders, insomnia, epilepsy, seizures, speech difficulties, hearing loss and personality changes.

Table 1. Characteristics of the types/subtypes of MPS described to date.

MPS type/subtype	Common name	Mutated gene	Stored GAG	Neurodegenerative component
MPS I	Hurler, Scheie or Hurler-Scheie syndrome	*IDUA*	Dermatan sulphate, heparan sulphate	Yes (severe cases)
MPS II	Hunter syndrome	*IDS*	Dermatan sulphate, heparan sulphate	Yes (severe cases)
MPS IIIA	Sanfilippo syndrome	*SGSH*	Heparan sulphate	Yes
MPS IIIB		*NAGLU*		
MPS IIIC		*HGSNAT*		
MPS IIID		*GNS*		
MPS IVA	Morquio syndrome	*GALNS*	Keratan sulphate	May occur as a result of second- and third-order disorders ( )
MPS IVB		*GLB1*	Keratan sulphate, chondroitin sulphate	
MPS VI	Maroteaux-Lamy syndrome	*ARSB*	Dermatan sulphate	No
MPS VII	Sly syndrome	*GUSB*	Dermatan sulphate, heparan sulphate, chondroitin sulphate	Yes
MPS IX	Natowicz syndrome	*HYAL1*	Hyaluronic acid	No
MPS X	-	*ARSK*	Dermatan sulphate	Inconclusive data

Diagnosis of MPS, after noticing symptoms that may suggest a metabolic disorder, begins with a biochemical analysis to detect excess GAG excreted in the urine. Confirmation of the diagnosis requires assessment of the activity of specific lysosomal enzymes in the patient's leukocytes or fibroblasts. Modern molecular techniques enable the identification of mutations in the genes whose dysfunction causes each type of MPS, allowing for precise determination of the type/subtype of the disease and initiation of appropriate treatment, if available. However, the diagnostic process is extremely difficult and time-consuming due to the rare occurrence of these diseases, the varied clinical picture (both between individual types and within a single type), and the progressive and multi-organ/systemic nature of the disease. Due to their rarity, the development of symptoms at different times and their similarity to other (more common) diseases, MPSs are often misdiagnosed. They are most often diagnosed as neurological disorders (i.e. autism spectrum disorders, psychomotor hyperactivity or intellectual disability), rheumatological and orthopaedic diseases (i.e. juvenile idiopathic arthritis, Perthes disease, rickets or muscular dystrophy). It can take years to make a correct diagnosis, and the diagnosis of MPS itself is still only half the battle. An important part of the diagnostic process is the proper identification of the type/subtype of the disease, which has a significant impact on planning and treatment availability. Diagnostic problems in patients with MPS, diseases with which they may be confused, and ways to prevent this phenomenon are described by

my co-authors and I in a review article, Wiśniewska K, Wolski J, Gaffke L, Cyske Z, Pierzynowska K, Węgrzyn G. (2022) Misdiagnosis in mucopolysaccharidoses. *Journal of Applied Genetics;* 63(3):475-495.

Registered MPS treatment methods primarily include *enzyme replacement therapy* (ERT*)* and *haematopoietic stem cell transplantation* (HSCT*)*. ERT involves administering the missing enzyme to reduce GAG storage, which alleviates some somatic symptoms. It is used in clinical practice in MPS I, MPS II, MPS IVA, MPS VI and MPS VII. However, it does not affect neurological symptoms (due to the inability of the enzyme molecule to cross the blood-brain barrier) or musculoskeletal disorders (due to the inability of the enzyme to reach bones and cartilage). HSCT, which involves transplanting healthy haematopoietic stem cells to rebuild the patient's haematopoietic and immune systems, is most commonly used in the youngest patients with MPS I and II. Research is being conducted on many other therapeutic approaches, which are currently in the clinical or preclinical trial phase. These include gene therapy, *substrate reduction* therapy (S*RT)* and molecular chaperone therapy. It should be noted that the available therapeutic methods are symptomatic treatments (with the exception of gene therapy), and their capabilities are severely limited and the effects are not entirely satisfactory.

It has long been believed that stored GAGs are the main, or even the only, cause of MPS. However, the fact that none of the therapies aimed at reducing GAG levels (ERT, SRT, gene therapy) led to a complete correction of symptoms began to raise doubts about the mechanisms of the disorders observed in the course of MPS. The wide spectrum of symptoms occurring among patients of different types also attracted attention. An example of such clinical variability is MPS III, characterised by neurodegeneration, severe neuropsychiatric symptoms and a relatively mild somatic component, and MPS IV, characterised by normal development of the central nervous system (CNS) but at the same time severe bone and joint disorders. Even if such a wide variability of symptoms may result from the storage of different types of GAGs (which cannot be ruled out), in MPS I and MPS II, where the same types of GAGs (dermatan sulphate and heparan sulphate) accumulate, their biochemical characteristics and symptoms may differ significantly. For example, corneal clouding occurs in patients with MPS I, but not in those with MPS II. On the other hand, skin changes (characteristic papules) are present in MPS II. Still, absent in MPS I. Hyperactivity and aggressive behaviour are also distinctive features of patients with the neuropathic type of MPS II, but not MPS I, where, even if there is significant cognitive impairment, patients are gentle and calm.

Attention should also be paid to MPS type III (Sanfilippo syndrome), which was given particular emphasis in the doctoral dissertation presented. MPS III, as one of two types of MPS (alongside MPS IV), has been divided into subtypes (MPS III A, B, C and D) due to its genetic basis, a deficiency of a different lysosomal enzyme, but leading to the storage of the same GAG (heparan sulphate in MPS III). As in the case of MPS I and II described above, patients with different subtypes of MPS III can exhibit significant clinical variations. Autism spectrum disorders occur in almost 30% of patients with MPS IIIA and only 8% of patients with MPS IIIC. Epilepsy occurs in 17% of patients with MPS IIIA and 8% of patients with MPS IIIC. Similarly, facial dysmorphic features and hepatomegaly occur in 94% and 56% of patients with MPS IIIB and 85% and 39% of patients with MPS IIIC, respectively. These subtypes also differ markedly in the age of onset of specific symptoms. For example, the average age of diagnosis for MPS IIIB is 2.5 to 5 years, and for MPS IIIC it is 4.5 to 19 years. Life expectancy also varies considerably, ranging from 15 to 18 years for MPS IIIA, 17 to 19 years for MPS IIIB, and 19 to 34 years for MPS IIIC (no data available for MPS IIID).

All observations to date indicate that the accumulation of glycosaminoglycans (GAGs) is a key process in the pathogenesis of MPS; however, it cannot be the sole factor determining the clinical picture. Similarly, differences in the type of GAGs accumulated are a biochemical fact, but do not in themselves explain why individual types and subtypes of MPS manifest themselves with such a wide range and dynamics. Similarly, the differences in the type of GAGs accumulated are a biochemical fact, but do not in themselves explain why different types and subtypes of MPS manifest such a wide range and dynamics of symptoms, particularly concerning the nervous system.

A growing body of research indicates that the course of MPS is modulated by additional cellular and molecular processes that may act independently of GAG accumulation or develop secondary to it. These include, among others, redox imbalance, chronic activation of the inflammatory response, oxidative stress, and disturbances in gene expression regulation. These mechanisms can significantly modify the course of the disease, affecting the variety of symptoms and the rate of their progression.

Therefore, **my doctoral thesis aimed to identify genetic, biochemical, and cellular factors that may modulate the course of MPS, with particular emphasis on Sanfilippo syndrome.**

This approach seeks to understand better why patients with theoretically the same enzyme defect and type of stored GAG may present with such a different clinical picture.

I conducted my research mainly on a model of skin fibroblasts taken from patients with all types/subtypes of MPS described up to 2020 (when I began my research). The characteristics of the cell lines used are presented in **Table 2**.

Table 2. Characteristics of the cell lines used

MPS type	**Defective enzyme**	**Type of mutation**	**Catalogue number ****
MPS I	α-L-iduronidase	p.Trp402Ter/p.Trp402Te	GM00798
MPS II	2-iduronate sulfatase	p.His70ProfsTer29	GM13203
MPS IIIA	N-sulfoglucosamine sulfhydrolase	p.Glu447Lys/p.Arg245His	GM00879
MPS IIIB	α-N-acetylglucosaminidase	p.Arg626Ter/p.Arg626Ter	GM00156
MPS IIIC	Acetyl-CoA:α-glycosaminide acetyltransferase	p.Gly262Arg/pArg509Asp	GM05157
MPS IIID	N-acetylglucosamine 6-sulfatase	p.Arg355Ter/p.Arg355Ter	GM05093
MPS IVA	N-acetylglucosamine-6-sulfate sulfatase	p.Arg386Cys/p.Phe285Ter	GM00593
MPS IVB	β-galactosidase	p.Trp273Leu/p.Trp509Cys	GM03251
MPS VI	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	Not determined	GM03722
MPS VII	N-acetylgalactosamine 4-sulfatase	p.Trp627Cys/p.Arg356Ter	GM00121
MPS IX	Hyaluronidase	p.Glu268Lys/c.37bp-del;14bp-ins at nt 1361	GM17494
Control line	N/A	N/A	N/A

*Catalogue numbers are consistent with the cell line description at the Coriell Institute.

Although fibroblasts may seem an unlikely model for studying neurodegeneration, they share several important characteristics with neurons. Both cell types originate from the ectoderm, suggesting that they share common molecular mechanisms, including signalling pathways and gene expression regulation processes. This feature is crucial for elucidating the pathogenesis of neurodegenerative diseases. Additionally, fibroblasts

exhibit abnormalities in cellular pathways, including oxidative stress, autophagy, and metabolism, which are also essential factors in neurodegeneration. Fibroblasts may reflect systemic cellular changes that affect neuronal function, especially in genetic disorders where the consequences of mutations remain unclear. Furthermore, fibroblasts are located near blood vessels, in the meninges, and in the choroid plexus of the brain and spinal cord, where they play a key role in maintaining CNS function. It is also worth noting that fibroblasts are easily accessible through minimally invasive procedures, allowing for regular monitoring of patients. Their stable cultures facilitate long-term studies, making them a valuable model for research into neurodegenerative mechanisms. I also conducted some of the research using tissues obtained from a mouse model of MPS IIIB.

I did not include MPS X, a type of MPS discovered only after I began my research, or MPS-plus- syndrome in my studies. Access to cells from patients suffering from these two types of MPS, even after the start of the project, was limited due to the very small number of patients (no more than 30 worldwide) and the fact that most of them come from Russia, which is affected by armed conflict.

As mentioned earlier, MPS is divided into 12 types/subtypes based on the type of GAG that is stored and the defective enzyme. In addition, MPS can be divided into neuronopathic types (MPS I, II, IIIA, IIIB, IIIC, IIID, VII) and non-neuronopathic types (MPS IV, VI, IX, X) based on the presence/absence of neurodegenerative features. This is an interesting division because, despite the seemingly identical cause of the disease, which is GAG storage, there is a clear difference in the occurrence of somatic symptoms and those related to neurodegeneration.

Currently, it is not possible to determine with certainty whether a patient with MPS will develop neurological symptoms. If they do, it is difficult to predict the rate of their progression or severity. The ability to predict this component would be of significant importance to patients and their families. Firstly, it would enable the early use of therapies aimed at protecting neurological functions, including intra-canal or intraventricular ERT and intra-canal gene therapy, which can target the central nervous system directly. Second, it would enable better planning of specialist care and rehabilitation, which could improve quality of life and slow the progression of symptoms. Early diagnosis of neurodegeneration risk would also help to adjust expectations and prepare carers for the specific challenges associated with progressive neurological changes. This is why it is so important to search for markers of neurodegeneration in MPS.

Using the RNA-seq library developed in previous years by the team I was working with, I performed a transcriptomic analysis to select genes whose expression levels, compared to control cells, are specific only to neuropathic types/subtypes of MPS (I, II, IIIA, IIIB, IIIC, IIID and VII), while showing no changes in expression levels in non-neuronopathic types. The results collectively showed over 300 transcripts that may be associated with nervous system disorders (specifically, 322 unique genes whose expression is significantly altered in at least two neuropathic MPS types/subtypes, but not in non-neuropathic types). Transcripts with altered expression levels in at least five neuropathic MPS types/subtypes (without simultaneous expression level alterations in non-neuropathic types) include transcripts with increased expression, e.g. *PDIA3* (encoding protein disulphide isomerase A3, PDIA3) and those with decreased expression, e.g. *ARL6IP6 (*encoding a protein interacting with GTPase 6 similar to ARF 6, ARL6IP6). Disruption of ARL6IP6 function can lead to abnormal neuron differentiation and disturbances in the homeostasis of the mitochondria and endoplasmic reticulum. Additionally, this protein plays a crucial role in the processing of APP into β-amyloid. A reduction in its level may therefore explain the increase in the level of β-amyloid and its precursors, as well as their tendency to aggregate. Literature data on studies using the *PDIA3*($^{-/-}$) mouse model have shown that the absence of *PDIA3* significantly reduces the effectiveness of apoptosis, alleviates inflammation and oxidative stress, and also improves cognitive function and reduces the volume of contusion caused by trauma in a traumatic brain injury model. In addition, an age-dependent increase in PDIA3 levels in the brains of mice with Alzheimer's disease was demonstrated, in contrast to its decline with age in healthy mice. These data strongly suggest that the high level of *PDIA3* expression detected in the analysis of neuropathic cell types/subtypes of MPS may also contribute in part to neurodegeneration.

The above-described results of research on *ARL6IP6* and *PDIA3* and other genes, suggesting their possible use as biomarkers of neurodegeneration in MPS, are described in the article: Wiśniewska K, Żabińska M, Szulc A, Gaffke L, Węgrzyn G, Pierzynowska K. (2024) The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease. *International Journal of Molecular Sciences;* 25(24):13447.

As I was interested in research on the functioning of the entire cell, I performed a similar analysis of neurodegeneration mechanisms, dividing it into the functioning of

individual cell organelles. Using transcriptomic methods, I identified genes associated with the function and structure of individual cell organelles (based on the Ensembl database) whose expression levels are altered in neuropathic types of MPS compared to control cells, while remaining unchanged in non-neuropathic types of MPS. This analysis indicated that genes whose expression is disrupted in the neuropathic type of MPS are often associated with the structures or functions of the cell nucleus, endoplasmic reticulum or Golgi apparatus. Experiments using fluorescence and electron microscopy confirmed abnormalities mainly within the Golgi apparatus, which appeared significantly more frequently in cells taken from neuropathic patients. The results of these analyses were discussed and compared with those found in other neurodegenerative diseases and neurological disorders in the article: Wiśniewska K, Gaffke L, Żabińska M, Węgrzyn G, Pierzynowska K. (2024) Cellular Organelle-Related Transcriptomic Profile Abnormalities in Neuronopathic Types of Mucopolysaccharidosis: A Comparison with Other Neurodegenerative Diseases. *Current Issues in Molecular Biology;* 46(3):2678-2700.

In the next stage of my research, I focused on two types of MPS – MPS III and MPS IV. Several factors dictated this choice. These are the only forms of MPS in which the division into subtypes is based on differences in the molecular defect, and not, as in the case of MPS I, on differences in the clinical course.

I found MPS III, in which CNS symptoms predominate, particularly intriguing. In this type, only heparan sulphate (HS) is accumulated – a compound whose accumulation is associated with neurodegeneration in other diseases, such as Alzheimer's disease. Neurodegeneration is also what distinguishes types IVA and IVB, among others. Nervous system disorders may occur in the course of MPS IVB, although skeletal musculoskeletal disorders predominate in this type. For these reasons, MPS III and IV are an extremely valuable reference point in research on neurodegeneration in MPS.

First, using transcriptomic data obtained from a cellular model, I selected genes that showed significantly different expression between MPS III subtypes. The number of transcripts showing differential expression was highest between subtypes IIIA vs. IIIB, IIIA vs. IIID, IIIB vs. IIID, and IIIC vs. IIID, totalling over 400. I performed a similar analysis between MPS IV subtypes (IVA and IVB), in which the number of transcripts showing differential expression was less than 100. The cellular processes in which the products of these transcripts participate largely involve cellular metabolism and its regulation, as well as the cell's response to stimulation or cellular communication

(according to the Ensembl database). Some of these transcripts showed very large, more than 16-fold differences in expression levels ($\log_{(2)}$fold change [FC]) > 4 or < -4), particularly between MPS IIIA and IIID, MPS IIIB and IIIC, and MPS IIIB and IIID. These transcripts include mRNA molecules derived from genes whose products are associated with ribosome functions (*RPLP2, RPL23, RPL10)*, maintenance of normal connective tissue structure (*COL4A1, COL4A2, COMP)*, involved in intracellular signalling (*NME2, WISP2, SRFP1)* and cell receptors and factors (*RARRES2, CRLF1, IGFBP5, TFPI2)*.

While the high variability in the expression of genes determining the proper function of connective tissue is not surprising (it has already been described in the context of MPS pathogenesis), the significant number of such genes involved in ribosome functions is a new discovery. Ribosomes are essential organelles responsible for protein synthesis, a key step in the gene expression process. The results obtained suggest significant variation in protein synthesis efficiency, which may contribute to the differing disease progression observed in patients with the four subtypes of MPS III. Furthermore, *RPL10* gene expression abnormalities have also been identified in other neurological disorders, such as autism spectrum disorders, X-linked microcephaly, X-linked synodromic intellectual disability, and epilepsy. Therefore, the reduced expression of these genes detected in MPS IIIA compared to MPS IIIC and IIID could, to some extent, explain the more frequent occurrence of autistic behaviours and more severe epilepsy in children with the former subtype of Sanfilippo syndrome than in other subtypes. Another example of a gene associated with epilepsy is *RARRES2,* which encodes chemerin, high levels of which are detected in the blood of children with idiopathic epilepsy as a prognostic factor. My transcriptomic analyses have shown that the expression level of this gene is elevated in MPS IIIA compared to IIID, and epilepsy is more common in the former subtype than in the latter. As previously mentioned, no significant changes in the expression levels of individual genes were observed between MPS IVA and IVB fibroblasts. In fact, the phenotypic differences between patients with different MPS IV subtypes are much smaller than between different MPS III subtypes. I therefore suggest that changes in gene expression patterns may determine the diversity of disease progression in patients suffering from different MPS III subtypes. Article by Wiśniewska K, Gaffke L, Krzelowska K, Węgrzyn G, Pierzynowska K. (2022) Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes. *Gene;* 812:146090 describes the above-summarised research results and points to the possibility

of a correlation between global gene expression patterns and the development of different symptoms in patients suffering from various subtypes of MPS III.

In the second stage of the study, I identified transcripts that undergo common changes in expression levels relative to control cells (taken from healthy individuals) in all subtypes of MPS III (IIIA, IIIB, IIIC, and IIID) and IV (IVA and IVB). No analysis of gene expression abnormalities common to all subtypes of MPS III and MPS IV had ever been conducted before. The results of this analysis identified 45 transcripts with similar changes in expression levels across all subtypes of MPS III, as well as up to 150 such transcripts in both subtypes of MPS IV, in relation to control cells. By identifying those transcripts with the highest fold change in expression ($log_2FC > 3$ or < -3), I noticed three that showed high levels of expression changes in all subtypes of both MPS III and MPS IV compared to control cells. These are the overexpressed genes *PFN1* and *MFAP5*, as well as the underexpressed gene *MMP12*. These genes encode profilin (PFN1), microfibril-associated protein 5 (MFAP5) and matrix metallopeptidase 12 (MMP12), respectively. In cells of all MPS III and IV subtypes, I performed immunodetection of these three proteins using immunofluorescence and Western blotting techniques, which indicated an increase in PFN1 and MFAP5 levels in MPS III and IV cells and a decrease in MMP12 levels in MPS III cells. Since these transcripts changed expression levels in all types/subtypes of MPS III, it seemed interesting to investigate the relationship between their levels and the levels of stored GAGs. The level of GAG in MPS cells was reduced by one of the flavonoids, genistein, which inhibits the autophosphorylation of the epidermal growth factor receptor (EGFR), ultimately leading to the inhibition of gene expression encoding GAG synthetases. Under these conditions, the levels of PFN1, MFAP5, and MMP12 proteins were re-evaluated, and the results of this analysis indicated that as GAG levels decreased, PFN1 levels also decreased. Furthermore, silencing *PFN1* gene expression also resulted in a decrease in GAG levels, suggesting a possible new therapeutic pathway.

Profilins encoded by the *PFN1* gene are small proteins that interact with the cytoskeleton, influencing actin polymerisation. They are also involved in membrane transport, cell signalling, transcription and autophagy. The cytoskeleton plays a vital role in synaptogenesis and neurotransmission, and is also involved in the development of the nervous system and the plasticity of the mature brain. Cytoskeleton disorders are often observed in mental illnesses such as schizophrenia, bipolar disorder, autism or severe depression. This is very important because behavioural problems are one of the neuropathic

symptoms of MPS. Furthermore, patients with MPS III are sometimes misdiagnosed as having autism spectrum disorders.

Mutations in the *PFN1* gene are also detected in patients with other neurodegenerative diseases, including amyotrophic lateral sclerosis, fragile X syndrome, spinal muscular atrophy, Huntington's disease, Parkinson's disease, and adrenoleukodystrophy. This fact seems particularly interesting, as protein aggregates are a common feature in many of these diseases. Some reports in the literature suggest that an increase in PFN1 expression may contribute to their formation. It has been demonstrated that some mutated PFN1 proteins exhibit prion-like properties and function as factors that trigger the conversion of TDP-43 protein into toxic conformational states.

In summary, the genes listed above and their products may be involved in common pathways of pathogenesis of different types/subtypes of MPS, with PFN1 levels appearing to be related to GAG levels. The above-presented research results concerning mainly PFN1 level disorders in MPS III and MPS IV cells are described in the article: Wiśniewska K, Żabińska M, Gaffke L, Szulc A, Walter BM, Węgrzyn G, Pierzynowska K. (2024) Shared Gene Expression Dysregulation Across Subtypes of Sanfilippo and Morquio Diseases: The Role of PFN1 in Regulating Glycosaminoglycan Levels. *Frontiers in Bioscience (Landmark Ed.);* 29(12):415.

The results mentioned above concerning profilin level disorders in MPS cells and their involvement in the pathogenesis of other neurological diseases prompted me to perform a comprehensive analysis of the levels of proteins known for their tendency to form aggregates in various neurodegenerative diseases. In MPS III cells of all subtypes, I performed immunodetection using immunofluorescence techniques and western blotting of beta-amyloid and its precursor (APP protein) levels, tau protein and its hyperphosphorylated form (p-tau), as well as alpha-synuclein and TDP-43 protein (b TDP-43 protein has never before been studied in the context of MPS pathogenesis). The results of these analyses indicated elevated levels of APP, β-amyloid, tau, and TDP-43 proteins in all MPS III subtypes, and elevated levels of p-tau and α-synuclein in all subtypes except MPS IIIC. Furthermore, aggregates formed by β-amyloid and tau, visible under a fluorescence microscope, were present in all MPS III subtypes, and aggregates formed by p-tau, TDP-43 and α-synuclein were present in all MPS III subtypes except IIIC. Elevated levels of the proteins mentioned above were also observed in the brains of mice modelling MPS IIIB.

Since the GAG level marked in the cells was the lowest of all the lines studied in MPS IIIC (although still significantly higher than in the control cells), the question arose as to the involvement of GAG in the formation of protein aggregates. Therefore, as before, the GAG level in MPS cells was reduced using genistein, and the levels of APP, β-amyloid, tau, p-tau, TDP-43, and α-synuclein, as well as their aggregates, were re-examined. Surprisingly, the results of these experiments indicated a reduction in the levels of all the above-mentioned proteins and the aggregates they form in cells treated with genistein, except α-synuclein, whose elevated level remained independent of GAG levels. This suggests a clear link between the formation of aggregates of certain proteins and GAG levels, which raises a number of questions about the role of GAG in the formation of such aggregates. The results of these experiments are described in the article : Wiśniewska K, Rintz E, Żabińska M, Gaffke L, Podlacha M, Cyske Z, Węgrzyn G, Pierzynowska K. (2024) Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients. *Biochemical and Biophysical Research Communications;* 733:150718.

The final element of my doctoral thesis was a literature review on behavioural disorders and sleep problems in MPS III, i.e. symptoms that are particularly characteristic of this condition and highly burdensome to both patients and their families. By analysing the available data, I sought to explain the underlying causes of these disorders, which involve overlapping pathological processes in OUN. Potential mechanisms include HS accumulation and the resulting neurodegeneration, chronic inflammation, oxidative stress, mitochondrial dysfunction, and disturbances in neurotransmitter metabolism. All these factors lead to the disruption of brain functions and structures responsible for controlling behaviour, emotions, and regulating sleep-wake cycles.

The most characteristic symptoms are psychomotor hyperactivity, impulsivity, aggressive outbursts, stereotypical movements, anxiety and sleep problems, such as difficulty falling asleep, frequent awakenings, reduced total sleep time or reversal of the circadian rhythm. Importantly, these symptoms very often appear in the early stages of the disease, before apparent somatic symptoms develop. In the review, I also discussed the possibilities of alleviating these disorders – both pharmacological (including antidepressants, antipsychotics, melatonin) and non-pharmacological, such as environmental modifications, maintaining consistent sleep rituals, behavioural therapies, and psychological support. However, I pointed out that the effectiveness of these methods

varies and that there is still a need for well-designed clinical trials in this area. In this paper, I aim to focus primarily on the daily problems associated with the disease that patients' families face, highlight the most significant ones, and discuss potential ways of dealing with them. This article is a paper by: Wiśniewska K, Wolski J, Anikiej-Wiczenbach P, Żabińska M, Węgrzyn G, Pierzynowska K. (2025) Behavioural disorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications. *Eur Child Adolesc Psychiatry;* 34(6):1795-1816. This work is particularly important to me because it highlights an aspect that is often overlooked in research on the disease's mechanisms and potential causal therapies. Although the search for effective causal treatments remains a priority, it should not be forgotten that until such therapies are available, it is necessary to simultaneously develop and improve symptomatic treatments that currently improve the quality of life of patients and their families.

To summarise the research presented, it can be said that the discoveries described in my doctoral thesis provide new insights into the molecular mechanisms underlying the pathogenesis of MPS and the mechanisms of differentiation between the neuropathic and non-neuropathic course of the disease. Furthermore, my research has revealed numerous differences and similarities between the various subtypes of MPS III at every level. In addition, the research as a whole points to numerous similarities between neuropathic types of MPS and other neurodegenerative diseases, which may indicate new directions for research into treatment methods.

List of publications included in the doctoral dissertation

1. Wiśniewska, Karolina et al. “Misdiagnosis in mucopolysaccharidoses.” *Journal of applied genetics* vol. 63,3 (2022): 475-495. doi:10.1007/s13353-022-00703-1
2. Wiśniewska, Karolina et al. “The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease.” *International journal of molecular sciences* vol. 25,24 13447. 15 Dec. 2024, doi:10.3390/ijms252413447
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HUMAN GENETICS • REVIEW

Misdiagnosis in mucopolysaccharidoses

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Abstract
Mucopolysaccharidosis (MPS) is a group of 13 hereditary metabolic diseases identified in humans (or 14 diseases if considering one MPS type described to date only in mice) in which an enzymatic defect results in the accumulation of glycosaminoglycans (GAG) in the lysosomes of cells. First of all, as a result of GAG storage, the proper functioning of the lysosome is disturbed; then, the cells, and finally, tissue, organs, and the whole organism malfunctions are observed. Due to the rarity, heterogeneity, and multi-systemic and progressive nature of MPS, they present a major diagnostic challenge. Due to the wide variation in symptoms and their similarity to other diseases, MPS is often misdiagnosed, usually as neurological diseases (like autism spectrum disorders, psychomotor hyperactivity, and intellectual disability) or rheumatology and orthopedic disorders (like juvenile idiopathic arthritis, Perthes disease, rickets, and muscular dystrophy). In this review article, we present the problems associated with the possibility of misdiagnosing MPS, discuss what diseases they can be confused with, and suggest ways to reduce these problems in the future.

Keywords Mucopolysaccharidosis · Diagnostic procedures · Misdiagnosis

Introduction

Lysosomal storage diseases (LSD) is a group of over 50 metabolic diseases in which the defect of a single gene encoding a protein necessary for the proper functioning of the lysosome is the basic cause of numerous disorders both at the cell and the whole organism level. LSDs can be classified according to the type of dysfunctional proteins and the nature of the accumulated material (Rintz et al. 2020).

Mucopolysaccharidosis (MPS) is a type of LSDs in which the complete or significant lack of activity of specific acid hydrolases or other enzymes leads to the accumulation of glycosaminoglycans (GAGs) within lysosomes of cells, disrupting their proper functioning. Due to the type of stored GAG and the enzyme responsible for its degradation, there are 13 types and subtypes of MPS in humans (Węgrzyn et al., 2022) plus one more, described to date only in mice (Kowalewski et al., 2012). Current classification of MPS types is presented in Table 1.

Undegraded GAGs also affect the abnormal course of other cellular processes, i.e., autophagy, apoptosis, or processes related to cell functioning (Gaffke et al. 2019, 2020, 2021; Brokowska et al. 2021). The abovementioned changes lead to disturbance of an intracellular homeostasis and multi-organ features including coarse facial features, dysostosis multiplex, hepatosplenomegaly, cardiovascular disorders, cognitive impairment (in neuronopathic forms), and many others (Zhou et al. 2020; Galzerano et al. 2021).

MPS is inherited in an autosomal recessive manner, with the exception of MPS type II (Hunter's disease), the inheritance of which is X-linked. It is estimated that the overall incidence of MPS is approximately 3.5 per 100,000 live births (Baehner et al. 2005); however, the prevalence of particular types and subtypes varies between countries. In Asia, the most commonly diagnosed type of MPS is Hunter's disease (MPS II), accounting for approximately 50% of confirmed cases. In Europe, the two most commonly diagnosed MPS are Hurler's disease (MPS I) and Sanfilippo's disease (MPS III) (Celik et al. 2021). However, the prevalence rates may differ significantly between various European countries. For example, the Sanfilippo disease is the most frequent

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Table 1 MPS classification

MPS type	Inheritance[a]	Stored GAG[b]	Defective gene	Deficient enzyme	OMIM number	Frequency (cases per 100,000 live births)[c]
MPS I-H	AR	HS,DS	*IDUA*	α-L-iduronidase	607,014	1.0
MPS I-S					607,016	
MPS I-HS					607,015	
MPS II	X-linked recessive	HS,DS	*IDS*	Iduronidase-2-sulfatase	309,900	0.3 to 0.71
MPS IIIA	AR	HS	*SGSH*	Heparan-N-sulfatase	252,900	0.17–2.35 collectively for all subtypes
MPS IIIB			*NAGLU*	α-Nacetylglucosaminidase	252,920	
MPS IIIC			*HGSNAT*	Heparan α-Glucosaminide N-acetyltransferase	252,930	
MPS IIID			*GNS*	N-Acetylglucosamine-6-sulfatase	252,940	
MPS IIIE[d]			*ARSG*	Arylsulfatase G	-	-
MPS IVA	AR	C6S, KS	*GALNS*	N-Acetylglucosamine-6-sulfate sulfatase	253,000	0.06 collectively for both subtypes
MPS IVB		KS	*GLB1*	b-Galactosidase	253,010	
MPS VI	AR	DS, C4S	*ARSB*	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	253,200	0.36–0.13
MPS VII	AR	HS, DS, C4S, C6S	*GUSB*	b-Glucuronidase	253,220	< 0.01
MPS IX	AR	Hyaluronan	*HYAL1*	Hyaluronidase	601,492	4 cases worldwide
MPS X	AR	DS	*ARSK*	Arylsulfatase K	619,698	4 cases worldwide
MPSPS[e]	AR	HS,DS	*VPS33A*	VPS33A	617,303	20 cases worldwide

[a]Abbreviation: *AR* autosomal recessive

[b]Abbreviations of glycosaminoglycan (GAG) names: *C4S* chondroitin 4,6-sulfate; *C6S* chondroitin 6-sulfate; *DS* dermatan sulfate; *HS* heparan sulfate; *KS* keratan sulfate

[c]The frequency depends on the subtype, population, and geographical region

[d]MPS IIIE was described to date only in an animal model

[e]Abbreviation: *MPSPS* MPS-plus syndrome

mucopolysaccharidosis in Poland, followed by Hunter's disease. Moreover, the prevalence of MPS in Poland is lower than in the Netherlands, Czech Republic, or Germany, while similar to that in Sweden and Denmark (Jurecka et al. 2015). In the USA, Hurler's disease, Hunter's disease, and Sanfilippo's disease are the most commonly confirmed cases of MPS (Celik et al. 2021, Puckett et al. 2021).

In fact, the actual incidence of MPS is difficult to assess, and many cases may be misdiagnosed or go undiagnosed. MPS diagnosis is difficult; the underlying problem is that it is a group of rare (only 0.1% of all genetic diseases) and heterogeneous diseases (Kubaski et al. 2020; Zhou et al. 2020). Thus, symptoms may be different not only among patients with different types of MPS, but also between people with the same type of disease (Kubaski et al. 2020; Zhou et al. 2020; Wiśniewska et al. 2022). Another difficulty is their multi-system and progressive nature. Very often, the early development of children with MPS is normal, problems appear after a few months or years and do not have to be immediately visible and obvious. The development of the disease causes not only the deepening of existing abnormalities, but also the appearance of new ones (Celik et al. 2021 (Mirella et al. 2018). More or less visible, they may be taken as another symptom of the present disease or be treated as the first symptom of another. Therefore, an effective and quick diagnosis requires the cooperation of specialists from various fields (Rigoldi et al. 2018). This is important because the effectiveness of the therapies used depends not only on the type of disease, but also largely on the stage of the disease (Zhou et al. 2020).

The aim of this review is to collect and present information on the most common difficulties and diagnostic pitfalls causing misdiagnosis and/or late diagnosis of mucopolysaccharidosis and to discuss the causes of these problems and the possibilities of preventing them in the future.

Mucopolysaccharidoses and problems with correct diagnosis—clinical picture

MPS diagnosis is difficult and it often takes a few or several years to make a correct diagnosis. Meanwhile, patients are examined by numerous specialists and treated for diseases

whose symptoms may largely resemble those of MPS (Rigoldi et al. 2018). The procedures associated with the performance of biochemical tests to assess GAG levels in urine also have a significant impact on the diagnostic process (Chih-Kuang et al. 2002).

The multi-system and progressive nature of MPS is one of the main diagnostic difficulties. There are different kinds of GAGs which cause various clinical problems when accumulated inside and outside of cells. Following GAGs accumulate in MPS (while their different kinds are stored in particular types of the disease; see Table 1 for details): chondroitin 4,6-sulfate (C4S), chondroitin 6-sulfate (C6S), dermatan sulfate (DS), heparan sulfate (HS), hyaluronate, and keratan sulfate (KS) (for an overview, see Węgrzyn et al. 2022). DS accumulation is associated with connective tissue damage and is observed in non-neuronopathic forms. Similarly, KS storage does not result in neuronal dysfunctions; however, it causes severe damage to bones and joints. The bone disorders, caused by DS or KS storage, arise from a lack of skeletal remodeling, impaired endochondral and intramembranous ossification, disruption of normal elastogenesis, and infiltration by GAGs of the ligaments, tendons, and joint capsules. Importantly, the disease starts already in utero while clinical signs and symptoms are observed later in life; moreover, some disorders are irreversible at the time of symptom appearance, like bone disease resulting from impairment of elastogenesis (Hinek and Wilson 2000). HS accumulation is associated with central nervous system (CNS) damage and observed in neuronopathic forms. Unlike the other MPS types that present with extensive somatic involvement, patients with MPS III (in which HS is the only primary stored GAG) typically present with mainly cognitive and neurological signs and symptoms. Accumulations of both DS and HS are observed in MPS I, II, and VII and are associated with neuronopathic disease and characteristic phenotype. Therefore, neuronopathic forms of MPS include types I, II, III, and VII. Cognitive impairment, psychomotor retardation, and neurologic regression are observed in patients with these MPS types. Types I, II, and VII also include changes in the bones and soft tissues. In patients with MPS IV and VI, the main symptoms are related to disorders of the skeletal system (Reichert et al. 2016; Biswas et al. 2017). Type IX MPS is the rarest one, and only on the basis of the biological function of hyaluronic acid (the accumulation of which is the cause of the disease), we can assume that this type of MPS is primarily bone tissue changes (Kiykim et al. 2015). Recently, works by two teams have led to the discovery of a previously unknown type of MPS (Trabszo et al. 2020; Verheyen et al. 2021). Each of the teams proposed a different classification; Trabszo et al. (2020) suggested classifying the new disorder as MPS IIB; however, considering the genetic defect and kinds of accumulated GAGs, we are inclined to the proposal of Verheyen et al. (2021) and the extension of the existing classification to MPS X. Another disease, caused by a defect in the *VPS33A* gene, coding for a protein whose function is not directly involved in GAG degradation but nevertheless leads to massive accumulation of DS and HS, has been assigned as MPS-plus syndrome (MPSPS) (Vasilev et al. 2020). Interestingly, a MPSPS-like disease has been described recently as an effect of a biallelic intronic variant (possibly causing alteration in splicing) of the *VPS16* gene (Yıldız et al. 2021). Patients suffering from this disease do not excrete elevated amounts of GAG (the urinary GAG levels are at the upper-normal values) while they develop symptoms resembling MPS, like short stature, coarse facial features, delay in development, peripheral neuropathy, enlarged spleen, spondylar dysplasia, and neutropenia (Yıldız et al. 2021).

A review of the available literature suggests that the similarity of MPS, in particular, to other neurological, rheumatological, and orthopedic disorders is one of the reasons for the late correct diagnosis. However, a review of the literature shows that there is relatively little work describing the diagnostic problems of MPS related to the incorrect qualification of patients to other disease entities. There were 128 items in the PubMed database (https://pubmed.ncbi.nlm.nih.gov) under the heading "mucopolysaccharidosis and misdiagnosis" (as of March 9, 2022), of which as few as 25 described or summarized actual problems with misdiagnosis of MPS. Below we present a summary of these problems, broken down into individual types of MPS.

MPS I (Hurler disease)

MPS I is associated with impaired activity of α-L-iduronidase (IDUA), the enzyme responsible for the degradation of HS and DS (Hampe et al., 2020). Due to the varied severity of symptoms, the disease is classified as mild (called also Scheie's disease), intermediate (Hurler-Scheie's disease), and severe (Hurler's disease); however, the biochemical differences between these forms are unclear, apart from the commonly accepted feature of a total lack of the enzyme activity in the severe form (Chakraborty et al. 2016). Hurler disease is characterized by severe neurological impairment. Cognitive functions quickly deteriorate with the simultaneous development of disorders of the skeletal system (dysostosis multiplex) cardiac system, or respiratory system. Other common symptoms are vision problems, hearing loss, and facial deformities (Zhou et al. 2020). The first symptoms of the disease appear in the first 6 months of life, but are not clear enough to prompt a diagnosis. By the time the child is 1 year old, the developing abnormalities become more and more pronounced. Problems with hearing, vision (corneal opacity), psychomotor retardation, musculoskeletal disorders, altered facial features, and enlarged tongue are starting to become noticeable (Hampe et al. 2020; Zhou

et al. 2020). Hurler-Scheie and Scheie syndromes are often asymptomatic for the first few years of life, and neurological features characteristic of Hurler disease are absent (Hampe et al. 2020). No major phenotypic changes (Chakraborty et al. 2016) including no altered facial features (Cimaz et al. 2006) might occur in Scheie syndrome, while severe somatic symptoms can occur in Hurler-Scheie syndrome which is distinguished from Hurler disease due to mild- or no mental changes and cognitive deficits.

Depending on the phenotype, patients may die even in the first decade of life if adequate treatment is not given or lead a relatively normal life if afflicted with a mild form of the disease (Hampe et al. 2020). Due to its prevalence, severe MPS I is relatively well understood, and therefore patients with this form are diagnosed rather early. The available treatment (enzyme replacement therapy) also influenced the more widely performed screening for MPS I (Clarke et al. 2020). In the case of mild forms, diagnosis is much more difficult. Due to non-specific symptoms resulting from the phenotype, patients with Hurler-Scheie and Scheie syndromes may remain undiagnosed or misdiagnosed as rheumatological cases for years (Hoseinzadeh Moghadam et al. 2019). However, Żuber et al. (2015) found that ultrasonography of hip joints might be useful in facilitating the differential diagnosis of MPS I and II with other rheumatic diseases. Walking problems resulting from valgus knees, abnormally dilated joints, and a number of other skeletal symptoms may suggest rickets, autoimmune diseases, idiopathic arthritis, or spinal-epiphyseal dysplasia (Grewal and Muranjan 2020). Other diseases diagnosed in the course of mild forms of MPS I may include muscular dystrophy, Perthes disease (an idiopathic osteonecrosis of the femoral head in children), dermatoses, growing pains, or rheumatic fever (Leo et al. 2020). Congenital bone fragility or polyneuropathy can also be misdiagnosed in MPS I (Cimaz et al., 2009).

Sherwood et al. (2021) described a case of the extremely mild form of MPS I, where one of the leading symptoms was cardiac abnormality. A 32-year-old patient with developmental delay saw a doctor due to increasing exercise dyspnea. The physical examination revealed a number of irregularities, i.e., thickened scratches on the face, stiffness and limited mobility of the arms and legs, and abnormal gait. Further studies revealed retinal degeneration, optic disc edema, and stenosis of the mitral and aortic valves. Rheumatic heart disease, congenital anomalies, and valve defects associated with Marfan or Jacobsen syndrome were considered as possible causes. Ultimately, histopathological analysis revealed a significant accumulation of GAG, which, along with other symptoms, identified MPS as the underlying disease (Sherwood et al., 2021).

Cimaz et al. (2006) collected and presented the medical histories of 13 patients with MPS I. Two of them were diagnosed within 1 year of reporting to the doctor. Four patients were left without a diagnosis for years (the time from the first symptoms to correct diagnosis was from 1 to 33 years). The first symptoms were stiffness and contractures in the hands, stiffness in the fingers, and limited mobility in the arms. Other skeletal disorders included scoliosis, kyphosis, dysostosis multiplex, and short stature. The most common symptoms unrelated to the skeletal system were corneal opacity and cardiac disorders. Seven patients were initially misdiagnosed as cases of juvenile idiopathic arthritis (first symptoms were finger stiffness and cramps), scleroderma (stiffness and pain in the hands, neck stiffness, and lameness were the first notable symptoms), and arthrogryposis (diagnosed due to hand stiffness, limited arm mobility, and inguinal hernia). The skeletal abnormalities worsened over time, and the patients suffered from recurrent diarrhea and ear infections. Over time, disorders related to the cardiovascular system, corneal opacity, and umbilical hernias appeared. A comprehensive evaluation of the symptoms that developed over the years eventually allowed the diagnosis of MPS I within 3 to 50 years.

Ten years later, the case of a 16-year-old boy diagnosed in childhood with rickets was described (Charkraborty et al. 2016). The initial development of the child was normal; at the age of 6 years, the parents noticed a curvature of the knees, which was the basis for the first diagnosis. After years of ineffective therapy, the boy underwent re-examinations, showing numerous skeletal abnormalities, short stature, chest deformity, carpal tunnel syndrome, corneal opacity, altered facial features, and aortic regurgitation with normal intelligence. Spondyloepiphyseal dysplasia and MPS have been considered as possible causes of numerous abnormalities. Only biochemical tests in conjunction with the results of radiological tests and clinical evaluation allowed for the diagnosis of MPS I and the implementation of appropriate therapy (Charkraborty et al. 2016).

Saraf et al. (2021) described the case of a 36-year-old patient in whom psychiatric treatment was introduced due to excessive talkativeness, sleep disorders, and auditory hallucinations. Six months after discontinuation of medications, the patient was hospitalized. The conducted research has shown, among others, too short stature, heart abnormalities, and mild cognitive impairment. The results obtained after performing magnetic resonance imaging (MRI) together with the patient's medical history allowed for the diagnosis of the mild form of MPS I (Saraf et al. 2021).

An uncertainty of diagnosis between MPS I and Fabry disease has been described by Langereis et al. (2013). Fabry's disease is a lysosomal storage disease in which, as a result of a disturbed effect of α-galactosidase A (α-Gal A), glycosylated molecules (mainly glycosphingolipids) accumulate in lysosomes. Patients most often suffer from chronic pain, hypersensitivity to mechanical stimuli, heat and cold intolerance, and other pain symptoms (Burand Jr et al. 2021).

It was noted that a certain percentage of patients with renal failure, left ventricular dystrophy, or stroke cryptogenic has a pathogenic variant of the gene conditioning Fabry disease (Langereis et al. 2013). A case of a 32-year-old man who, due to ischemic stroke and the lack of predisposing factors, had a cryptogenic stroke and the diagnosis of Febry disease was started. The tests showed a normal level of α-Gal A activity, but the level of activity of the reference enzyme, IDUA, was well below the normal level. The disease was therefore suspected Scheie syndrome. Further tests (including the assessment of urinary GAG levels) and a review of medical records did not reveal any abnormalities that could be related to MPS. Genetic analysis revealed two previously unknown variants of the *IDUA* gene, but due to the lack of clinical symptoms, the diagnosis of Scheie disease was not confirmed (Langereis et al. 2013).

The summary of symptoms that may indicate MPS I and possible misdiagnoses is presented in Table 2.

MPS II (Hunter's disease)

MPS II is associated with the dysfunction of iduronate-2-sulfatase (I2S) that catalyzes DS and HS degradation. As with MPS I, Hunter disease is clinically classified as severe (neuropathic) and attenuated (non-neuropathic), depending on whether or not there is cognitive impairment. Additionally, the non-neuropathic form may present with somatic symptoms as severe as those found in the neuronopathic form (Porter 2018).

Children with MPS II usually develop properly until the age of 2–4 years; however, in the case of the severe course, symptoms may appear slightly earlier (D'Avanzo et al. 2020). In fact, it was reported that mean values for birth body length and weight for MPS I, II, and VI were greater than in the general population (Różdźyńska-Świątkowska et al. 2016). Therefore, high birth weight and/or large-for-gestational age can be suggestive of these MPS types and should raise suspicion aiding early disease recognition. Moreover, in boys with MPS II, almost half of laryngological procedures (including ventilation drainage and adenotonsillectomy) are performed before the diagnosis of Hunter disease is made (Muhlebach et al. 2011). The next-appearing symptoms mainly include developmental delay, mental retardation, and behavior problems (aggression, over-excitability) (Porter 2018). Initially, normal growth is stunted. Features such as macrocephaly, thickened lips, abnormal nostrils, and a short neck become more and more visible. Marucha et al. (2012) described an early joint range of motion impairment in children with MPS II. In fact, restrictions in shoulder joints were the earliest being observed already before the second year of life. Over time, abnormalities related to the skeletal system become more and more emphasized, i.e., abnormal bone thickness, joint stiffness, and abnormal shape of the chest and spine (Racoma et al. 2021; D'Avanzo et al. 2021). Patients with the mild form usually live to adulthood, while those with the severe form usually survive only the first two decades of life (Amartino 2015). Behavioral problems, like aggression, hyperactivity, and sleep problems, are often the first noticeable symptoms resulting from the progressive central nervous system (CNS) damage and somatic complaints that are misdiagnosed as psychomotor hyperactivity (ADHD), autism spectrum disorders, intellectual disability (Ozbay et al. 2020), or Kluver-Bucy syndrome (Ozbay et al., 2020). CNS disorders can also lead to epilepsy as the underlying disease (D Chiong et al. 2017). The attenuated forms may be so mild that they will not cause symptoms that require a diagnosis. It may be that non-neuropathic MPS II will be diagnosed as Legg-Calve-Perthes disease or rheumatic disease (Chakraborty et al. 2018).

Chakraborty et al. (2018) described the case of a 7-year-old boy admitted to the hospital with pain in the hip joints and difficulties with walking. The medical interview showed the proper development of the child with satisfactory learning progress. There were no perinatal abnormalities. Further studies showed short stature, shortened torso (invisible at first glance), and umbilical hernia. The cardiological examination revealed mitral regurgitation, while the biochemical examination revealed the presence of azurophilic granules in white blood cells. Despite the absence of elevated levels of GAGs in the urine, the enzymatic test showed a reduced activity of iduronate-6-sulfatase. Eventually, a mild form of MPS II was diagnosed (Chakraborty et al. 2018).

The possibility of diagnosing a mild form of Hunter disease as an autism spectrum disorder was indicated on the basis of the medical history of a 2.5-year-old boy reported to a psychiatric clinic due to speech impairment (Ozbay et al. 2020). The boy did not develop properly, he was reluctant to play with other children, he was unresponsive when called by his name, and he limited eye contact. The child's mother noted excessive tearfulness and tantrums. She also reported that the boy showed excessive anxiety when his pencils were removed. On the autism behavior checklist (ABC) scale, he scored 91 points. As further examinations revealed numerous bone deformities, enlarged head circumference, shortened neck, protruding tongue, and hearing loss, MRI and biochemical tests were additionally performed. All the results obtained allowed the boy to be diagnosed with Hunter's syndrome (Ozbay et al., 2020).

Due to the inheritance mode, MPS II is a disease that occurs primarily in males, but it may also occur in females (Sohn et al., 2010). In the case of carriers of pathogenic gene variants, Hunter syndrome may develop due to non-random inactivation of the X chromosome, with the non-active correct version of the *IDS* gene, chromosomal translocation, or chromosome X monosomy due to a partial de novo deletion of the long arm of chromosome X. So far, there have been

Table 2 Symptoms that may indicate specific MPS types and possible misdiagnoses

MPS type	Typical symptoms	Potential misdiagnosis	Comments
MPS I-H (Hurler syndrome)	Progressive neurological disorders Skeletal disorders (dysostosis multiplex) Coarse facial features Macroglossia Hepatosplenomegaly Corneal clouding Cardiac abnormalities Joint stiffness Diarrhea	Rickets Autoimmune diseases Idiopathic arthritis Rheumatoid arthritis Legg-Perthes disease Spinal-epiphyseal dysplasia Polyneuropathy Growing pains Congenital fragility of bones Dermatomyositis Muscular dystrophy Systemic scleroderma Arthrogryposis Fabry disease	• The division of MPS I into subtypes is based only on differences in the clinical picture • The symptoms of MPS I may mostly resemble those that occur in the course of various types of rheumatological diseases • Skeletal abnormalities (especially in the case of benign forms) do not have to be the dominant symptoms
MPS I-H/S (Hurler-Scheie syndrome) MPS I-S (Scheie syndrome)	No neurological disturbances Significant phenotypic changes		
MPS II (Hunter syndrome)	Developmental delay Mental retardation Behavior problems (aggression, hyperactivity) Coarse facial features Skeletal disorders Abnormal shape of the chest Joint stiffness Short stature Incorrect body proportions Sleep disturbance Cardiac abnormalities Hearing loss Mild form is without cognitive impairment	ADHD Autism spectrum disorders Intellectual disability Epilepsy Turner's syndrome Legg-Calve-Perthes disease Rheumatic diseases	• The division of MPS II into severe and mild form is based on the absence/presence of symptoms of cognitive impairment. At the same time, the somatic symptoms in the case of the mild form may be as serious as in the case of the severe course of the disease • Symptoms and disorders developing in the MPS II stage can often lead to the diagnosis of mental/neurological disorders or rheumatological/orthopedic disorders in the milder forms • Skeletal abnormalities (especially in benign forms) may not be the dominant symptoms
MPS IIIA MPS IIIB (Sanfilippo syndrome)	Developmental delay Cognitive impairment Behavioral disorders (impulsivity, aggression, anxiety disorders, autistic behavior) Coarse facial features Frequent ears and upper respiratory infection Joint stiffness Skeletal disorders Diarrhea	Autism spectrum disorders ADHD Landau-Kleffner syndrome Juvenile idiopathic arthritis Rett syndrome	• The division of MPS III into subtypes results from the deficiency of the activity of different enzymes • So far, MPS IIIE has not been recognized in humans • Due to the type of symptoms, MPS III can be misdiagnosed as psychiatric/neurological disorders, less frequently rheumatologic
MPS IIIC MPS IIID (Sanfilippo syndrome)	Symptoms similar to types IIIA and IIIB, but milder and with a later onset		
MPS IIIE (described to date only in mice)	Cognitive impairment Behavioral disorders Ataxia		

Table 2 (continued)

MPS type	Typical symptoms	Potential misdiagnosis	Comments
MPS IVA (Morquio syndrome)	Excessive joint mobility Absence or mild neurological disorders Skeletal deformation Short stature Pigeon chest Short neck Coarse facial features Excessive joint mobility Absence or mild neurological disorders Skeletal deformation Short stature Pigeon chest Short neck Coarse facial features	Epiphyseal dysplasia Spondyloepiphyseal dysplasia Bilateral Perthes Spondylometaphyseal dysplasia Brachyolmia Perthes disease Juvenile idiopathic arthritis Rickets Skeletal dysplasia Ehler-Danlos syndrome Sots' syndrome	• The division of MPS IV into subtypes is due to the deficiency of the activity of 2 different enzymes • Because MPS IV symptoms mainly concern the skeletal system, the potential diagnosis may refer to diseases in the field of rheumatology/orthopedics • In patients with Morquio syndrome, other (non-skeletal) somatic symptoms are usually milder than with other types of MPS
MPS IVB (Morquio syndrome)	MPS IVB is milder in course, skeletal abnormalities and other somatic disorders are not so obvious		
MPS VI (Maroteaux-Lamy syndrome)	Skeletal deformation Dysostosis multiplex Coarse facial features Skeletal disorders Corneal clouding Joint stiffness Short stature Frequent ears and upper respiratory Circulatory and respiratory failure	Epiphyseal dysplasia Spondyloepiphyseal dysplasia Stickler syndrome Hurler syndrome Morquio syndrome Mucolipidosis type II Multiple sulfatase deficiency Rheumatoid myocarditis	• Due to the phenotype, two forms of the disease can be distinguished. Rapidly progressive, with a severe course and early symptoms, and a slow course with mild and late-onset symptoms • Symptoms that appear in the course of MPS VI can most often lead to the diagnosis of rheumatology/orthopedic diseases • In MPS VI, neurological disorders are uncommon and, when present, are due to secondary disorders
MPS VII (Sly syndrome)	Non-immune hydrops fetalis Hydrops and the hydropic aspect of the placenta Macrocrania Hypertelorism Retrognathia Skeletal dysplasia Heart abnormalities Respiratory problems Coarse facial features Frequent ear infections Mental retardation Sleep disturbance Hepatosplenomegaly	ADHD Autism spectrum disorders Perthes disease Developmental delay	• Symptoms that appear in the course of MPS VII (as long as they do not lead to the early death of the child) most often resemble diseases in the field of psychiatry/neurology • MPS VII is one of the rarer types, with less than 200 patients currently diagnosed

Table 2 (continued)

MPS type	Typical symptoms	Potential misdiagnosis	Comments
MPS IX (Natowicz syndrome)	Short stature Frequent ear infections Cleft palate Joint stiffness Development of soft-tissue masses Numerous cysts	Juvenile idiopathic arthritis	• Extremely rare type of MPS, 4 cases were diagnosed to date
MPS X	Skeletal dysplasia Frequent ear infections Coarse facial features Sleep disturbance Frequent ear infections Short structure Ophthalmological abnormalities Heart abnormalities	Morquio disease	• The first patients with this type of MPS were described at the end of 2021 • Given the symptoms described, a potential diagnosis may include various types of orthopedic disorders
MPSPS (MPS-plus syndrome)	Skeletal deformation Frequent upper respiratory infections Cough Short, noisy breathing Coarse facial features Psychomotor retardation Hepatosplenomegaly Joint contraction Proteinuria Disorders of hematopoiesis Congenital heart defects	Other types of MPS Mucolipodosis types I and II Fucosidosis Mannosidosis Sialidosis Multiple sulfatase deficiency Gaucher disease Niemann-Pick syndrome	• MPSPS has so far been recognized primarily among Turkish ethnic groups

several cases of MPS II diagnosed in women (Semyachkina et al. 2019). The spectrum of symptoms, as in men, can range from mild to severe (Tuschl et al. 2005).

Tuschl et al. (2005) described a severe MPS II case in a 4-year-old girl. The patient had altered facial features, macroglossia, hepatosplenomegaly, and short stature. Impairment of psychomotor functions and speech was also visible. The child's parents reported progressive deterioration of hearing and behavioral and sleeping problems. No cardiac abnormalities were noted. The girl was the youngest child of unrelated parents. Her older siblings (brother and sister) did not show similar symptoms (Tuschl et al. 2005).

Cudry et al. (2000) described two female patients diagnosed with Hunter syndrome. One of the girls, the daughter of related parents, developed a severe form of the disease. At 3.5 years of age, she was diagnosed with abnormal facial features, hearing loss, hepatosplenomegaly, skeletal disorders, limited joint mobility, and mental retardation. Due to the inactivation of one of the X chromosomes, the girl developed a clinical manifestation of Hunter syndrome. The second case was a patient with a mild form of MPS II. At the age of 11, the child was diagnosed with hepatomegaly and short stature. There were no other symptoms typical of MPS, such as abnormal facial features or skeletal abnormalities. Mental retardation, cardiac abnormalities, lens opacities, or other changes developing in the course of MPS II were also not observed. Interestingly, the patient turned out to have a pathogenic variant in both alleles of the *IDS* gene (Cudry et al. 2000).

A case of a 28-year-old woman admitted for examination due to abnormal facial features and short stature was reported by Sohn et al. (2010). The interview showed that the woman had an older brother diagnosed with MPS II. The patient's medical records also contained information about a short neck and slight mental retardation. Further examinations did not reveal any symptoms typical of MPS, i.e., skeletal system abnormalities, cardiological abnormalities, hearing loss, and others. The enzymatic activity tests showed a decrease in iduronate-2-sulfatase levels, pointing to MPS II as the cause of the observed abnormalities. Molecular studies revealed a pathogenic variant of the *IDS* gene and skewed inactivation of one of the X chromosomes (Sohn et al., 2010).

A 4-year-old girl with MPS II was also reported who was admitted to the hospital with slow growth, pain in the lower limbs, and joint stiffness (Semyachkina et al. 2019). In the medical history of the child, there was information about a slight delay in the development of motor functions and speech. The initial diagnosis was Turner syndrome; the karyotype analysis showed a partial deletion of the long arm of the X chromosome, but the changed features of the girl's face resembled the phenotype typical for patients with Hurler disease (MPS I). The results of urine GAG analysis (unidirectional electrophoresis was performed) indicated that the girl might suffer from MPS (the GAG storage pattern suggested MPS I, II, or VII). Further examinations revealed cardiological disorders and abnormalities of the skeletal system. Ophthalmological examination indicated myopia and astigmatism in both eyes. The results of radiological examinations clearly suggested MPS I or II as the cause of the observed abnormalities. Molecular studies confirmed the reduction in iduronidase-2-sulfatase activity, which was the basis for the diagnosis of MPS II (Semyachkina et al. 2019).

It is worth stressing that female MPS II might be especially prone to misdiagnosis as this disease is generally not expected in girls. Therefore, even if MPS is suspected, when a female patient is investigated, other types are often considered rather than Hurler syndrome.

The summary of symptoms that may indicate MPS II and possible misdiagnoses is presented in Table 2.

MPS III (Sanfilippo disease)

In MPS III, the excessive accumulation of heparan sulfate (HS) may be due to the absence or decreased activity of four different enzymes. Therefore, there are 4 disease subtypes, depending on which enzyme is affected. MPS IIIA is associated with a defect of N-sulfoglucosamine sulfohydrolase (SGSH), MPS IIIB is a malfunction of N-acetyl-α-D-glucosaminidase (NAGLU), in MPS IIIC impaired activity of acetyl-CoA:α-glucosaminide N-acetyltransferase is observed, while MPS IIID results from damage to the gene encoding N-acetylglucosamine-6-sulfate sulfatase (GNS) (Heon-Roberts et al. 2020). Regardless of the disease subtype, patients with MPS III present similar disease symptoms; however, types IIIA and IIIB are more frequent and more severe than types IIIC and IIID. A characteristic feature of Sanfilippo disease is slight somatic changes with significant cognitive and neurological disorders (Heon-Roberts et al. 2020). There are also proposals to extend Sanfilippo disease to the IIIE subtype, in which the accumulation of HS is associated with a pathogenic variant of the *ARSG* gene, encoding arylsulfatase G (ARSG). However, so far, ARSG deficiency has not been identified in humans. On the other hand, animal models of the disease show features characteristic of MPS III, like ataxia, cognitive impairment, or behavioral disorders (Kowalewski et al., 2012).

The first symptoms of Sanfilippo disease appear between the ages of 1 and 3 years. In the first phase, development slowdown or inhibition is observed. Somatic abnormalities such as facial dysmorphia, frequent ear and upper respiratory tract infections, and diarrhea may be underestimated or not at all worrying (Wijburg et al. 2013). In the second phase, occurring at the age of 3–4 years, cognitive functions deteriorate, sleep disorders appear, and behavioral problems (impulsivity, aggression, anxiety disorders, or autistic-like

behavior appear) become evident (Urgancı et al. 2020; Ozbay et al. 2020). Skeletal abnormalities may become more evident in this phase. Calmness, dementia, and deterioration of motor functions are the third phase of the disease. Over time, patients lose the ability to move and function independently. This stage begins usually in the teenage years (Wijburg et al. 2013).

In the case of a milder form, symptoms develop later (the first phase of the disease occurs around 4 years of age) and are less severe. Therefore, the diagnostic process itself may not begin until adulthood (Heon-Roberts et al., 2020). People suffering from MPS IIIA or IIIB die in the second decade of life. People with a milder phenotype (types IIIC and IIID) sometimes live to the fourth or even sixth decade (Wijburg et al. 2013).

Speech disorders or delays that cause both emotional and social problems, or cognitive impairment are misdiagnosed as autism spectrum disorders (ASD) (Wolfenden et al. 2017). On the other hand, attention problems, tantrums, and hyperactivity can be the basis for the diagnosis of ADHD (Anikiej-Wiczenbach et al. 2020; Escolar et al. 2020). It can be difficult to distinguish behavioral difficulties in the course of MPS III from ADHD or ASD. However, the lack of response to standard stimulant medications for ADHD is a key diagnostic clue. The presence of developmental or speech delay associated with any characteristic somatic sign(s) or symptom(s) or with behavioral difficulties should prompt diagnostic testing (Wijburg et al., 2013). Speech disorders, cognitive deficits, impaired social behavior, or aggression may be the cause of the diagnosis of the Landau-Kleffner syndrome (Ahmed et al. 2020). Excessive stiffness of the joints with slight neurological deficits can be diagnosed as juvenile idiopathic arthritis (JIA) (Cimaz et al. 2006). It is also intriguing that in the case of mothers of children with MPS IIIA, a large number of miscarriages are observed. Moreover, caesarean section, performed due to the lack of progress of labor, macrocephaly, or the so-called fetal bracket position, is common in children with MPS IIIA (Krawiec et al. 2014).

Urgancı et al. (2020) reported cases of 4 patients diagnosed with MPS IIIA, in three of them the first diagnosis was ADHD, one of the children had not been previously diagnosed, and MPS IIIA was diagnosed several years after the first abnormalities appeared. Sleep problems, speech delay and impairment, hyperactivity, mental retardation, and altered facial features were symptoms that developed in all of the patients. Three children experienced hirsutism, hearing impairment or loss, and skeletal disorders. A definitive diagnosis was made on the basis of the abnormalities observed and confirmed by enzyme activity testing indicating disturbances in heparan N-sulphatase activity (Urgancı et al, 2020).

Rezayi et al. (2019) presented a case of MPS III diagnosis as Landau-Kleffner syndrome (LKS), an age-related epileptic encephalopathy. In LKS, the early development of a child is normal, and symptoms appear between the age of 3 and 7 years (Ahmed et al., 2020). Hyperactivity (similar to mild forms of MPS) and usually problems with attention and behavior, auditory agnosia, and speech regression are observed. In addition, the EEG test shows a reading whose pattern is similar to the results obtained in patients with epilepsy. The patient described by Rezayi et al. (2019) was admitted to the hospital at the age of 9 years due to speech regression, seizure, and ataxia. The interview revealed information about problems with focusing attention, auditory agnosia, difficulties in communication, and tonic-clinic seizures. Based on the performed tests (EEG and SPECT), LKS was diagnosed. However, the applied treatment did not bring any results and the symptoms worsened. Genome sequencing was commissioned which revealed the MPS IIIB due to the presence of the pathogenic variants of *NAGLU* (Rezayi et al., 2019).

It may also happen that the patient remains undiagnosed due to an atypical phenotype, as described by Zeng et al. (2019). A 9-year-old boy with no typical clinical symptoms was diagnosed with MPS IIIB based on genome sequencing and enzyme activity tests. The boy was developing correctly; the first noticeable symptom was a gradual loss of speech at the age of 3 years and rude behavior. Due to the lack of other abnormalities, he was initially suspected of Rett syndrome and a carnitine deficiency, but studies did not confirm any of the diseases. Only sequencing revealed pathogenic variants of the gene encoding N-acetyl-α-D-glucosaminidase, which prompted physicians to diagnose MPS IIIB. Ultimately, the diagnosis was confirmed by an enzyme activity test (Zeng et al., 2017).

Dalpiaz Irigonhê et al. (2020) described an MPS IIIB patient recognized as ASD. Until the age of 2 years, the patient developed normally, the first disturbing change was her deteriorating ability to speak, which was eventually lost within 1.5 years. Psychomotor agitation, sleep problems, aggression, stubbornness, difficulty concentrating, and other behavioral disorders began to appear. At the age of 6, the patient was diagnosed with ASD. Despite the therapy used, the behavior problems worsened. The initial balance problems that made it difficult to walk turned into a complete loss of ability to move. Serious problems with swallowing developed, and the initial hyperactivity and aggression gradually turned into drowsiness and apathy. In the interview, there was information about recurring infections of the upper respiratory tract. Studies have shown numerous abnormalities such as macroglossia, altered facial features, short neck, joint stiffness, and other skeletal disorders. Cardiovascular abnormalities were also noted. Laboratory tests

have finally confirmed the diagnosis of MPS IIIB (Dalpiaz Irigonhê et al. 2020).

It was indicated that the diagnosis of metabolic diseases (including MPS) is not a rare mistake. In one analysis, among 179 patients diagnosed with ASD, 3.3% were later diagnosed with metabolic diseases, including MPS IIID (Cakar et al. 2021). The summary of symptoms that may indicate MPS III and possible misdiagnoses is presented in Table 2.

MPS IV (Morquio disease)

MPS IV, like Sanfilippo disease, is divided into subtypes. Depending on whether the activity deficit concerns cetylgalactosamine-6-sulfate sulfatase (GALNS) or β-galactosidase (GLB1), one can distinguish the type of IVA, with the accumulation of keratan sulfate and chondroitin-6-sulfate, and IVB, with the accumulation of keratan sulfate, respectively. In both cases, the development of numerous skeletal abnormalities is observed with the simultaneous absence of neurological disorders (Zhou et al. 2020). Patients with Morquio syndrome live about 20–30 years, but in the case of an extremely mild phenotype, life expectancy may not differ from the average (Peretz et al. 2020).

Symptoms of MPS IV appear between 1 and 3 years of age with socket hypoplasia, growth retardation, thickened facial features, pectus carinatum, or a short neck being among the first symptoms. A characteristic feature of patients with Morquio syndrome is the laxity of the joints, in contrast to other types of MPS, where, as the disease progresses, the joints become stiff, which may cause difficulties with walking (Zhou et al. 2020). By 7–8 years of age, growth is completely stopped. Type IVB is slightly milder than IVA, and short stature or abnormal facial features are not as visible. Skeletal abnormalities, hearing loss, valve defects, and other organ abnormalities are also less severe (Galimberti et al. 2018).

Due to the fact that the disorders mainly affect the skeletal system, making a correct diagnosis is not easy. Back pain, scoliosis, excessive joint mobility, abnormal growth, impaired motor function, and altered shape of the chest may suggest epiphyseal dysplasia (MED), spondyloepiphyseal dysplasia (SED), or bilateral Perthes disease. Other disease entities to which MPS IV may be similar due to skeletal disorders with normal intelligence include spondylometaphyseal dysplasia, brachyolmia, Perthes disease, juvenile idiopathic arthritis, or rickets (Biswas et al. 2017; Lachman et al. 2014). On the other hand, Różdżyńska-Świątkowska et al. (2020) created an anthropometric pattern of face and body stature of MPS IVA patient. Dwarfism occurred with age as a result of the relatively short trunk and lower extremities. The head and neck were relatively elongated, in comparison to body height, and tucked between narrow shoulders. The head had dolichocephalic shape, while the nose was short with wide nostrils (Różdżyńska-Świątkowska et al. 2020).

Colmenares-Bonilla et al. (2017) described the medical history of 50 patients with confirmed MPS IV. The diagnostic process took from 1 to 15 years. Ten patients were initially misdiagnosed, with the incorrect diagnosis being skeletal dysplasia, achondroplasia, Ehler-Danlos syndrome, Sotos' syndrome, scoliosis, and even chronic bronchitis. Twenty-nine patients never received an initial diagnosis. The first noticeable symptoms were chest deformity, knee valgus, and joint abnormalities, including restriction of mobility and deformation. All patients had a severe disease phenotype that should allow for early diagnosis. Interestingly, in patients with a positive family history, the diagnosis was placed later than in the case of the no-loaders family (Colmenares-Bonilla et al., 2017).

Biswas et al. (2017) discussed the case of a 19-year-old boy diagnosed in childhood with spinal-epiphyseal dysplasia. Until the age of 11 years, the child's development was normal, only later there were valgus of the legs and deformation of the limbs and the spine, which led to the loss of mobility. At the same time, no abnormalities in intellectual development were observed. Clinical trials conducted at a later time showed, among others, thickening of facial features, short stature, deformation of the chest and wrists, and hypermobility of the joints. At that stage, compression of the spinal cord was suspected. The examination of the whole body skeleton revealed dysostosis multiplex which is typical for MPS. The positive result of the urine GAG test was the basis for the enzymatic activity test confirming the diagnosis of MPS IVA (Biswas et al. 2017).

Early observations of Fang-Kircher et al. (1995) led them to describe the case of a boy who was admitted to the hospital with pain, mainly in his left hip, at the age of 13 years. On the basis of the obtained test results and the clinical picture, the boy was diagnosed with Pethry's disease. As treatment was ineffective and the walking problems progressed, the patient underwent a hip replacement at 51 years of age. Further examinations revealed a short neck, altered hair structure, and corneal clouding (the patient only complained of poor color vision). However, there were no problems with hearing and no features of hepatosplenomegaly or heart abnormalities. Imaging studies showed slight changes in the skeletal system. After 38 years, the patient was diagnosed with MPS IVA. Interestingly, no ligament laxity or hypermobility of the joints, typical of this MPS type, was observed, while the stiffness of the right elbow, hip, and knee joints was noted (Fang-Kircher et al. 1995).

The summary of symptoms that may indicate MPS IV and possible misdiagnoses is presented in Table 2.

MPS VI (Maroteaux-Lamy disease)

MPS VI is caused by the deficiency in the activity of N-acetylgalactosamine-4-sulfatase, an enzyme involved in the degradation of DS and C6S (D'Avanzo et al. 2021). As in Morquio syndrome, MPS VI is primarily a skeletal disorder, as neurological dysfunctions, if they occur, are the result of secondary abnormalities (Galimberti et al. 2018; Reichert et al., 2016). Clinical features, age of symptom onset, and the speed with which the disease progresses allow us to distinguish a rapidly progressive (severe, with early symptoms, around 2 years of age) and a slow (milder) phenotypes. However, an intermediate form between slowly and rapidly progressing MPS VI also exists (Akyol et al. 2019). Depending on their phenotype, patients may live between 20 and 50 years (Zhou et al. 2020; Khan et al. 2017). Typical symptoms of MPS IV include thickened facial features, dysostosis multiplex, short stature, motor dysfunction, eye defects (corneal clouding), ENT (ear, nose, throat) symptoms, or circulatory and respiratory failure (D'Avanzo et al. 2021). It is worth to indicate that the severe forms may have clinical onset from birth, while milder patients can be diagnosed at the 2nd or 3rd decade of life. Despite a lack of intellectual deficiency, some CNS problems may appear, like cervical cord compression, meningeal thickening, communicating hydrocephalus, and optic nerve atrophy (Valayannopoulos et al. 2010). Interestingly, an attenuated phenotype can be associated with specific variants of the *ARSB* gene, like p.R152W which is especially frequent in the Lithuanian population, suggesting the founder effect (Jurecka et al. 2014).

Skeletal symptoms typical of MPS VI may be misinterpreted as multiple epiphyseal dysplasia (MED), spondyloepiphyseal dysplasia congenita (SEDC), or Stickler dysplasia (Lachman et al. 2014). With severe phenotype, Maroteaux-Lamy syndrome may be misdiagnosed as Hurler disease. MPS VI may also show some similarities to mucolipidosis II or multiple sulfatase deficiency (Al-Sannaa et al. 2017).

Choy et al. (2015) presented the medical history of 8 patients diagnosed (sooner or later) with MPS VI. In the first described case, fetal swelling in the 18th week of pregnancy was the earliest manifestation of the disease. Despite the symptoms, including development slowdown, progressive limitation of limb mobility, diagnosed hepatosplenomegaly, and heart valve abnormalities, MPS VI was not diagnosed until the age of 13 months when bold facial features began to become visible. In two patients, the first diagnosis was rheumatoid myocarditis (diagnosis made at 5 and 9 years of age). The first of them often suffered from recurrent upper respiratory tract infections and progressive joint stiffness. At the age of 5 years, the boy's cardiologist noticed thickened features and clawed hands. After identifying hepatosplenomegaly, MPS VI diagnostics was started. The second patient (diagnosed with rheumatoid arthritis at 9 years of age) also developed joint stiffness, short stature, and vision problems. Despite the mild thickening of the facial features, MPS was not suspected. Only when the patient started cardiological treatment at the doctor who previously treated a boy with a similar history of the disease, appropriate diagnostics were implemented. Carpal tunnel syndrome and heart abnormalities were found during the examination. An ophthalmological examination showed corneal clouding which is the cause of the patient's reported vision problems (Choy et al. 2015).

In two other patients, the motor delay was the leading symptom indicative of MPS (Choy et al. 2015). In the case of the first patient, diagnostic measures were taken due to the child's "reluctance to use their hands in everyday activities." The study showed low growth for age, altered facial features, and skin melanocytosis. The delay in walking did not cause any concern to the parents due to the similar duration of the older siblings. Apart from mild scoliosis and abnormalities of the metacarpals and phalanges, the patient had no other abnormalities of the skeletal system. An ophthalmological examination showed bilateral corneal opacity. Ultimately, the urine GAG test identified MPS as the cause of the observed disorders. The second patient went to the doctor because at the age of 15 months she was still not walking. In the interview, there was information about frequent diarrhea between 5 and 6 days of age. Radiological examination revealed dysostosis multiplex and numerous abnormalities of the skeletal system. At the age of 16 months, the diagnosis of MPS VI was made (Choy et al. 2015). An interesting case is a 50-year-old patient with a normal level of intelligence and physically active. At the age of 45, aortic valve replacement and mitral valve surgery were performed. Five years later, an ophthalmological examination revealed corneal opacity. The ophthalmologist ordered a urine GAG analysis, which turned out to be positive. Eventually, a woman (aged 50 years) was diagnosed with MPS VI (Choy et al., 2015).

Vairo et al. (2018) described the cases of 4 patients; in two of them, the first diagnosis was Morquio syndrome, while the remaining two remained without a diagnosis for a few years. Patients diagnosed at 5 years of age with Morquio syndrome were cousins who have been diagnosed with Maroteaux-Lamy syndrome more than 30 years after the initial diagnosis. Both men suffered from the pain of joint stiffness (with MPS IV excessive joint mobility is observed) (Galimberti et al. 2018). They were diagnosed with dysostosis multiplex, corneal opacity, cardiac disorders, deteriorating lung function, and altered facial features. In 2014, enzyme replacement therapy (ERT) was registered as a treatment method for MPS IVA, so both men were referred for a study to assess whether they would be able to benefit from the therapy. An enzyme activity test ordered by a physician showed a deficiency in enzyme activity which is the determinant of the Maroteaux-Lamy syndrome. The diagnosis was confirmed by the detection of pathogenic variants

of the gene encoding ARSB. Another described case was a 12-year-old female patient referred for examination due to progressive pain and contracture of the hand joints, wrist bone abnormalities, and corneal clouding. The girl's medical history contained information about a urine GAG test performed at the age of 7 years, but the results showed no abnormalities. Due to the deteriorating condition of the patient, re-tests were ordered, which revealed a slightly elevated level of DS in the urine. Genome sequencing was performed, which only after the second analysis allowed for the identification of the pathogenic variants of the gene. In order to confirm the obtained results, an analysis of enzymatic activity was performed, which clearly indicated MPS VI as a diagnosis (Vairo et al. 2018).

The studies described above pointed out two important things. Firstly, they emphasized the importance of expanding the knowledge and awareness of doctors about rare diseases, and secondly, they pointed to the need to perform biochemical tests in centers experienced in the diagnosis of MPS.

The summary of symptoms that may indicate MPS VI and possible misdiagnoses is presented in Table 2.

MPS VII (Sly disease)

Accumulation of GAGs containing glucuronic acid (C4S, C6S, DS, and HS) due to deficiency in β-glucuronidase (GUSB) activity is the major cause of MPS VII. Sly syndrome is extremely rare (< 1:1,000,000 live births) (Morrison et al. 2019), and there are currently fewer than 200 patients with a confirmed diagnosis. Exceptional low prevalence and high perinatal mortality (Shapiro et al. 2021) mean that little information is available about the clinical picture. As in the case of other MPS, patients may show a wide range of symptoms with varying degrees of severity and disease progression. What distinguishes MPS VII from others is that patients have visible symptoms at birth. Non-immune hydrops fetalis (NIHF), characterized by ascites, hepatosplenomegaly, limb edema, brain development delay, lung hypoplasia, and dilatation of the ventricles, is one of them (Montaño et al. 2016). Although NIHF can occur in the course of many metabolic diseases (including MPS I and II) (Gort et al. 2012), it is the most characteristic for MPS VII (Hizem et al. 2021). Fetal features such as macrocranes, hypertelorism, and retnogratia are typical for MPS VII, and the edema and the hydropic aspect of the placenta should be an argument to consider Sly syndrome (Hizem et al. 2021). It should be noted that a patient with NIHF, if surviving infancy, does not have to develop severe Sly syndrome (Shapiro et al. 2021). Other distinctive features, already visible in newborn babies, are thickened facial features, skeletal dysplasia, cardiac and respiratory dysplasia, hepatosplenomegaly, frequent ear infections, and handicap (Montaño et al. 2016).

An interesting cohort of MPS VII patients was found in Brazil (Giugliani et al. 2021). They expressed a relatively mild phenotype, and the diagnosis was made at the age of 5 years on average. The patients had a neurodevelopmental delay and cognitive impairment. Knee deformities and dysostosis multiplex were also found, and cardiomyopathy developed in some of them. Interestingly, the vast majority of patients from this cohort were homozygous for the c.526C > T (p.Leu176Phe) variant of the *GUSB* gene, suggesting the founder effect (Giugliani et al. 2021).

In the most severe cases of MPS VII, the baby is stillborn or dies in the first few weeks of life, most often as a result of NIHF. Patients with milder severity usually live to the second or third decade. Life expectancy is shortened due to recurrent upper respiratory tract infections, neurological complications, and gastrointestinal abnormalities. In rare cases, it may happen that the patient lives up to 50 years (Morrison et al 2019).

Patients with NIHF are diagnosed early, on average before the age of 2 years. People with a later diagnosis (between 6 and 14 years of age) are most often characterized by altered facial features, hernias, sleep disorders, recurrent infections ear, enlarged liver and/or spleen, thick hair/eyebrows, and an enlarged head circumference. The misdiagnosis was usually ASD, Perthes disease, ADHD, or developmental delay (Morrison et al. 2019).

The summary of symptoms that may indicate MPS VII and possible misdiagnoses is presented in Table 2.

MPS IX (Natowicz disease)

MPS IX is caused by a deficiency in hyaluronidase activity, resulting in the accumulation of hyaluronic acid in the cells (Zhou et al., 2020). Natowicz syndrome is one of the two rarest types of MPS, as until now, only 4 cases were described. Based on them, it can be said that the typical symptoms include short stature, numerous cysts, frequent ear infections, cleft palate, and the development of soft-tissue masses. Reported in three siblings, later diagnosed with MPS IX, joint stiffness was initially misdiagnosed as juvenile idiopathic arthritis (JIA). Taking into account the biological function of hyaluronan, it can be assumed that abnormalities of the skeletal system and joints will be part of the typical picture of patients with this syndrome (Kiykim et al. 2015; Zhou et al. 2020; Reichert et al. 2016).

The summary of symptoms that may indicate MPS IX and possible misdiagnoses is presented in Table 2.

MPS X

In 2020, Trabszo et al. published a paper on the biallelic mouse model of arylsulfatase K deficiency (ARSK). In mice deficient in this enzyme, accumulation of HS and DS was observed in many tissues in the absence of elevated urinary GAG levels. Mild behavioral changes were not associated with skeletal abnormalities or neurological disorders. A classification of the deficiency in arylsulfatase K activity as MPS IIB has been suggested (Trabszo et al. 2020).

Recently, Verheyen et al. (2021) described 4 patients, from two unrelated families, with arylsulfatase K deficiency. The first pair of siblings were the only children of the couple. The children of the second pair had healthy older siblings. Sick children from both families were diagnosed with skeletal dysplasia with signs of spinal-epiphyseal dysplasia and thickened facial features. Radiological tests showed flat feet, widening of the ribs and collarbones, reduction of bone epiphyses, and hypo-plasticity of the bones of the wrist. Typical heart abnormalities (murmurs, regurgitation, valve abnormalities, and others) have been found in children without older siblings. These children also had short stature and short torso. Over time, recurrent ear infections, difficulty sleeping, and macrocephaly seen at birth have been associated with ARSK deficiency. There were also mild clouding of the lens and vitreous body and slight pigment changes (the eye was examined for changes known in the course of MPS). None of the four children had cognitive impairment, neurological abnormalities, or learning difficulties. Urinary GAG examination showed no abnormalities, but electrophoresis showed increased levels of keratan sulfate and chondroitin sulfate. The possible diagnosis was Morquio syndrome, but the activity of galactosamine-6-sulfatase (MPS IVA) and β-galactosidase (MPS IVB) was within the norms. Analysis using liquid chromatography/mass spectrometry (LC–MS/MS) showed a significantly increased level of DS in two children. Genetic studies confirmed in all four children a biallelic pathogenic variant of the gene encoding ARSK (Verheyen et al. 2021).

In summary, in the case of patients with skeletal dysplasia of unclear origin and the clinical symptom of MPS with a simultaneous normal level of GAG in the urine, diagnostics for the enzymatic activity deficit should be considered. Trabszo et al. (2020) proposed to classify ARSK activity deficit as MPS IIB. We, however, agree with Verheyen et al. (2021) that it would be more appropriate to treat this phenomenon as a new type of MPS, named MPS X. The phenotype presented by the mice did not include the skeletal changes seen in the children studied. Moreover, neither behavior abnormalities nor heparan sulfate accumulation was observed in human patients, contrary to what was the case in the mouse model.

The summary of symptoms that may indicate MPS X and possible misdiagnosis is presented in Table 2.

MPSPS (MPS-plus syndrome)

MPS-plus syndrome (MPSPS) is a specific form of MPS in which despite the normal activity of lysosomal enzymes and the absence of autophagy and endocytosis disorders, GAG accumulation occurs. The pathomechanism of this disease is not explained, while the role of the p.R498W variant of the *VPS33A* gene was postulated when detected in patients with MPS features with normal results of enzymatic activity tests (Kondo et al. 2017). Like most MPS types, MPSPS is inherited autosomal recessively, while GAG accumulation mainly concerns HS and DS (Vasilev et al. 2020).

Patients, children between 2 and 6 months of age, are frequently hospitalized due to recurrent infections of the upper respiratory tract, accompanied by coughing and shortened and noisy breathing. MPSPS develops a number of symptoms typical of MPS, such as altered facial features, macroglosses, skeletal abnormalities (including dysostosis multiplex and joint stiffness), cardiovascular abnormalities, psychomotor retardation, and developmental delay. Moreover, proteinuria occurs due to kidney damage, and hematopoiesis disorders (including anemia, leukopenia, and thrombocytopenia) and congenital heart defects are found, which are not observed in patients with MPS (Vasilev et al. 2020). Immunological features, like decreased serum IgG concentrations, were documented in some patients (Vasilev et al. 2020).

MPSPS is a rare and severe disease with only 20 cases reported to date (Vasilev et al. 2020; Faraguna et al. 2022). Most patients died between 10 and 20 months of age from heart failure. The differential diagnosis should include other types of MPS and lysosomal storage diseases, like MPS I and II, mucolipidosis, fucosidosis, mannosidosis, Gaucher disease, Niemann-Pick syndrome, sialidosis, or multiple sulfatase deficiency (Vasilev et al. 2020).

Kondo et al. (2016) described the results of the analysis of 13 cases with diagnosed MPSPS. All patients showed the features typical of MPS, including recurrent respiratory infections, thickened facial features, macroglosses, chest abnormalities, dysostosis multiplex, joint contractures, progressive skeletal dysplasia, and psychomotor retardation. Blood tests showed leukopenia, thrombocytopenia, and anemia. Urine tests, in addition to high GAG levels, showed proteinuria. Most of the patients suffered from hypertrophic cardiomyopathy, and 11 children died before the age of 2 years due to circulatory and respiratory failure (Kondo et al. 2017).

Dursun et al. (2017) described the disease history of two children diagnosed with MPSPS. Both girls had a number of symptoms typical of lysosomal storage diseases, in one

of them mainly abnormalities of the skeletal system, in the other altered facial features, developmental delay, and deterioration of motor functions and speech. Both patients developed anemia and proteinuria, and breathing difficulties due to lung infection were the predominant symptoms. Later studies revealed acute respiratory distress syndrome (ARDS). Enzymatic analyses and exon sequencing ruled out all known lysosomal storage diseases, but gene mapping and family exome sequencing indicated a pathogenic variant of the *VPS33A* gene as the cause of the disease (Dursun et al. 2017).

Finally, during the clinical examination of a 5-month-old patient, the following symptoms were observed: thickened facial features, a short neck, abnormalities in the chest and spine, joint stiffness, and a slight enlargement of the abdomen (Vasilev et al., 2020). From the third month of life, the baby's shallow and loud breathing was noticeable; from the age of 12 months, she started recurring respiratory infections. Echocardiography revealed a patent foramen ovale (PFO), blood tests showed anemia, but urine analysis was normal. With time, the child's condition worsened, and at the age of 1 year and 9 months, the patient presented with severe developmental delays, hepatosplenomegaly, nephromegaly, numerous heart abnormalities, and pulmonary hypertension. Multiple organ failure led to the patient's death before 2 years of age, and MPSPS was suspected on the basis of the clinical picture. The diagnosis was confirmed by genetic testing revealing the p.R498W allele of the *VPS33A* gene (Vasilev et al. 2020).

The summary of symptoms that may indicate MPSPS and possible misdiagnoses is presented in Table 2.

Mucopolysaccharidoses and problems with correct diagnosis—biochemical tests

Since the main cause of GAG storage in MPS is a defect in the activity of certain lysosomal enzymes, the primary diagnosis of MPS is made on the basis of the enzymatic activity test. However, it is not the first test to be performed when MPS is suspected as diagnostics often begins with the performance of non-enzymatic tests (Monachesia et al. 2018). The Berry test and the acid turbidity test were the first methods used to screen for MPS; however, both tests showed high rates of both false-negative and positive results (Chih-Kuang et al. 2002). In a study by Chih-Kuang et al. (2002), 492 samples were tested with these methods for congenital disorders of metabolism. Despite a positive Berry test, 68 samples revealed negative results in the acid turbidity test. Of these subjects, 48 patients were diagnosed with MPS (types I, II, III, IV, and VI). In the case of types I, II, and VI, positive results were obtained in both tests, but about half of the patients with MPS III and MPS IV would not be recognized (test results were false negative) if the samples were analyzed only using the abovementioned tests (Chih-Kuang et al. 2002).

Currently, the dimethylmethylene blue (DMB) assay is usually the first test to be performed when MPS is suspected. DMB allows estimating the level of GAG in the patient's urine. However, the limitations of this test should be kept in mind, as the DMB index (GAG mg/mmol creatinine) is age-dependent and differs between the types of MPS, and the assay is not suitable for determining the type of disease (Chih-Kuang et al. 2002). Due to the fact that the quantification of urinary GAGs may give false-negative results in patients with MPS III and IV, when performing the DMB test, simultaneous unidirectional electrophoresis is recommended (Mahalingam et al. 2004). This combination provides both high sensitivity and specificity (Monachesia et al. 2018). In the differential diagnosis, bidirectional electrophoresis (2D-EP) works best, as precise GAG separation allows to determine the type of MPS based on the obtained electrophoretic pattern (Chih-Kuang et al. 2002).

An issue as important as the selection of the test is the appropriate method of GAG isolation. Pyrimidine chloride is most often used for this, but it was suggested to use Alacian blue due to its ability to detect KS (which may be undetectable when employing other methods). Additionally, pyrimidine chloride, unlike blue, does not detect GAGs with a molecular weight of less than 3000 (Mahalingam et al. 2004).

Appropriate diagnostic management at the stage of biochemical analysis is as important as the clinical evaluation of the patient. When choosing a test, one should be guided by its specificity and sensitivity. It should also be remembered that the GAG assessment should be made both in terms of quantity and quality. Obviously, biochemical and genetic analyses should be performed to confirm the diagnosis; however, these methods are still too expensive to be used in the routine practice of physicians which should make a primary diagnosis seeing patients with unusual symptoms.

When and what to look for?

MPS is a rare disease; therefore, even a patient with obvious symptoms may initially be misdiagnosed due to their similarity to more common diseases (Kubaski et al. 2020). Despite the diverse clinical picture, both between types and within subtypes of the same disease (Wiśniewska et al. 2021), there are a number of symptoms common to all MPS, which include, among others, thickened facial features, recurrent infections of the upper respiratory tract, lower than average height, joint stiffness (in all types except MPS IV which is characterized by excessive joint mobility), abnormal dentition, heart abnormalities (e.g., valve defects), and hepatospelnomegaly (Galimberti et al. 2018, Hoseinzadeh Moghadam et al. 2019).

The diagnosis of MPS should be considered when the patient develops joint stiffness or contractures without signs of swelling or local inflammation. An additional indication may be the lack of inflammatory markers and the lack of response to standard treatment in the case of inflammation. It also happens that in MPS heart murmurs and vision problems are associated with joint stiffness (Rigoldi et al. 2018). In the case of neuropathic forms, the earliest symptom may be developmental delay or regression of skills. Behavioral disorders, such as overactivity or aggression, may appear later (Kubaski et al. 2020). The rationale of performing tests for metabolic diseases in patients with intellectual disability (due to the low diagnostic efficiency of the tests and the rarity of this group of diseases) is being discussed, but many protocols recommend the differential diagnosis (Campistol et al. 2016; Kubaski et al. 2020). Non-immune fetal edema also may be an indication for considering MPS as a potential diagnosis (Iyer et al. 2021). Characteristic changes in the skeletal system and even neurological changes within the brain can be seen in imaging studies (Reichert et al. 2016; Galimberti et al. 2018; Zhou et al. 2020).

In patients with mild phenotypes where the onset of symptoms is significantly extended over time, the correct diagnosis of the disease is even more difficult, but mild skeletal manifestations appear over time (Rocha Siqueira et al. 2016). It seems almost impossible that a person with a single symptom, even typical of MPS, was diagnosed quickly and correctly (Kubaski et al. 2020). Therefore, a reasonable approach seems to be to initiate MPS diagnostics in the event of several symptoms indicative of MPS (Omar et al. 2019).

In addition to symptoms that may indicate MPS, there are disorders unusual for this group of diseases, like hematopoiesis disorders, blood tests showing anemia, leukopenia and thrombocytopenia, and kidney involvement manifested by proteinuria and increased excretion of uric acid. Brain imaging studies showed delayed myelination of white matter and calcification of the basal ganglia (Kondo et al. 2017).

A well-collected interview and careful analysis of the patient's medical history is the most important stage of the diagnostic process (Zhou et al. 2020). A relative may have similar symptoms or a family history of MPS might be confirmed previously.

A simplified model for proposed procedures of MPS diagnosis, indicating symptoms which may suggest this disease and biochemical and genetic tests to confirm or reject the suspected diagnosis, is presented in Fig. 1.

Diagnostics and therapy

Since the malfunction of the enzymes, which is the primary cause of MPS, causes excessive accumulation of GAGs, the usual first test for the diagnosis of MPS is to assess the amount of GAG in the urine. However, it should be remembered that the result of such examination is not the basis for confirmation or rejection of the diagnosis. Bidirectional electrophoresis is also recommended to better define the problem (Chih-Kuang et al. 2002). For adults with a mild phenotype or early stage of the disease, GAG levels may be normal and the test may be false negative (Zhou et al. 2020; Rigoldi et al. 2018). In neonates, maternal estrogen may interfere with testing performed, resulting in false-positive results (Monachesia et al. 2018).

The gold standard in the diagnosis of MPS is the measurement of enzymatic activity from leukocytes or fibroblast cultures. A dry blood drop (DBS) test, although useful in primary screening, cannot be used as the basis for diagnosis (Kubaski et al., 2020). Performing molecular tests to identify pathogenic variant(s) of the relevant gene is also recommended when confirming the diagnosis (Rigoldi et al. 2018). This allows us to determine what phenotype of the disease may develop in the patient; it is possible to better adjust the therapy or check whether we are dealing with a syndrome similar to MPS (Kubaski et al. 2020).

Importantly, it is possible to perform prenatal and preimplantation tests for MPS (Gaber et al. 2015). It is especially important and recommended if MPS is diagnosed in older children from a previous pregnancy. Moreover, if any of the relatives (especially in closely related couples) have been diagnosed with MPS, genetic testing is recommended (Vasilev et al. 2020; Metcalfe et al. 2011, Merten 2019).

Early diagnosis is extremely important for therapy, as diagnosis in the asymptomatic or early stage of the disease allows us to achieve better therapeutic effects, including longer maintenance of the efficiency and normal functions of organs (Zhou et al. 2020). There is currently no effective causal therapy (Fecarotta et al. 2018), and treatment of MPS is symptomatic and requires the cooperation of many specialists. In addition to rehabilitation and surgery, enzyme replacement therapy (ERT) or hematopoietic stem cell transplantation (HSCT) is possible for some MPS types (Mitrovic et al. 2017). ERT is about delivering a properly functioning enzyme to the body and is available primarily in MPS I, II, IVA, VI, and VII. The most important limitation of ERT is its ineffectiveness in the case of symptoms from the central nervous system (CNS), as without appropriate modification (the use of a vector or direct administration) the enzyme is not able to cross the blood–brain barrier (Rintz et al. 2020; Fecarotta et al. 2018). HSCT is the transplant of cells from the blood (umbilical cord or peripheral) or bone marrow into the patient's body. This therapy is based on the assumption that cells introduced in this way will penetrate into tissues and organs, where they will secrete a properly functioning enzyme (Gaffke et al. 2019). Although this therapy is used in MPS I, II, IV, VI, and VII, finding matching donors is

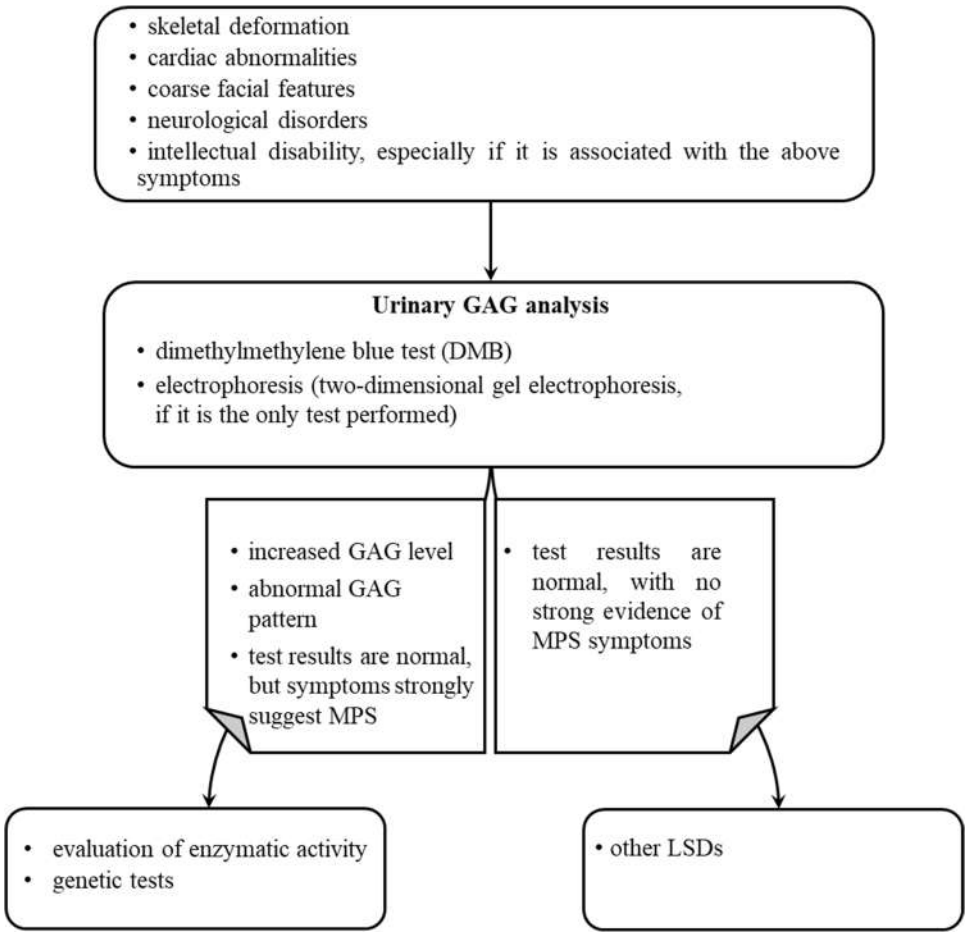

Fig. 1 Simplified diagram of the diagnostic procedures towards MPS. Abbreviations: DMB, dimethylmethylene blue; GAG, glycosaminoglycans; LSD, lysosomal storage diseases; MPS, mucopolysaccharidosis

an obvious and significant limitation. However, neither of these methods is effective for MPS III, where the main symptoms are related to CNS disorders (Zhou et al., 2020).

Other emerging therapies that may have a significant impact on the treatment of MPS include substrate reduction therapy (SRT) and gene therapy. The first one consists in limiting the production of compounds that, due to the lack of functional enzymes, cannot be properly degraded (Gaffke et al. 2017), while gene therapy consists in introducing the correct version of the gene encoding the appropriate enzyme into the patient's cells. Both treatments are still under investigation, though some clinical trials are ongoing (Zhou et al. 2020).

Currently, there are no defined biomarkers for MPSPS. The presence of typical clinical features and an excessive amount of GAG in urine, with the normal activity of lysosomal enzymes, are only grounds for suspecting MPS-plus syndrome. Confirmation of the diagnosis requires DNA analysis to detect the p.R498W variant of the *VPS33A* gene. Treatment is limited to relieving symptoms, and since MPSPS is a multi-system disease, therapy requires the participation of specialists in various fields (Vasilev et al. 2020).

Concluding remarks

MPS is a group of lysosomal storage diseases in which GAG accumulation occurs in the lysosomes of cells due to the malfunction of certain enzymes (Kloska et al., 2011). The exception is MPSPS, in which GAG accumulation is not a result of a reduction or complete lack of activity of lysosomal hydrolases, but is related to a specific allele of the *VPS33A* gene. In both cases, due to the storage of unbranched polysaccharides, the proper functioning of the lysosome is disturbed, which leads to pathological changes in cells, tissues, and organs.

Difficulties in diagnosing MPS have many causes, but the main problem is that they are multi-system rare diseases of a progressive nature. Symptoms may differ not only between patients suffering from various MPS types, but also within subtypes of the same MPS. There are even differences between patients with the same diagnosis, depending on whether they develop severe or mild disease. In the case of a mild phenotype, the onset of symptoms is very long, and at first glance, they may seem unrelated and may be treated as manifestations of various diseases. Even among severe MPS patients with visible, distinctive features, late

diagnosis, resulting from an initial misdiagnosis, is common. A review of the available literature suggests that the similarity of MPS to neurological, rheumatological, and orthopedic disorders is the most common cause of late diagnosis.

Early diagnosis requires the cooperation of many specialists and is extremely important for successful therapy. The longer the disease goes undiagnosed, the more irreversible changes occur in the body and the potential treatments will not be effective.

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Declarations

Competing interests The authors declare no competing interests.

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The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease

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The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease

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Abstract: Mucopolysaccharidosis (MPS) comprises a group of inherited metabolic diseases. Each MPS type is caused by a deficiency in the activity of one kind of enzymes involved in glycosaminoglycan (GAG) degradation, resulting from the presence of pathogenic variant(s) of the corresponding gene. All types/subtypes of MPS, which are classified on the basis of all kinds of defective enzymes and accumulated GAG(s), are severe diseases. However, neuronopathy only occurs in some MPS types/subtypes (specifically severe forms of MPS I and MPS II, all subtypes of MPS III, and MPS VII), while in others, the symptoms related to central nervous system dysfunctions are either mild or absent. The early diagnosis of neuronopathy is important for the proper treatment and/or management of the disease; however, there are no specific markers that could be easily used for this in a clinical practice. Therefore, in this work, a comparative analysis of shared and specific gene expression alterations in neuronopathic and non-neuronopathic MPS types was performed using cultures of cells derived from patients. Using transcriptomic analyses (based on the RNA-seq method, confirmed by measuring the levels of a selected gene product), we identified genes (including *PFN1*, *ADAMTSL1*, and *ABHD5*) with dysregulated expression that are common for all, or almost all, types of MPS, suggesting their roles in MPS pathogenesis. Moreover, a distinct set of genes (including *ARL6IP6* and *PDIA3*) exhibited expression changes only in neuronopathic MPS types/subtypes, but not in non-neuronopathic ones, suggesting their possible applications as biomarkers for neurodegeneration in MPS. These findings provide new insights into both the molecular mechanisms of MPS pathogenesis and the development of differentiation method(s) between neuronopathic and non-neuronopathic courses of the disease.

Keywords: mucopolysaccharidosis; neurodegeneration; transcriptomics

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1. Introduction

Although fibroblasts might not initially appear to be an obvious model for studying neurodegeneration, they share several significant characteristics with neurons. Both cell types originate from the ectoderm, suggesting common molecular mechanisms, including signaling pathways and gene regulatory processes. This feature is critical for elucidating the pathogenesis of neurodegenerative diseases. Additionally, fibroblasts exhibit disruptions in cellular pathways, such as oxidative stress, autophagy, and metabolism, which are also essential factors in neurodegeneration. Fibroblasts can reflect systemic cellular changes that influence neuronal function, especially in genetic disorders where the consequences of mutations remain unclear. Furthermore, fibroblasts are found near blood vessels, in the meninges, and within the choroid plexus of the brain and spinal cord, where they

play critical roles in maintaining central nervous system (CNS) function. Importantly, fibroblasts are easily accessible through minimally invasive procedures, enabling regular patient monitoring, and their stable cultures facilitate long-term studies, making them a valuable model for research into neurodegenerative mechanisms [1,2].

One of the aforementioned genetic disorders is mucopolysaccharidosis (MPS). This group of metabolic diseases arises from the disruption (reduction or complete absence) of lysosomal hydrolase activity, leading to the accumulation of glycosaminoglycans (GAGs). This accumulation damages cells, tissues, and the entire organism. Based on the defective enzyme and the type of stored GAG(s), 12 to 14 types and subtypes of MPS are currently recognized, depending on the criteria adopted [3]. The characteristics of MPS types/subtypes are presented in Table 1.

Table 1. Characteristics of classical/conventional types of MPS [4,5].

MPS Type	Defective Gene	Deficient Enzyme	Stored GAG(s) [a]	Neurological Symptoms
MPS I	*IDUA*	α-L-iduronidase	HS, DS	Impaired cognitive function, language, speech abilities, behavioural abnormalities (excessive silencing), sleeping problems, and/or epileptic seizures
MPS II	*IDS*	Iduronidase-2-sulfatase	HS, DS	Developmental delay, mental retardation, and behaviour problems (aggression and over-excitability)
MPS IIIA	*SGSH*	Heparan-N-sulfatase	HS	Developmental delay, cognitive impairment, behavioural disorders (impulsivity, aggression, anxiety disorders, and autistic behaviour), and sleeping problems
MPS IIIB	*NAGLU*	α-Nacetylglucosaminidase		
MPS IIIC	*HGSNAT*	Heparan α-Glucosaminide N-acetyltransferase		
MPS IIID	*GNS*	N-Acetylglucosamine-6-sulfatase		
MPS IVA	*GLANS*	N-Acetylglucosamine-6-sulfate sulfatase	C6S, KS	Absence or mild neurological disorders as a consequence of secondary disturbances
MPS IVB	*GLB1*	b-Galactosidase	KS	
MPS VI	*ARSB*	N-acetylglucosamine4-sulfatase (arylsulfatase B)	DS, C4S	-
MPS VII	*GUSB*	β-Glucuronidase	HS, DS, C4S, C6S	Impaired cognitive, language, and speech abilities; behavioural abnormalities; sleep problems; and/or epileptic seizures
MPS IX	*HYAL1*	Hyaluronidase	Hyaluronan	-
MPS X	*ARSK*	Arylsulfatase K	DS	-

[a] HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; C6S, chondroitin 6-sulfate; C4S, chondroitin 4-sulfate; -, no or few neurological symptoms.

Although MPS represents a heterogeneous group of diseases, several symptoms are typical across all subtypes [4,5]. Special attention should be given to symptoms related to CNS which occur in some, but not all, MPS types/subtypes, as indicated in Table 1 (see Refs. [6–8]). Patients with neuronopathic forms of MPS usually require round-the-clock care [9]. Although patients with non-neuronopathic MPS types may experience some neurological problems, they are not dominant in clinical settings [5,10–12].

The availability of a specific therapy largely depends on the type of MPS [13]. One of the treatment methods is enzyme replacement therapy (ERT), which involves supplying the missing enzyme to the body. A similar approach is used in hematopoietic stem cell transplantation (HSCT) for young patients (up to 2 years old). The transplanted bone marrow or

blood cells (from either umbilical or peripheral blood) from healthy individuals migrate to the patient's organs and tissues, synthesizing properly functioning enzymes [14–17]. ERT and HSCT are registered for MPS types I, II, IVA, VI, and VII [4,5,18]. For type III MPS, where somatic symptoms are relatively mild but CNS disorders are especially severe, there is currently no registered treatment method [19,20].

Despite the current understanding of the etiology of MPS, available treatment methods can only alleviate the symptoms of the disease, and they do so to a limited extent [5,21]. The effectiveness of therapies largely depends on the patient's age (the older the patient, the more advanced the changes that cannot be reversed). Additionally, enzymes and stem cells significantly struggle to reach poorly vascularized tissues, such as bone tissue and heart valves, as well as the brain, which is protected by the blood–brain barrier. As a result, these tissues hardly respond to treatment [4,5,7,22–25]. On the other hand, the early diagnosis of MPS is essential for achieving the best therapeutic outcomes. Identifying whether the disease has a neurodegenerative component or not would be especially beneficial, as it would guide the choice of treatment and provide better insights into the patient's prognosis [5].

Research on gene expression regulation in genetic diseases, such as MPS, is crucial for understanding the pathogenesis of these disorders. Such studies allow for the identification of changes in gene activity that lead to abnormal cellular functions and the development of the disease. This kind of research also enables the development of new therapeutic strategies that could focus on correcting abnormal gene expression, which cannot be restored by ERT or HSCT. Moreover, understanding the mechanisms of gene expression regulation facilitates the advancement of molecular diagnostics, which can lead to the earlier detection of genetic diseases, the prompt initiation of treatment, and the effective monitoring of its course. In the case of MPS, it may also help with predicting the neuronopathic component. As a result, such research helps to improve the quality of life for patients and increase the effectiveness of therapies [26–28].

Since neuronopathic and non-neuronopathic types of MPS differ in symptoms, it is likely that these differences arise not only from the kind of accumulated GAG(s), but also from other molecular processes. In fact, the significant modulation of MPS pathomechanism has been demonstrated to be related to the dysregulation of expression of specific genes [21,29]. Therefore, the aim of this study was a comparative analysis of shared and specific gene expression alterations in neuronopathic and non-neuronopathic MPS types using fibroblasts derived from patients. This research should help us to identify potential disease markers and their specific symptoms, with a particular focus on neurodegeneration.

2. Results

2.1. Indentification of Transcripts with Changed Expression Levels in More than One MPS Type/Subtype

In the first stage of this study, we aimed to identify transcripts whose expression is disrupted more than once among the 11 tested MPS types/subtypes. The analysis of the number of genes with altered expression levels in MPS cells compared to control cells indicated approximately 300 such transcripts for MPS II, IVA, and VI, and almost 900 for MPS IIIA, IIIB, IVB, and IX. Furthermore, for each MPS type/subtype, expression disruptions in at least half of these genes were also observed in at least one other type/subtype of MPS (Figure 1). In general, the disruption of the expression of a large number of transcripts was observed not in an individual, but rather in multiple types/subtypes of MPS, both neuronopathic and non-neuronopathic, indicating common pathways in the pathogenesis of the MPS disease.

A more detailed analysis involved determining the number of transcripts with altered expression levels depending on the number of MPS types/subtypes. It revealed, for example, 399 transcripts with altered expression levels compared to the control cells in at least 2 different MPS types/subtypes, 85 transcripts with altered expression levels in at least 6 MPS types/subtypes, and 8 transcripts with altered expression levels in at least

10 MPS types/subtypes. It also revealed one transcript with altered expression levels in all 11 tested MPS types/subtypes compared to the control (Figure 2).

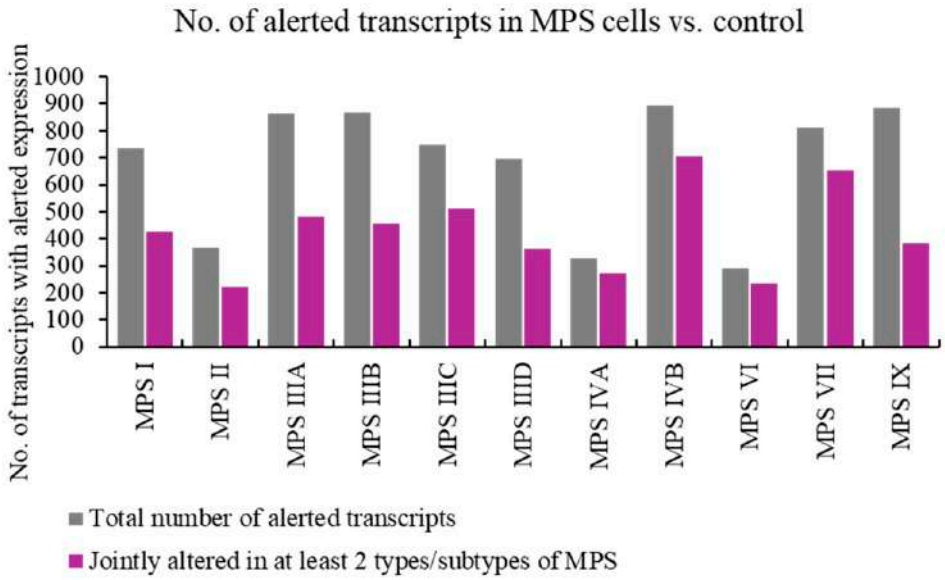

Figure 1. Total number of transcripts with altered levels of expression (at FDR < 0.1; $p < 0.1$) in cells of different MPS types/subtypes relative to control cells, and those concerning jointly neuronopathic and non-neuronopathic types.

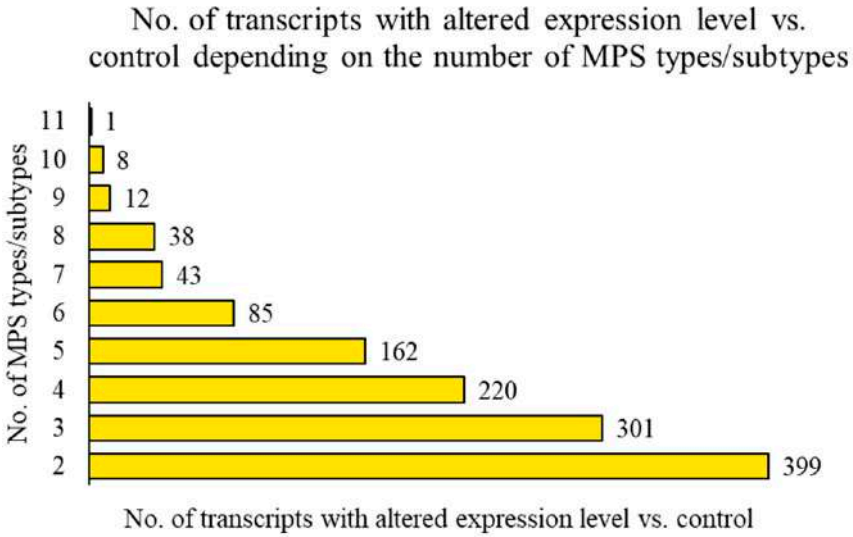

Figure 2. Number of transcripts with altered levels of expression (at FDR < 0.1; $p < 0.1$) in cells of different MPS types/subtypes relative to control cells in relation to the number of MPS types where such differences occur (1 transcript in 11 MPS types, 8 transcripts in 10 MPS types, and so on).

2.2. Transcripts with Altered Levels in Most of MPS Types/Subtypes

The list of transcripts with altered expression levels in at least 10 MPS types/subtypes, for which the $\log_2$ fold change value exceeded 2.5 or −2.5 ($\log_2$FC > 2.5 or <−2.5), is shown as a heatmap in Figure 3 and Table 2, with exact $\log_2$FC values. This list includes transcripts, such as the up-regulated *PFN1*, encoding the protein profilin, which showed increased expression in all MPS types/subtypes. Up-regulation was also observed for genes like *ADAMTSL1* (coding for ADAMTS Like 1 or Punctin-1, a putative secretory protein), *MFAP5* (coding for Microfibril-Associated Protein 5), *SH3BP5* (coding for SH3 Domain-Binding Protein 5), and *CAPG* (coding for Gelsolin-Like Capping Actin Protein). The analysis

also identified down-regulated genes, including *C1D* (coding for C1D Nuclear Receptor Corepressor), *ABHD5* (coding for Abhydrolase Domain Containing 5, Lysophosphatidic Acid Acyltransferase), *LY6K* (coding for Lymphocyte Antigen 6 Family Member K), and *PLCB4* (coding for Phospholipase C Beta 4).

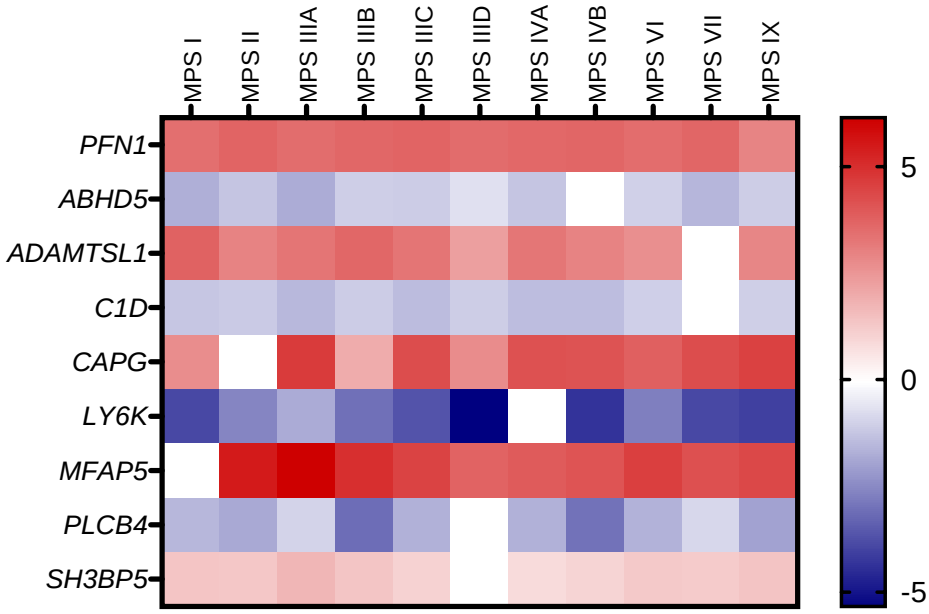

Figure 3. A heatmap presentation (created with HeatMapper software v. 2.8) of genes with altered expression levels in at least 10 MPS types/subtypes, for which the $\log_2$ fold change value exceeded 2.5 or −2.5 ($\log_2$FC > 2.5 or <−2.5).

Table 2. Transcripts with altered expression levels in at least 10 types/subtypes of MPS compared to control cells, with $\log_2$FC > 2.5 or <−2.5 (X, changes smaller than $\log_2$FC > 2.5 or <−2.5).

MPS Type	Changes in Levels (in $\log_2$FC) in Specific Transcripts (MPS vs. Control)								
	PFN1	***ABHD5***	***ADAMTSL1***	***C1D***	***CAPG***	***LY6K***	***MFAP5***	***PLCB4***	***SH3BP5***
MPS I	3.4	−1.7	3.8	−1.2	2.7	−3.8	X	−1.5	1.4
MPS II	3.7	−1.2	3.0	−1.1	X	−2.6	5.5	−1.8	1.3
MPS IIIA	3.5	−1.7	3.3	−1.5	4.7	−1.8	6.1	−0.9	1.8
MPS IIIB	3.6	−1.0	3.6	−1.1	2.0	−3.0	5.0	−3.1	1.4
MPS IIIC	3.7	−1.1	3.3	−1.4	4.3	−3.6	4.5	−1.7	1.1
MPS IIID	3.5	−0.7	2.3	−1.1	2.8	−5.3	3.8	X	X
MPS IVA	3.6	−1.2	3.3	−1.4	4.2	X	3.9	−1.7	0.9
MPS IVB	3.7	X	3.0	−1.4	4.1	−4.3	4.1	−3.0	1.0
MPS VI	3.5	−1.0	2.7	−1.0	3.8	−2.7	4.6	−1.6	1.3
MPS VII	3.7	−1.5	X	X	4.3	−3.8	4.2	−0.9	1.2
MPS IX	2.9	−1.1	2.9	−1.0	4.6	−4.0	4.4	−2.0	1.4

2.3. PFN1—A Gene Whose Expression Is Enhanced in All MPS Types/Subtypes

Since *PFN1* was the only gene showing increased expression levels in all MPS types/subtypes, the immunodetection of the encoded protein, profilin, was performed in MPS cells. The profilin levels were significantly increased in all MPS types, specifically from about three-fold to about seven-fold, depending on the MPS type/subtype

(Figure 4). This also confirmed the reliability of the transcriptomic results, obtained by an independent method.

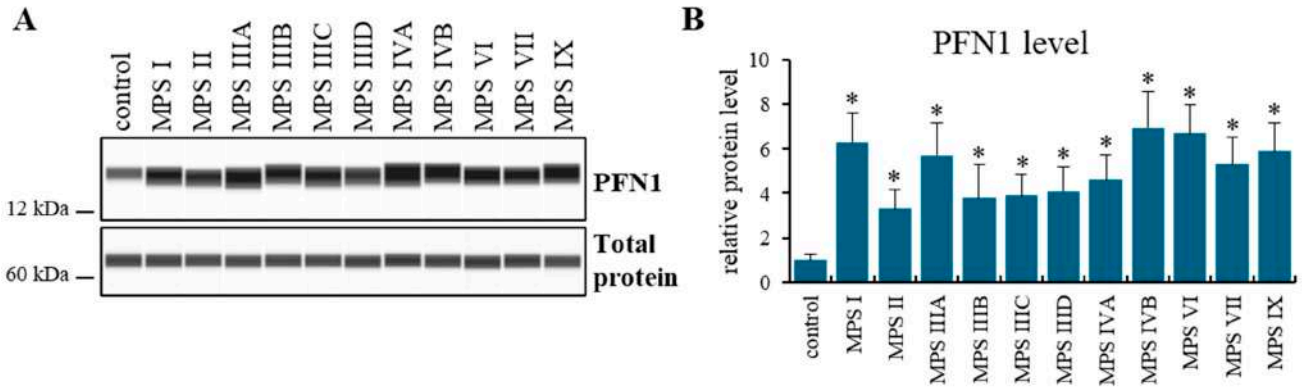

Figure 4. Levels of profilin-1 (PFN1 protein, the *PFN1* gene product) in control cells and in fibroblast derived from all tested MPS types/subtypes, as assessed by automatic Western blotting (the WES system, based on capillary electrophoresis and immunoblotting conducted inside each capillary). Representative blots (**A**) (the picture prepared using a piece of software which is an integrated part of the WES—Automated Western Blots with Simple Western; ProteinSimple, San Jose, CA, USA) and (**B**) (quantification of results, i.e., mean values from three independent biological experiments with error bars representing SD) are demonstrated. In panel (**A**), the Total Protein Module (#DM-TP01, Protein Simple, San Jose, CA, USA) was used to determine the loading control. Statistically significant differences (in two-way ANOVA) relative to the control (at $p < 0.05$) are indicated in panel (**B**) by asterisks.

2.4. Genes with Efficiency of Expression Changed Specifically Only in Neuronopathic MPS Types/Subtypes

The aim of the next stage of this study was to identify genetic factors that could potentially be responsible for the neuronopathic component of the described disease. An analysis was therefore conducted to isolate genes whose expression changes are specific only to the neuronopathic MPS types/subtypes (I, II, IIIA, IIIB, IIIC, IIID, and VII). Genes whose expression changes were also observed in non-neuronopathic MPS types (IVA, IVB, VI, and IX) were excluded from this analysis. The results demonstrated that among hundreds of genes with altered expression levels in each of the neuronopathic MPS types/subtypes, between 30 and 50% of the transcripts with changed expression levels also appeared in at least one other neuronopathic MPS type/subtype. In total, between 140 and approximately 400 genes (depending on the type/subtype of the disease) were found to possibly be associated with nervous system disorders (exactly 418 unique genes whose expression is significantly altered in neuronopathic MPS types/subtypes but not in non-neuronopathic types) (Figure 5).

The analysis of the number of transcripts with altered expression levels depending on the number of neuronopathic MPS types/subtypes showed values ranging from 322 (for at least two types/subtypes) to 1 (for six types/subtypes). There was no single gene whose expression was altered in all seven neuronopathic MPS types (Figure 6).

The list of genes whose expression was altered compared to control cells in at least five neuronopathic MPS types/subtypes, without changes in expression levels in non-neuronopathic MPS types/subtypes, is shown in Figure 7 and Table 3. A significant decrease in the expression of the *ARL6IP6* gene, which encodes ADP Ribosylation Factor Like GTPase 6 Interacting Protein 6, was observed in six out of seven MPS neuronopathic types/subtypes (no significant changes were observed in type IIID). Other genes that exhibited altered expression levels compared to control cells in five neuronopathic MPS types/subtypes included down-regulated *C11orf58* (corresponding to Chromosome 11 Open Reading Frame 58) and *MINOS1* (coding for Myosin X), and up-regulated *RPN2* (cod-

ing for Ribophorin II), *PDIA3* (coding for Protein Disulfide Isomerase Family A Member 3), and *VASN* (coding for Vasorin).

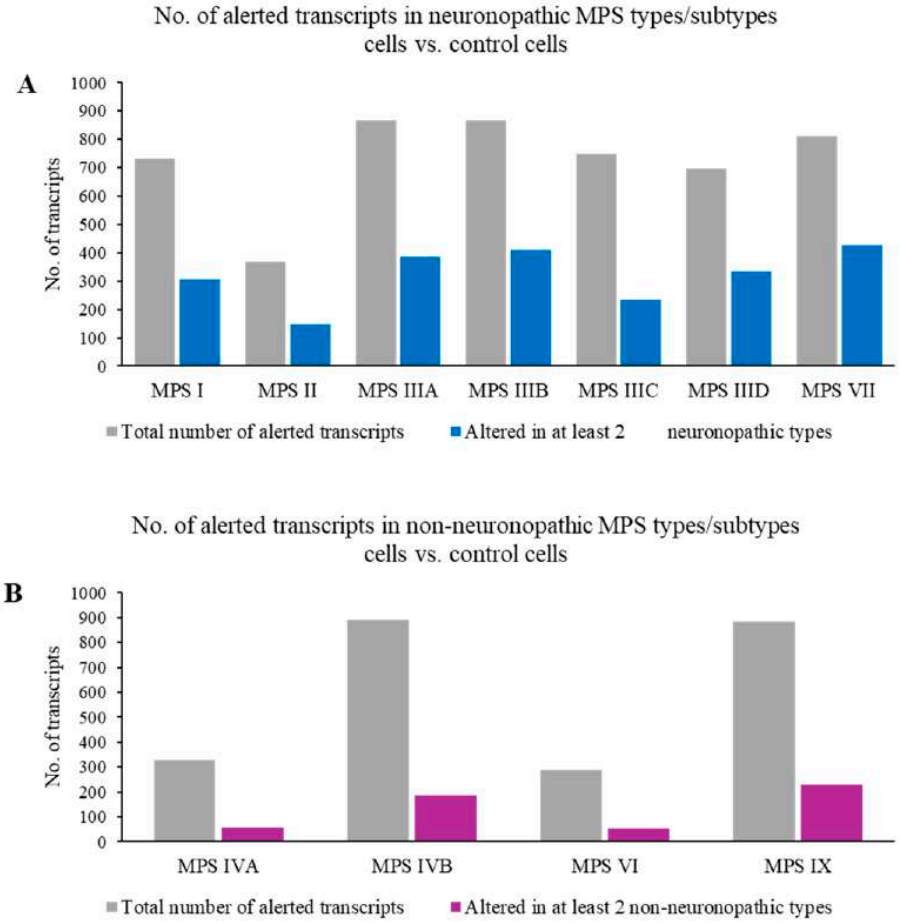

Figure 5. Number of transcripts with altered levels of expression (at FDR < 0.1; $p < 0.1$) in cells of different neuronopathic (**A**) and non-neuronopathic (**B**) MPS types/subtypes relative to control cells, with an indication of the number of specific transcripts altered in at least two neuronopathic (**A**) or non-neuronopathic (**B**) MPS types/subtypes.

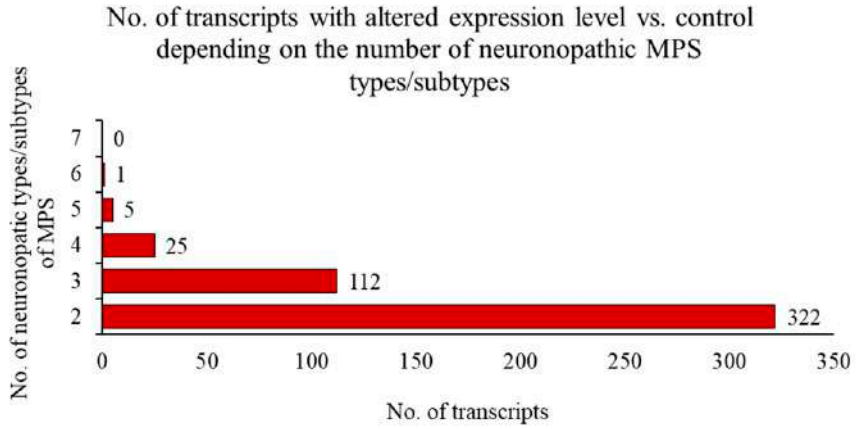

Figure 6. Number of transcripts with altered levels of expression (at FDR < 0.1; $p < 0.1$) in cells of different neuronopathic MPS types/subtypes relative to control cells in relation to the number of neuronopathic MPS types/subtypes where such differences occur (no (0) transcripts in 7 neuronopathic MPS types, 1 transcript in 6 neuronopathic MPS types, 5 transcripts in 5 neuronopathic MPS types, and so on).

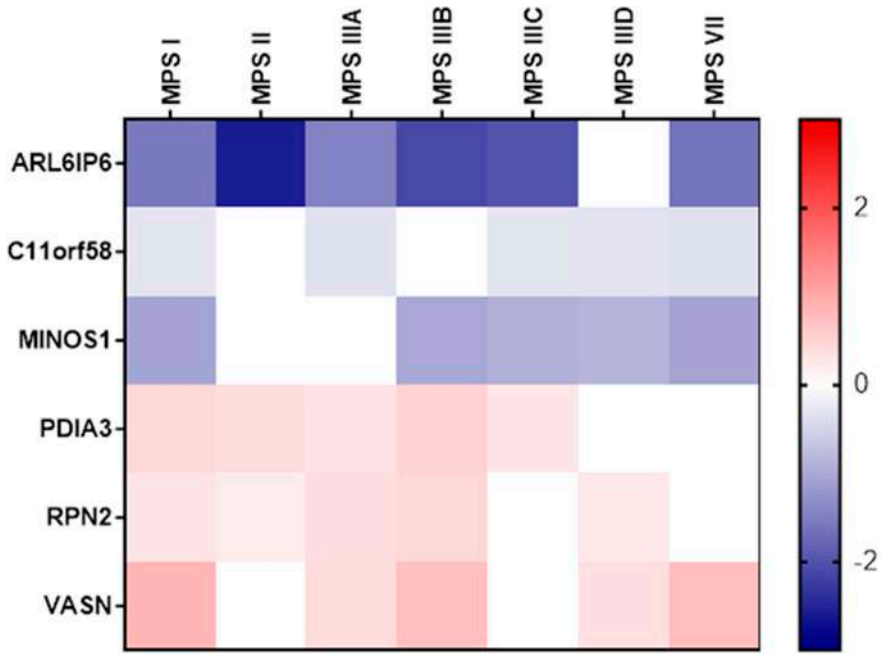

Figure 7. A heatmap presentation (created with HeatMapper software v. 2.8) of genes whose expression was altered compared to control cells in at least 5 neuronopathic MPS types/subtypes, without changes in expression in non-neuronopathic MPS types/subtypes.

Table 3. Transcripts with altered expression levels in at least 5 neuronopathic types/subtypes of MPS compared to control cells, without concurrent expression changes in non-neuropathic MPS types/subtypes.

Transkcript	Number of Transcripts with Common Expression Changes in at Least 5 Neuronopathic MPS Types vs. Control Cells	Up (↑) or Down (↓) Regulation	Neuronopathic MPS Types
ARL6IP6	6	↓	I, II, IIIA, IIIB, IIIC, VII
C11orf58	5	↓	I, IIIA, IIIC, IIID, VII
RPN2	5	↑	I, II, IIIA, IIIB, IIID
PDIA3	5	↑	I, II, IIIA, IIIB, IIIC
VASN	5	↑	I, IIIA, IIIB, IIID, VII
MINOS1	5	↓	I, IIIB, IIIC, IIID, VII

An analysis was also conducted to determine the number of transcripts whose expression was changed (either up- or down-regulated) based on the $\log_2$ fold change ($\log_2$FC) in expression relative to the control cells (Figure S1). The list of transcripts showing a three-fold increase/decrease in MPS cells compared to control cells is presented as a heatmap in Figure S2 and Table S1, along with the exact fold change values. High fold change values ($\log_2$FC > 3 or <−3) in the expression were frequently observed in individual MPS types/subtypes. Notable down-regulated genes in MPS compared to controls included *RARRES2* (coding for Retinoic Acid Receptor Responder 2) (MPS IIIA and IIIB) and *CXCL8* (coding for C-X-C Motif Chemokine Ligand 8) (MPS I and IIIA), while up-regulated genes compared to controls included *MFGE8* (coding for Protein Containing Milk Fat Globule EGF and Factor V/VIII Domain) (MPS I and IIIA) and *RPLP2* (coding for Ribosomal Protein Lateral Stalk Subunit P2) (MPS I and IIIB).

3. Discussion

MPS is a group of lysosomal storage diseases associated with impaired GAG degradation, leading to their accumulation in various tissues. Different types/subtypes of MPS exhibit significant clinical variability—some patients have severe neurodegenerative symptoms, while others mainly experience peripheral organ involvement (Table 1).

Currently, it is not possible to predict whether, and if yes—to what extent, a patient will develop neurological symptoms, as the neurodegenerative component can occur with varying severity even within the same type/subtype of MPS [30]. The ability to predict such a component would be of significant importance for patients and their families. First, it would allow for the early implementation of therapies targeting the protection of neurological functions, such as intrathecal or intracerebroventricular ERT or gene therapy delivered intrathecally to target the brain directly. Second, it would enable better planning of specialized and rehabilitative care, which could improve the quality of life and slow down the progression of symptoms. Early recognition of the risk of neurodegeneration would also help with adjusting expectations and preparing caregivers for the specific challenges associated with progressing neurological changes, influencing a comprehensive approach to long-term care. Unfortunately, despite attempts to find specific markers for neuronopathy in MPS, only complex bioinformatic analyses could be offered to date, which, despite their utility in theoretical analyses, are hardly applicable in clinical practice [31]. Therefore, further research is needed to better understand which factors may influence the development of neurodegenerative symptoms in MPS.

Since finding minimally invasive markers of neurodegeneration is of significant scientific and medical importance, the aim of this study was to conduct a comparative analysis of shared and specific gene expression alterations in neuronopathic and non-neuronopathic types of MPS using fibroblasts derived from patients. Such studies enabled (i) the selection of transcripts/genes whose expression is altered in multiple MPS types/subtypes, allowing for the determination of their involvement in common disease mechanisms, and (ii) the selection of genes whose expression is altered in comparison to control cells only in neuronopathic types/subtypes, which could be considered potential markers of neurological disorders.

The use of fibroblasts derived from patients in neurodegeneration research may seem surprising, but it is highly valuable. Their collection is less invasive compared to obtaining nerve tissue cells. Furthermore, as mentioned earlier, fibroblasts and neurons originate from the same embryonic germ layer, which means they can reflect some key molecular changes typical of neurodegeneration in MPS. Recent studies also indicated that fibroblasts are present around blood vessels, in the meninges, and in the choroid plexus of the brain and spinal cord, where they play important roles for CNS. Fibroblasts in the meninges support the immune response by secreting cytokines, and perivascular fibroblasts play a crucial role in scar formation after nerve inflammation. Limiting their proliferation can reduce scarring and improve motor functions [1,2]. Hence, fibroblasts can serve as a practical and more accessible alternative model to nerve cells for studies on pathogenic mechanisms and potential therapeutic targets in neurodegenerative diseases [32]. Such studies for rare metabolic diseases with a neurodegenerative component are commonly conducted using fibroblast models [33–37].

Transcriptomic analysis conducted with fibroblasts derived from patients with neuronopathic and non-neuronopathic types of MPS (Table 2) revealed that expression alterations of many genes (relative to controls) occur in various MPS types. Some of these gene expression alterations are shared among all/multiple types/subtypes of the disease, while others are specific only to neuronopathic types/subtypes, making them potential markers for neurodegeneration.

The analyses conducted in this work showed that at least half of the genes with altered expression levels compared to control cells in a given MPS type also appear in other types/subtypes of the disease (Figures 1 and 2). The list of genes whose expression is altered in at least 10 types/subtypes of MPS, with a $\log_2$ fold change ($\log_2$FC) greater than

2.5 or less than −2.5, includes up-regulated genes *PFN1, ADAMTSL1, MFAP5, SH3BP5*, and *CAPG*, as well as down-regulated genes *C1D, ABHD5, LY6K*, and *PLCB4* (Figures 3 and 4; Table 3).

The *PFN1* gene is the only gene that exhibits increased expression levels in the cells of all MPS types/subtypes studied in this work when compared to control cells. It plays a crucial role in the development of the skeletal system and the maintenance of bone homeostasis. Previous studies demonstrated that disturbances in the levels of profilin-1, the protein encoded by *PFN1*, have been detected in individuals with skeletal disorders, such as dwarfism, facial deformities, and altered bone structure and size. These findings highlighted the importance of PFN1 function in bone health and its potential connection to the pathophysiology of MPS [38]. A heterozygous deletion of the *PFN1* gene has been detected in patients with Paget's disease of bone, a chronic progressive bone disorder of late onset. Paget's disease of bone is characterized by the abnormal activation of osteoclasts, which leads to bone pain, deformities, and fractures [39]. Heterozygous deletions of the PFN1 gene have also been observed in patients with a severe phenotype of Miller–Dieker syndrome, a rare genetic disorder caused by a contiguous gene deletion. This syndrome is characterized by lissencephaly type 1 (a condition resulting from impaired neuronal migration), facial dysmorphism, seizures, and severe intellectual disability. These findings suggest that profilin-1 may play a critical role not only in the proper development of the skeletal system but also in the nervous system, supporting its involvement in both bone formation and neurogenesis [40]. In zebrafish models with *PFN1* mutations, motor deficits are observed at early life stages, associated with changes in the structure of motor neurons. It has also been shown that mutations in *PFN1* lead to disruptions in actin dynamics, resulting in protein aggregation and the degeneration of motor neurons in *Drosophila* models. Studies on mice with PFN1 mutations demonstrate that alterations in this protein function cause symptoms resembling amyotrophic lateral sclerosis in humans. These mice exhibit progressive muscle weakness, coordination problems, and the degeneration of motor neurons in the spinal cord. Histological analyses also revealed disruptions in the cytoskeleton of neurons, indicating damage to the mechanisms stabilizing actin. Furthermore, certain mutated forms of profilin-1 exhibit prion-like properties and can induce the transformation of natural TDP43 into toxic conformational structures upon entering cells with unmutated prifilin-1 [41–45]. It would be consistent with recent data on the pathogenesis of MPS III, where the accumulation of protein aggregates such as TDP43, APP, beta-amyloid, and alpha-synuclein was observed in cells obtained from patients [46]. Dysregulation of or mutations in the *PFN1* gene have also been implicated in Fragile X syndrome, spinal muscular atrophy, Huntington's disease, Parkinson's disease, and adrenoleukodystrophy [47].

The *ADAMTSL1* gene, encoding ADAMTS Like 1 or Punctin-1, also appears to be of interest in terms of developmental disorders. Mutations in this gene have also been observed in Microcephaly Facial, Dysmorphism Ocular, and Multiple Congenital Anomaly Syndrome. This disease is characterized by symptoms similar to MPS, particularly in terms of facial dysmorphia (square face with a prominent jaw, wide and flat nasal bridge, short philtrum, and protruding ears). Additionally, symptoms of this syndrome include congenital glaucoma, myopia, retinal detachment, hearing loss, dental abnormalities, kidney anomalies, brain vessel malformations, hypothyroidism, and distal limb deformities [48].

Mutations in the *ABHD5* gene, encoding Abhydrolase Domain Containing 5, Lysophosphatidic Acid Acyltransferase, have been identified in patients with Chanarin–Dorfman Syndrome, a rare autosomal recessive disorder characterized by triglyceride accumulation in various tissues. Patients with this syndrome display a range of symptoms that overlap with those seen in MPS, such as hepatomegaly, liver dysfunction, muscle weakness, growth delay, cataracts, hearing loss, and intellectual disability [49].

On the basis of above facts, the association of genes identified in this work, which show altered expression levels in MPS cells with the clinical features observed in patients with both MPS and other mentioned diseases, appears indisputable. However, at the current

stage of our knowledge, it is difficult to deduce whether a disturbance in the expression levels of a particular gene, or perhaps an entire group of genes, is responsible for the appearance of characteristic symptoms.

In the next phase of the study, an analysis was conducted to identify genes with altered expression levels exclusively in neuronopathic types/subtypes of MPS (MPS I, II, IIIA, IIIB, IIIC, IIID, and VII) compared to control cells, which were not altered in non-neuronopathic types/subtypes. This analysis revealed that between 30 and 50% of the transcripts with changed expression levels were also present in at least one other neuropathic type/subtype of MPS (Figures 5 and 6). In total, 418 genes were identified whose expression was significantly altered in the neuronopathic MPS types/subtypes but not in non-neuronopathic types, suggesting their potential involvement in CNS disorders. The list of genes with altered expression levels in at least five neuropathic MPS types/subtypes includes up-regulated *RPN2*, *PDIA3*, and *VASN*, as well as down-regulated *ARL6IP6*, *C11orf58*, and *MINOS1* (Figure 7; Table 3).

The *ARL6IP6* gene is involved in key functions related to the regulation of alternative splicing, which is crucial for the proper functioning of the nervous system. Disruptions in its function can lead to abnormal neuronal differentiation and disturbances in mitochondrial and endoplasmic reticulum homeostasis. Additionally, the delivery of a correct copy of this gene via gene therapy has been shown to reduce neuroinflammation and neurodegeneration, as demonstrated in in vivo and in vitro models of hereditary spastic paraplegia [50]. Moreover, the product of this gene has been found to play a role in regulating the activity of BACE1, the beta-site amyloid precursor protein (APP) cleaving enzyme-1, which is involved in the processing of APP into beta-amyloid [51]. The reduction in its levels, as demonstrated in this study, could therefore explain the increase in beta-amyloid and its precursor levels, as well as their tendency to aggregate, which has been observed in previous research on MPS [46,52,53].

Protein disulfide-isomerase A3, encoded by the *PDIA3* gene, also plays a significant role in protein proteostasis by facilitating proper protein folding. A product of mutated *PDIA3* is prone to aggregate formation, which aberrantly interacts with ER chaperones. This disrupts the biogenesis and signaling of integrins, essential adhesive molecules that support synaptic activity [54]. An interesting piece of research on the role of *PDIA3* in nervous system damage has been conducted using a mouse model of traumatic brain injury. Findings from studies on wild-type mice ($PDIA3^{+/+}$) and *PDIA3* knockout mice ($PDIA3^{-/-}$) revealed that the absence of *PDIA3* significantly improved cognitive function and reduced the contusion volume caused by trauma. Additionally, *PDIA3* deletion was associated with decreased apoptosis, mitigated neuroinflammation, and reduced oxidative stress, highlighting its potential role in modulating these processes during brain injury [55]. Similar conclusions were reached by researchers who demonstrated an age-dependent increase in PDIA3 levels in the amygdala, entorhinal cortex, and ventral hippocampus of Alzheimer's disease model mice (3×Tg-AD) [56]. In contrast, healthy mice exhibited an age-dependent decrease in PDIA3 levels. Moreover, immunohistochemical analysis revealed a direct correlation between cellular levels of beta-amyloid and PDIA3 across all examined brain regions, suggesting a potential interaction in the pathogenesis of Alzheimer's disease [56].

The most important limitation of this study was using only one cell line per MPS type/subtype. However, MPS is a very rare disease with poor availability of biological materials. For example, there are only single fibroblast lines of MPS IIIC and MPS IX, and only two commercially available MPS IIID fibroblast lines (https://www.coriell.org/1/NIGMS/Additional-Resources/Available-Products#cell; last accessed on 30 November 2024). On the other hand, one should note that, in most cases, the results were similar in groups of all neuronopathic and all non-neuronopathic types of MPS in the cell lines tested, which indicates their reliability. Moreover, each experiment was repeated several times (three for Western blotting and four for transcriptomics). Indeed, such a limitation arising for the ultra-rarity of some genetic diseases, including MPS, has been explained previously,

indicating that the results based on analyses of single cell line can be reliable under certain conditions, especially when using a suitable number of repeats of experiments and obtaining data showing similar tendency of changes in similar kinds of diseases [21,29,31,46]. One might also suggest that more cell lines should be investigated in the cases of MPS types where such lines are available. However, comparing transcriptomic results of experiments where some types are represented by several cell lines while others are represented by single cell lines would be incoherent and statistical analyses would be unreliable. Therefore, we decided to stay with a single cell line per each MPS type/subtype.

One might also ask whether factors unrelated to MPS type/subtype could influence the results of our transcriptomic analysis. Indeed, patients being donors of fibroblasts used in this study differed in sex, race, and age of the biological material withdrawal, as indicated in Table 4. However, we were not able to find any correlations between these parameters and transcriptomic results. Thus, in light of the identified correlations between the expression of certain genes and MPS neuronopathy (discussed above), we conclude that it is likely that the observed effects are related to neuronopathic and non-neuronopathic types/subtypes of MPS rather than other factors, like the sex, race, or age of the patient.

Table 4. Characteristics of cell lines used in the study.

MPS Type	Stored GAG(s) *	Defective Enzyme	Mutation(s)	Catalog Number of the Cell Line **/Patient's Characteristics: Sex ***/Race ****/Age (in Years) at the Time of Sample Collection
MPS I	HS, DS	α-L-iduronidase	p.Trp402Ter/ p.Trp402Ter	GM00798/F/C/1
MPS II		2-iduronate sulfatase	p.His70ProfsTer29	GM13203/M/C-H/3
MPS IIIA	HS	N-sulfoglucosamine sulfhydrolase	p.Glu447Lys/ p.Arg245His	GM00879/F/C/3
MPS IIIB		α-N-acetylglucosaminidase	p.Arg626Ter/ p.Arg626Ter	GM00156/M/C/7
MPS IIIC		Acetyl-CoA:α-glycosaminide acetyltransferas	p.Gly262Arg/ pArg509Asp	GM05157/M/U/8
MPS IIID		N-acetylglucosamine 6-sulfatase	p.Arg355Ter/ p.Arg355Ter	GM05093/M/A-I/7
MPS IVA	KS, CS	N-acetylglucosamine-6-sulfate sulfatase	p.Arg386Cys/ p.Phe285Ter	GM00593/F/C-M/7
MPS IVB		β-galactosidase	p.Trp273Leu/ p.Trp509Cys	GM03251/F/C/4
MPS VI	DS, C4S	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	Not determined	GM03722/F/B/3
MPS VII	HS, DS, CS	N-acetylgalactosamine 4-sulfatase	p.Trp627Cys/ p.Arg356Ter	GM00121/M/A-A/3
MPS IX	HA	Hyaluronidase	p.Glu268Lys/ c.37bp-del;14bp-ins at nt 1361	GM17494/F/U/14
Control line (HDFa)	None	N/A	N/A	N/A

* Abbreviations: HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; C6S, chondroitin 6-sulfate; C4S, chondroitin 4-sulfate. ** According to the Coriell Institute; N/A, not applicable. *** Abbreviations: F, female; M, male; U, unknown. **** Abbreviations: A-A, African-American; A-I, Asian-Indian; B, Black; C, Caucasian; C-H, Caucasian-Haitian; C-M, Caucasian-Mexican; U, unknown.

In summary, our study identified a set of genes with dysregulated expression common across all types of MPS, such as *PFN1*, *ADAMTSL1*, and *ABHD5*, emphasizing their role in the disease's pathogenesis. Additionally, a distinct set of genes, including *ARL6IP6* and *PDIA3*, exhibited expression changes specific to neuronopathic MPS types/subtypes, suggesting their possible applications as biomarkers for neurodegeneration in MPS. These findings provide new insights into the molecular mechanisms underlying MPS pathogenesis and differentiation between neuronopathic and non-neuronopathic courses of the disease, the latter being of special significance in MPS types where patients may represent one of two such courses, like MPS I and MPS II. They also highlight opportunities for developing more precise diagnostic and therapeutic strategies.

4. Materials and Methods

4.1. Cell Lines and Cell Cultures

Cell lines (skin fibroblasts) of MPS patients were purchased commercially (Coriell Institute for Medical Research, Camden, NJ, USA) (Table 4). The HDFa cells were used as controls. Cultures of cells were incubated in the DMEM medium with 10% Fetal Bovine Serum and a standard mixture of antibiotics. A temperature of 37 °C, humidity of 95%, and CO_2 saturation of 5% were kept throughout the experiments.

4.2. Transcriptomic Analyses

4.2.1. RNA Isolation and Purification

To isolate and purify total RNA, 5×10^5 cells were passaged and incubated in the medium (Section 4.1) overnight. Cell lysis was conducted via homogenization with a QIAshredder column in the presence of guanidinium isothiocyanate and β-mercaptoethanol. An RNeasy Mini Kit (Qiagen, Hilden, Germany) and Turbo DNase (Life Technologies, Life Technologies, Carls- bad, CA, USA) were used to extract RNA molecules. Nano Chip RNA and the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) were employed to assess the RNA sample quality. Samples from 4 independent biological repeats (i.e., 4 independent cell cultures with independent RNA isolation procedures) were used in further analyses.

4.2.2. Transcriptomic (RNA-Seq) Analysis

An Illumina TruSeq Stranded mRNA Library Prep Kit was used for obtaining mRNA libraries. Following reverse transcription, the HiSeq4000 system (Illumina, San Diego, CA, USA) was used to sequence cDNA libraries. After sequencing the parameters of the reactions, we used FastQC version v0.11.7 to determine the following values: 150 bp paired-end, 40 million raw reads, and 12 Gb of the raw data/sample. The mapping of raw readings was performed to the GRCh38 human reference genome (Ensembl; Hisat2 v. 2.1.0 software). The Cuffquant and Cuffmerge software (v. 2.2.1) and the GTF Homo_sapiens.GRCh38.94.gtf file (Ensembl database; https://www.ensembl.org/index.html, as of 19 February 2019) were used to calculate the levels of transcripts. To normalize the expression values by means of the FPKM algorithm, Cuffmerge software was employed with the library-norm-method classic-fpkm parameter. The BioMart interface for the Ensembl gene database (https://www.ensembl.org/info/data/biomart/index.html, as of 19 February 2019) was used for the annotation and classification of transcripts. Raw RNA-seq data were deposited in the NCBI Sequence Read Archive (SRA) database (accession no. PRJNA562649).

4.2.3. Statistical Analysis

R v3.4.3 software was used to perform statistical analysis. One-way analysis of variance (ANOVA) on $\log_2(1 + x)$ values was employed for data which had a continuous normal distribution. Student's post hoc t-test with Bonferroni correction was employed to analyze statistical significance between two groups. The Benjamini–Hochberg method was used to calculate the false discovery rate (FDR). The Ensembl database (BioMart interface)

(https://www.ensembl.org/info/data/biomart/index.html, as of 19 February 2019) was used for transcript classification.

4.3. Analysis of Protein Levels via Western Blotting

Fibroblasts from cell cultures (6×10^5 cells; Section 4.1) were lysed using the buffer consisting of 1% Triton X-100, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris (pH 7.5). A mixture of protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany; #05892791001 and #11873580001) was employed to protect proteins from degradation. Following centrifugation for 10 min, at 12,000 rpm and temperature of 4 °C, the lysates were used for the analysis of protein levels. The WES system (WES—Automated Western Blots with Simple Western; ProteinSimple, San Jose, CA, USA) was used for automatic Western blotting. This system employs the capillary electrophoresis-based separation of proteins and immunological detection direction in each capillary (without using traditional SDS-PAGE and transfer to the membrane). The 12–230 kDa separation module (#SM-W003) was used for separating proteins. The profilin-1 protein was detected with the Anti-Mouse (#DM-002) or Anti-Rabbit (#DM-001) detection module and anti-profilin-1 antibody (#PA5-17444, Thermo Fisher Scientific, Waltham, MA, USA). As a loading control, the Total Protein Module (#DM-TP01, ProteinSimple, San Jose, CA, USA) was used.

The capillary electrophoresis-based automatic Western blotting system (the WES system) allows for the use of highly standardized procedure, resulting in the high reproducibility of results and the more precise quantitative determination of levels of investigated proteins than in traditional Western blotting. Moreover, the method is significantly shorter (3–4 h from loading samples to obtaining the final results) than traditional Western blotting and requires significantly lower amounts of studied proteins and antibodies [57–66].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms252413447/s1.

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Supplementary

	MPS I	MPS II	MPS IIIA	MPS IIIB	MPS IIIC	MPS IIID	MPS VII
$log_2FC > 5$	0	0	1	0	1	0	0
$log_2FC > 4$	0	0	1	0	1	1	1
$log_2FC > 3$	2	1	2	2	3	2	6
$log_2FC < -3$	2	0	5	6	0	4	2
$log_2FC < -4$	1	0	3	1	0	2	2
$log_2FC < -5$	0	0	2	0	0	1	0

Figure S1. Number of transcripts with altered expression levels depending on log_2FC values in individual neuronopathic types/subtypes of MPS vs. control.

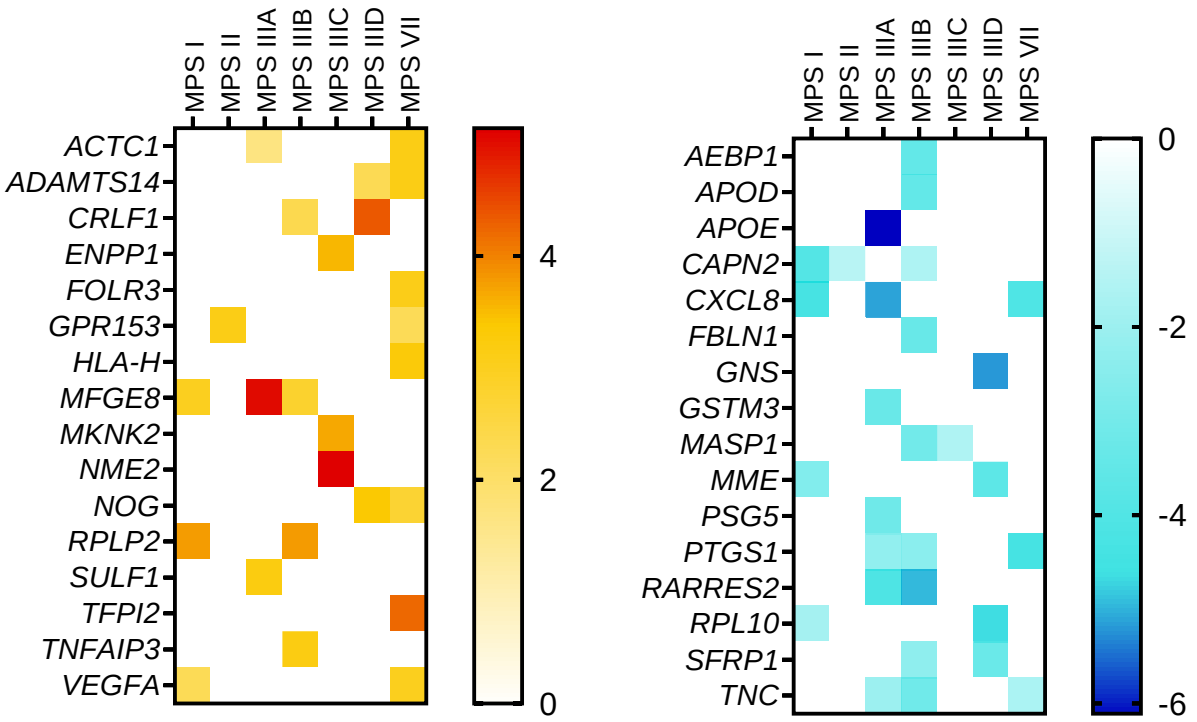

Figure S2. Genes revealing at least 3-fold changes in transcript levels between neuronopathic MPS types/subtypes compared to control cells.

Table S1. Genes revealing at least 3-fold changes (FC) in transcript levels between neuronopathic MPS types/subtypes compared to control cells, with exact FC values indicated (-, smaller changes).

log_2FC value of transcripts which fold change value greater than 3

Gene name	Transcript ID	MPS I	MPS II	MPS IIIA	MPS IIIB	MPS IIIC	MPS IIID	MPS VII
ACTC1	ENST00000650163	-	-	-	-	-	-	3.15
ADAMTS14	ENST00000373207	-	-	-	-	-	-	3.15
CRLF1	ENST00000392386	-	-	-	-	-	4.38	-
ENPP1	ENST00000647893	-	-	-	-	3.57	-	-
FOLR3	ENST00000442948	-	-	-	-	-	-	3.11
GPR153	ENST00000377893	-	3.14	-	-	-	-	-
HLA-H	ENST00000383620	-	-	-	-	-	-	3,32
MFGE8	ENST00000560937	3.03	-	5.05	-	-	-	-
MKNK2	ENST00000589534	-	-	-	-	3.70	-	-
NME2	ENST00000503064	-	-	-	-	5.14	-	-
NOG	ENST00000332822	-	-	-	-	-	3.42	-
RPLP2	ENST00000530797	3.80	-	-	3.81	-	-	-
SULF1	ENST00000529041	-	-	3.22	-	-	-	-
TFPI2	ENST00000222543	-	-	-	-	-	-	4.25
TNFAIP3	ENST00000237289	-	-	-	3.20	-	-	-
VEGFA	ENST00000497139	-	-	-	-	-	-	3.03

log_2FC value of transcripts which fold change value less than -3

Gene name	Transcript ID	MPS I	MPS II	MPS IIIA	MPS IIIB	MPS IIIC	MPS IIID	MPS VII
AEBP1	ENST00000450684	-	-	-	-3.43	-	-	-
APOD	ENST00000343267	-	-	-	-3.44	-	-	-
APOE	ENST00000252486	-	-	-6.11	-	-	-	-
CAPN2	ENST00000433674	-3.87	-	-	-	-	-	-
CXCL8	ENST00000307407	-4.35	-	-5.08	-	-	-	-4.04
FBLN1	ENST00000262722	-	-	-	-3.27	-	-	-
GNS	ENST00000258145	-	-	-	-	-	-5.16	-
GSTM3	ENST00000486823	-	-	-	-3.28	-	-	-
MASP1	ENST00000296280	-	-	-	-3.02	-	-	-
MME	ENST00000615825	-	-	-	-	-	-3.62	-
PSG5	ENST00000401992	-	-	-3.12	-	-	-	-
PTGS1	ENST00000223423	-	-	-	-	-	-	-4.32
RARRES2	ENST00000223271	-	-	-4.08	-4.91	-	-	-
RPL10	ENST00000406022	-	-	-	-	-	-4.61	-
SFRP1	ENST00000220772	-	-	-	-	-	-3.22	-
TNC	ENST00000350763	-	-	-	-3.08	-	-	-

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polegał na:

1. zaproponowaniu tematyki pracy
2. zaplanowaniu i przeprowadzeniu analiz transkryptomicznych
3. interpretacji wyników
4. przygotowaniu figur i tabeli
5. asyście w przygotowaniu odpowiedzi na uwagi recenzentów

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polegał na:

1. przygotowaniu biblioteki RNA-seq do analiz transkryptomicznych
2. przygotowaniu preparatów do mikroskopii elektronowej i fluorescencyjnej
3. pomocy w przygotowaniu pierwotnej wersji manuskryptu

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polegał na:

1. recenzji wewnętrznej manuskryptu
2. udziale w interpretacji wyników
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2. interpretacji i analizie wyników
3. rewizji manuskryptu po uwagach recenzentów
4. przygotowaniu odpowiedzi na uwagi recenzentów

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Cellular Organelle-Related Transcriptomic Profile Abnormalities in Neuronopathic Types of Mucopolysaccharidosis: A Comparison with Other Neurodegenerative Diseases

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Article

Cellular Organelle-Related Transcriptomic Profile Abnormalities in Neuronopathic Types of Mucopolysaccharidosis: A Comparison with Other Neurodegenerative Diseases

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Abstract: Mucopolysaccharidoses (MPS) are a group of diseases caused by mutations in genes encoding lysosomal enzymes that catalyze reactions of glycosaminoglycan (GAG) degradation. As a result, GAGs accumulate in lysosomes, impairing the proper functioning of entire cells and tissues. There are 14 types/subtypes of MPS, which are differentiated by the kind(s) of accumulated GAG(s) and the type of a non-functional lysosomal enzyme. Some of these types (severe forms of MPS types I and II, MPS III, and MPS VII) are characterized by extensive central nervous system disorders. The aim of this work was to identify, using transcriptomic methods, organelle-related genes whose expression levels are changed in neuronopathic types of MPS compared to healthy cells while remaining unchanged in non-neuronopathic types of MPS. The study was conducted with fibroblast lines derived from patients with neuronopathic and non-neuronopathic types of MPS and control (healthy) fibroblasts. Transcriptomic analysis has identified genes related to cellular organelles whose expression is altered. Then, using fluorescence and electron microscopy, we assessed the morphology of selected structures. Our analyses indicated that the genes whose expression is affected in neuronopathic MPS are often associated with the structures or functions of the cell nucleus, endoplasmic reticulum, or Golgi apparatus. Electron microscopic studies confirmed disruptions in the structures of these organelles. Special attention was paid to up-regulated genes, such as *PDIA3* and *MFGE8*, and down-regulated genes, such as *ARL6IP6*, *ABHD5*, *PDE4DIP*, *YIPF5*, and *CLDN11*. Of particular interest is also the *GM130* (*GOLGA2*) gene, which encodes golgin A2, which revealed an increased expression in neuronopathic MPS types. We propose to consider the levels of mRNAs of these genes as candidates for biomarkers of neurodegeneration in MPS. These genes may also become potential targets for therapies under development for neurological disorders associated with MPS and candidates for markers of the effectiveness of these therapies. Although fibroblasts rather than nerve cells were used in this study, it is worth noting that potential genetic markers characteristic solely of neurons would be impractical in testing patients, contrary to somatic cells that can be relatively easily obtained from assessed persons.

Keywords: mucopolysaccharidosis; neurodegeneration; organelles; organelle-related genes; transcriptomics; Golgi apparatus fragmentation

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1. Introduction

Mucopolysaccharidoses (MPS) are a group of diseases caused by mutations in genes encoding lysosomal enzymes that carry out degradation reactions of glycosaminoglycans (GAGs); thus, these compounds accumulate in lysosomes, impairing the proper functioning of not only lysosomes but also whole cells and tissues [1]. This leads to a number of symptoms, such as short stature, facial dysmorphism, chronic joint pain, organomegaly, or

sensory problems [2]. The life expectancy of patients with MPS ranges from 10 to 20 years, depending on the severity of symptoms [2,3].

To date, 12–14 types/subtypes (depending on the MPS definition) of this disease have been described, which are differentiated by the type of GAG accumulated and the non-functional lysosomal enzyme. The characteristics of each type of MPS are shown in Table 1 [4]. Some of the MPS types/subtypes (severe cases of MPS I and MPS II, and all of MPS III and MPS VII) are characterized by neurological abnormalities. These symptoms can result from either (i) direct accumulation of GAGs in nerve cells and resulting damage to the central nervous system (CNS) or (ii) spinal cord compression or hydrocephalus that leads to brain damage. This results in aggression, sleep problems, hearing loss, speech difficulties, and personality changes in patients [5,6]. Patients lose previously acquired cognitive-motor skills and begin to regress, requiring round-the-clock care [7].

Table 1. Characteristics of different types/subtypes of MPS.

MPS Type [#]	Defective Gene	Defective Enzyme	Stored GAG(s) *
MPS I	*IDUA*	α-L-iduronidase	HS, DS
MPS II	*IDS*	2-iduronate sulfatase	
MPS IIIA	*SGSH*	N-sulfoglucosamine sulfhydrolase	HS
MPS IIIB	*NAGLU*	α-N-acetylglucosaminidase	
MPS IIIC	*HGSNAT*	Acetyl-CoA:α-glycosaminide acetyltransferas	
MPS IIID	*GNS*	N-acetylglucosamine 6-sulfatase	
MPS IIIE	*ARSG*	Arylusulfatase G	
MPS IVA	*GLANS*	N-Acetyloglucosaminase-6-sulfate sulfatase	C6S, KS
MPS IVB	*GLB1*	β-Galactosidase	KS
MPS VI	*ARSB*	Arylusulfatase B	DS, C4S
MPS VII	*GUSB*	N-acetylgalactosamine 4-sulfatase	HS, DS, CS
MPS IX	*HYAL1*	Hyaluronidase	HA
MPS X	*ARSK*	Arylusulfatase K	DS
MPS-PS	*VPS33A*	VPS33A	HS, DS

[#] MPS IIIE is not included into MPS by some researches, as studies conducted with patients' materials confirmed a decrease in the enzymatic activity, but not an increase in GAG levels. Moreover, the clinical picture did not resemble any of the MPS III subtypes, nor did it have the typical features of MPS. This gave rise to the diagnosis of atypical Usher's disease in the patients. On the other hand, classical MPS IIIE with *ARSG* mutation and increased GAG levels was identified in the mouse model. MPS-plus syndrome (MPS-PS) is also questioned to be an MPS type, as despite GAG accumulation and symptoms typical for MPS, there is no decrease in the activity of lysosomal enzymes responsible for GAG degradation. * Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate; KS, keratan sulfate.

Therapies such as hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) are used to alleviate symptoms and improve quality of life for patients with MPS. HSCT is a procedure in which hematopoietic cells (usually bone marrow cells) are transplanted into the patient's organism to replace abnormal cells (in this case, cells with a missing enzyme). ERT, on the other hand, involves delivering the recombinant enzyme that is missing in the patient's body. The effectiveness of both of these therapies depends on a number of factors, including the age of the patient, the type of MPS, existing symptoms of the disease, and, in the case of HSCT, also the status of the donor (carrying or not having the disease) and the source of the HSCT tissue. There is also a wide variety of responses to therapy in different tissues of the body. In highly vascularized organs such as the liver and spleen, there is a reduction in GAG levels and an improvement in organ function and size. However, both the transplanted stem cells and the administered enzyme have difficulty reaching tissues such as bone, cartilage, and heart valves. In addition, low penetration through the blood-brain barrier (BBB) and ineffective delivery to non-vascular tissues are problems. As a result, cognitive abilities, skeletal deformities, or visual acuity

are not improved. Some sources report that these symptoms may possibly be stabilized but not restored [8]. Thus, there is currently no treatment for neuronopathic types of MPS.

It is also worth noting the problem of MPS types I and II, as those types may (but not necessarily) have a neurodegenerative component. This depends largely on the type of mutation and, thus, the residual activity of the enzyme or its complete absence. However, there may be many more factors affecting enzyme activity, and these may include the efficiency of individual GAG synthesis, oxidative stress, endoplasmic reticulum stress, disruption of cell energetics, infection, defects in autophagy and lysosome biogenesis, disruption of calcium homeostasis, and many others [9].

Both treatment and diagnosis of neurological symptoms in MPS patients are difficult due to the lack of reaching the central nervous system (CNS) with current therapies and the lack of markers of neurodegeneration. Thus, it seems reasonable to identify pathogenic disorders present in the neuronopathic types of the disease (severe forms of MPS I and II, all MPS III and MPS VII) and thus not present in types without a neurodegenerative component (mild forms of MPS I and II, all MPS IV, MPS VI, and MPS IX). This approach, in addition to understanding the mechanisms of neurodegeneration itself, could lead to the designation of new therapeutic targets and diagnostic markers for neurodegeneration in MPS or to monitoring the effectiveness of the therapies used.

Organelle dysfunction is strongly associated with neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). High mitochondrial dysfunction, consisting of impaired mitochondrial bioenergetics, impaired respiratory chain, impaired mitochondrial gene expression, mitophagy dysregulation, altered mitochondrial DNA levels, and increased oxidative stress, has already been observed for some time in AD and PD patients [10,11]. Changes in other organelles such as the endoplasmic reticulum, lysosomes, endosomes, or Golgi apparatus have also been correlated with neurodegenerative diseases [12–15]. Recent data indicated that the levels of organelle-related transcripts were disrupted and the number and morphology of individual cellular organelles were also altered in MPS [16–19]. In addition to other processes whose performance is disrupted [1,16,20–30], changes in the morphology of cellular organelles or changes in the expression levels of genes related to organelle function or structure may provide a marker for differentiating the course of MPS into those with or without a neurodegenerative component or, in the future, provide a therapeutic target for currently untreatable neurological types of the disease.

Therefore, the aim of this work was to identify genes related to the function or structure of cellular organelles that show changes in expression levels in neuronopathic types of MPS compared to healthy cells, while not showing these changes in non-neuronopathic types of MPS. In addition, the data identified in this work were compared with data on similar changes in other neurodegenerative diseases.

2. Materials and Methods

2.1. Cell Lines and Cell Cultures

Fibroblast lines taken from patients with neuronopathic (MPS I, MPS II, MPS III, and MPS VII) and non-neuronopathic (MPS IV, MPS VI, and MPS IX) types of MPS and purchased from the Coriell Institute were used for the experiments (Table 2). The HDFa (Human Dermal Fibroblast, adult) line was used as wild-type (healthy and control) cells. Cells were cultured under standard conditions at 37 °C, 95% humidity, in an atmosphere saturated with 5% CO_2, in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS), and in the presence of antibiotics.

Table 2. Characteristics of MPS cell lines used in the study.

MPS Type	Stored GAG(s) *	Defective Enzyme	Mutation Type	Cat. Number of the Cell Line **
MPS I	HS, DS	α-L-iduronidase	p.Trp402Ter/p.Trp402Te	GM00798
MPS II		2-iduronate sulfatase	p.His70ProfsTer29	GM13203
MPS IIIA	HS	N-sulfoglucosamine sulfhydrolase	p.Glu447Lys/p.Arg245His	GM00879
MPS IIIB		α-N-acetylglucosaminidase	p.Arg626Ter/p.Arg626Ter	GM00156
MPS IIIC		Acetyl-CoA:α-glycosaminide acetyltransferas	p.Gly262Arg/pArg509Asp	GM05157
MPS IIID		N-acetylglucosamine 6-sulfatase	p.Arg355Ter/p.Arg355Ter	GM05093
MPS IVA	KS, CS	N-acetylglucosamine-6-sulfate sulfatase	p.Arg386Cys/p.Phe285Ter	GM00593
MPS IVB		β-galactosidase	p.Trp273Leu/p.Trp509Cys	GM03251
MPS VI	DS, C4S	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	Not determined	GM03722
MPS VII	HS, DS, CS	N-acetylgalactosamine 4-sulfatase	p.Trp627Cys/p.Arg356Ter	GM17494
MPS IX	HA	Hyaluronidase	p.Glu268Lys/c.37bp-del;14bp-ins at nt 1361	GM17494
Control (HDFa)	None	N/A	N/A	N/A

* Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate; KS, keratan sulfate; N/A, not applicable. ** Cat. numbers are according to the cell line description at the Coriell Institute.

2.2. Transcriptomic Analyses

2.2.1. RNA Isolation and Purification

Fibroblasts (5×10^5 cells) were passaged onto 10-centimeter-diameter plates and left overnight. To inactivate RNAases, cells were lysed with a solution containing guanidinium isothiocyanate and β-mercaptoethanol and then homogenized using a QIAshredder column. Subsequently, RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) and treated with Turbo DNase (Life Technologies, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the isolated RNA samples was tested using Nano Chips RNA (Agilent Technologies, Santa Clara, CA, USA) on an Agilent 2100 Bioanalyzer system. RNA from each cell line was isolated in 4 independent replicates (4 independent biological repeats).

2.2.2. RNA-Seq Analyses

The mRNA libraries were generated with the Illumina TruSeq Stranded mRNA Library Prep Kit. The cDNA libraries were sequenced on a HiSeq4000 (Illumina, San Diego, CA, USA). The following sequencing parameters were obtained: PE150 (150 bp paired-end) and a minimum of 40 million (40 M) of raw reads, which gave a minimum of 12 Gb of raw data per sample. Quality assessment was carried out by FastQC version v0.11.7. Raw readings were mapped to the GRCh38 human reference genome from the Ensembl database using the Hisat2 v. 2.1.0 program. To calculate the expression level of the transcripts, the Cuffquant and Cuffmerge programs in version 2.2.1 and the GTF Homo_sapiens.GRCh38.94.gtf file from the Ensembl database (https://www.ensembl.org/index.html, as of 19 February 2019) were used. The Cuffmerge program was started with the library-norm-method classic-fpkm parameter, normalizing the expression values by means of the FPKM algorithm. Transcript annotation and classification were performed using the BioMart interface for the Ensembl gene database (https://www.ensembl.org/info/data/biomart/index.html, as

of 19 February 2019). RNA-seq data were deposited in the NCBI Sequence Read Archive (SRA) database: PRJNA562649.

2.2.3. Statistical Analysis

Statistical analysis was performed using R v3.4.3 software using one-way analysis of variance (ANOVA) on $\log_2(1 + x)$ values, which have a continuous normal distribution, and post hoc Student's *t*-test with Bonferroni correction, as well as the Benjamini-Hochberg method for analyzing statistical significance between two groups and false discovery rate (FDR), respectively. Transcript classification was performed using the Ensembl database (BioMart interface) (https://www.ensembl.org/info/data/biomart/index.html, as of 19 February 2019). The procedure for RNA isolation and transcriptomic analysis using the RNA-seq technique has been described previously [23,31].

2.3. Electron Microscopy

Cells at 2×10^5 were passaged into 12-well plates. The next day, cells were washed with PBS buffer, fixed with 2.5% glutaraldehyde, followed by 1% osmium tetroxide and 1% potassium hexacyanoferrate, and then dehydrated with ethanol. In the next step, cells were embedded in Epon 812 resin (Fluka Chemie GmbH, Buchs, Switzerland) and stained with lead citrate and uranyl acetate. Microscopic analyses were carried out using a Philips CM100 microscope with Olympus Soft Imaging Solution 1.4. software. Electron micrographs were used to evaluate the morphology of Golgi apparatuses. At least 100 cells were included in each case. The fragmentation features of Golgi apparatuses were compared between control cells and MPS cells, taking into account the division into neuronopathic and non-neuronopathic MPS types/subtypes.

2.4. Fluorescence Microscopy

Cells at 5×10^4 were passaged on coverslips in 12-well plates. They were then fixed with 2% paraformaldehyde in PBS for 15 min, and sequentially in 0.1% Triton X-100 in PBS for 15 min. In the next step, the cells were washed 5 times with PBS. Golgi apparatuses were stained with CellLight™ Golgi-GFP BacMam 2.0 (#C10592, Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Cover slips were mounted on basal slides using ProLong™ Gold Antifade Mountant with DAPI closure medium (#P36935, Invitrogen). Microscopic analyses were carried out using a Leica DM4000 B fluorescence microscope (Leica Microsystems, Mannheim, Germany).

3. Results

Since disorders of the functions or structures of cellular organelles have increasingly been shown to be components of the pathogenesis of neurodegenerative diseases, this work set out to see if the phenomenon of disrupted organelle-related gene expression also occurs in neuronopathic types of MPS.

3.1. Transcriptomic Analyses

Transcriptomic studies indicating abnormal expression of genes whose products are involved in the functions or structures of cell organelles were carried out by RNA-seq technique with a model of fibroblasts taken from patients with neuronopathic (MPS I, II, IIIA, IIIB, IIIC, IIID, and VII) and non-neuronopathic types/subtypes of MPS (MPS IVA, IVB, VI, and IX), as well as with control cells (HDFa, or Human Dermal Fibroblasts, adults). On this basis, transcripts were selected whose expression levels are altered in neuronopathic types of MPS but are not altered in non-neuronopathic types relative to control cells and whose products are involved in organelle function or structure; the following organelles were taken into consideration: Golgi apparatus (GO:0005794); mitochondrion (GO:0005739); ribosome (GO:0005840); nucleus (GO:0005634); cytoskeleton (GO:0005856); endoplasmic reticulum (GO:0005783); and vacuole (GO:0005773) (selection was made based on the Ensembl database as of 1 August 2023).

In the first stage of the study, the number of transcripts involved in the above-mentioned processes was determined. A large number of transcripts whose expression is disrupted only in neuronopathic types/subtypes of MPS (while not being altered in non-neuronopathic types) relative to control cells were identified for the cell nucleus (142 transcripts), endoplasmic reticulum (ER) (91 transcripts), and Golgi apparatus (57 transcripts), with down-regulated transcripts accounting for the vast majority (Figure 1).

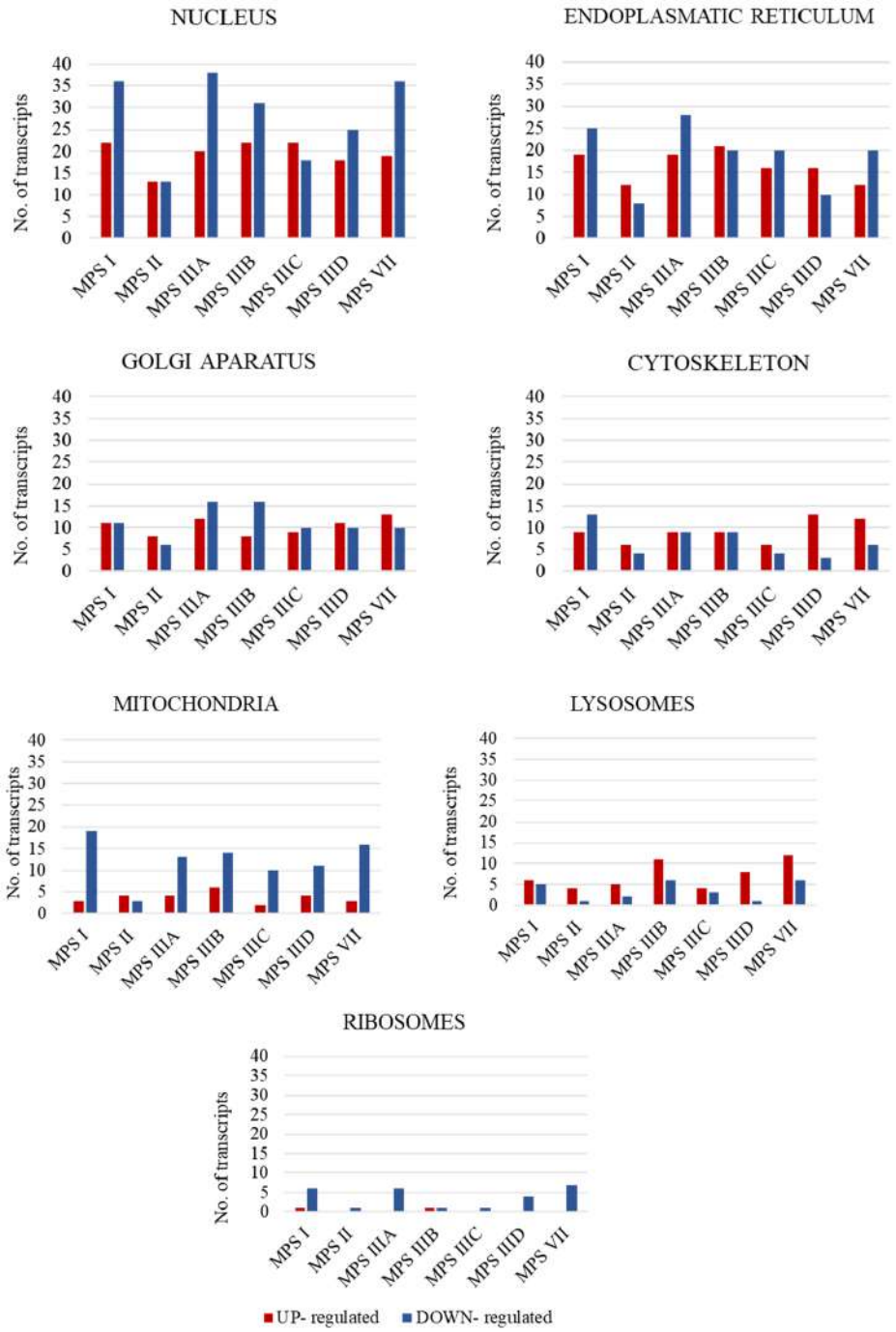

Figure 1. Number of statistically significant up- and down-regulated transcripts included in organelle-related GO terms, which are Golgi apparatus (GO:0005794); mitochondrion (GO:0005739); ribosome (GO: 0005840); nucleus (GO:0005634); cytoskeleton (GO:0005856); endoplasmic reticulum (GO:0005783); and vacuole (GO:0005773), in neuronopathic MPS types/subtypes relative to control cells (HDFa). Abnormal expression levels of these transcripts were not observed in non-neuronopathic MPS types/subtypes.

In order to identify specific transcripts whose expression was disrupted in the greatest number of neuronopathic types/subtypes of the disease, those transcripts that appear in at least four neuronopathic types/subtypes of MPS were selected. The results of this selection by specific organelles are shown in Table 3.

Table 3. List of transcripts whose expression is altered in at least four neuronopathic types of MPS.

Organellum	No. of Genes with Altered Expression in a Given Structure	Selected Genes	Number of MPS Types/Subtypes in Which Gene Expression Has Changed	Regulation of Expression vs. Control Cells	MPS Types/Subtypes in Which Altered Gene Expression Has Been Detected
Nucleus	142	*ARL6IP6*	6	↓	I, II, IIIA, IIIB, IIIC, VII
		PDIA3	5	↑	I, II, IIIA, IIIB, IIIC
		RPN2	5	↑	I, II, IIIA, IIIB, IIID
		ABHD5	4	↓	I, IIIA, IIIB, VII
		DMWD	4	↑	IIIB, IIIC, IIID, VII
		MCM4	4	↓	IIIA, IIIB, IIIC, IIID
		PDE4DIP	4	↓	I, II, IIIA, VII
		SORBS3	4	↑	I, IIIA, IIIB, IIID
		TIGAR	4	↓	I, IIIB, IIIC, VII
Endoplasmic reticulum	91	*ARL6IP6*	6	↓	I, II, IIIA, IIIB, IIIC, VII
		RPN2	5	↑	I, II, IIIA, IIIB, IIID
		PDIA3	5	↑	I, II, IIIA, IIIB, IIIC
		BCAP29	4	↓	I, IIIA, IIIB, IIIC
		CPED1	4	↓	I, IIIA, IIIB, VII
		SDC2	4	↓	I, IIIA, IIIC, VII
		STS	4	↓	IIIB, IIIC, IIID, VII
		YIPF5	4	↓	I, II, IIIA, VII
Golgi apparatus	57	*GOLGA2*	5	↑	I, IIIA, IIIB, IIIC, IIID
		CHPF	4	↑	IIIA, IIIB, IIID, VII
		KIF13A	4	↓	I, IIIB, IIID, VII
		PDE4DIP	4	↓	I, II, IIIA, VII
		S100A3	4	↓	IIIA, IIID, IIIC, VII
		SDC2	4	↓	I, IIIA, IIIC, VII
		STS	4	↓	IIIB, IIIC, IIID, VII
		YIPF5	4	↓	I, II, IIIA, VII
Mitochondrion	43	*MINOS1*	5	↓	I, IIIB, IIIC, IIID, VII
		VASN	5	↑	II, IIIA, IIIB, IIID, VII
		ABHD5	4	↓	I, IIIA, IIIB, VII
		CHPF	4	↑	IIIA, IIIB, IIID, VII
		SLC22A4	4	↓	I, IIIA, IIIC, VII
		TIGAR	4	↓	I, IIIB, IIIC, VII
Cytoskeleton	48	*KIF13A*	4	↓	I, IIIB, IIID, VII
		PDE4DIP	4	↓	I, II, IIIA, VII
		SORBS3	4	↑	I, IIIA, IIIB, IIID, VII
Lysosome	22	*VASN*	5	↑	II, IIIA, IIIB, IIID, VII
		CCZ1	4	↑(I, IIID, VII); ↓(IIIB)	I, IIIB, IIID, VII
		SDC2	4	↓	I, IIIA, IIIC, VII
		STS	4	↓	IIIB, IIIC, IIID, VII

Symbols: ↓, down-regulation of gene expression; ↑, up-regulation of gene expression.

Some of the selected transcripts appear frequently in different neuronopathic types/subtypes of the disease in question. Expression of the *ARL6IP6* gene, encoding ADP ribosylation factor like GTPase-interacting protein 6, was reduced in as many as six neuronopathic types/subtypes of MPS (out of seven tested). The ARL6IP6 protein is localized in the nuclear envelope and RE. It has the ability to shape high-curvature ER tubules, acts as a regulator of intracellular transport pathways in the ER membrane, and interacts with many proteins involved in membrane vesicle formation, also showing anti-apoptotic effects [32–34]. Among the ER-related genes selected were two more genes whose expression is up-regulated in five neuronopathic types/subtypes. These include *RPN2* and *PDIA3*. The *RPN2* gene encodes a highly conserved ribophorin II glycoprotein found in the membrane of the murine ER. The involvement of this glycoprotein has been confirmed by processes mediating the translocation of secretory proteins and maintaining the specificity of the murine ER, or N-glycosylating proteins. In contrast, *PDIA3* encodes the protein disulfide isomerase A3, which is a thioloxidoreductase found primarily in the RE endoplasm in the cytosol or cell nucleus [35]. The product of this gene, the PDIA3 protein, is responsible for regulating the folding of newly synthesized glycoproteins and promoting the re-folding of misfolded proteins. In addition, it protects the cell from entering the pathway of ER stress-induced apoptosis and interacts with lectin-binding chaperone proteins inside this organelle [36]. The *GOLGA2* gene, which encodes one of the golgins, a family of proteins located in the Golgi apparatus, was also within the scope of our interest. This protein plays an important role in the cisternae arrangement of the Golgi apparatus; vesicular transport interactions between the Golgi apparatus and microtubules are important for the reorganization of the Golgi apparatus after its fragmentation during mitosis [37].

Genes related to mitochondrial function and structure also often appeared on the list of selected genes. One of them is *MINOS1*, whose product is a protein of the inner mitochondrial membrane that affects its organization. In the MINOS1/Mio10 complex, it is responsible for maintaining the normal structure of mitochondria [38,39]. Another example is the *VASN* gene, which is associated with both mitochondria and lysosomes. The product of this gene is a type I trans-membrane glycoprotein that protects cells from apoptosis by regulating the activity of the mitochondrial antioxidant thioredoxin-2 [40,41].

In the next step, genes whose expression undergoes particularly high ($\log_2$FC > 1.5 or $\log_2$FC < −1.5) changes in expression in neuronopathic types/subtypes of MPS (while not undergoing changes in non-neuronopathic types) relative to healthy cells were selected. The selected transcripts are shown in Figure 2 as heat maps and in Supplementary Table S1 with the exact values of the fold change ($\log_2$FC).

Among the transcripts selected, there were both up-regulated (e.g., *MFGE8*, *HSPB7*, *SULF1*) and down-regulated (e.g., *ABHD5*, *PDE4DIP*, *YIPF5*, *CLDN11*). The *MFGE8* gene encodes milk fat globule-epidermal growth factor VIII, which maintains cellular homeostasis by alleviating ER stress [42]. Its role in phagocytosis of apoptotic cells, anti-inflammatory reactions, and tissue regeneration has also been reported [43]. Another example is the *HSPB7* gene up-regulated in MPS II, IIID, and VII, which encodes one of the most common small chaperone proteins located in the cell nucleus or cytoplasm. Recent reports indicate its major contribution to protection against neurodegeneration caused by protein aggregation in nerve cells [42]. Also associated with protein aggregates is the product of the *SULF1* gene synthesized in the Golgi apparatus, a surface sulfatase that removes specific 6-O-sulfate groups from heparan sulfate proteoglycans, thanks to which it can influence the modulation of various signaling pathways. Interest in this protein increased when its frequent occurrence in intracellular and extracellular protein aggregates was found. Studies indicated that heparan sulfate can interact with soluble proteins in different ways, causing them to fold abnormally into insoluble fibrils or aggregates [43].

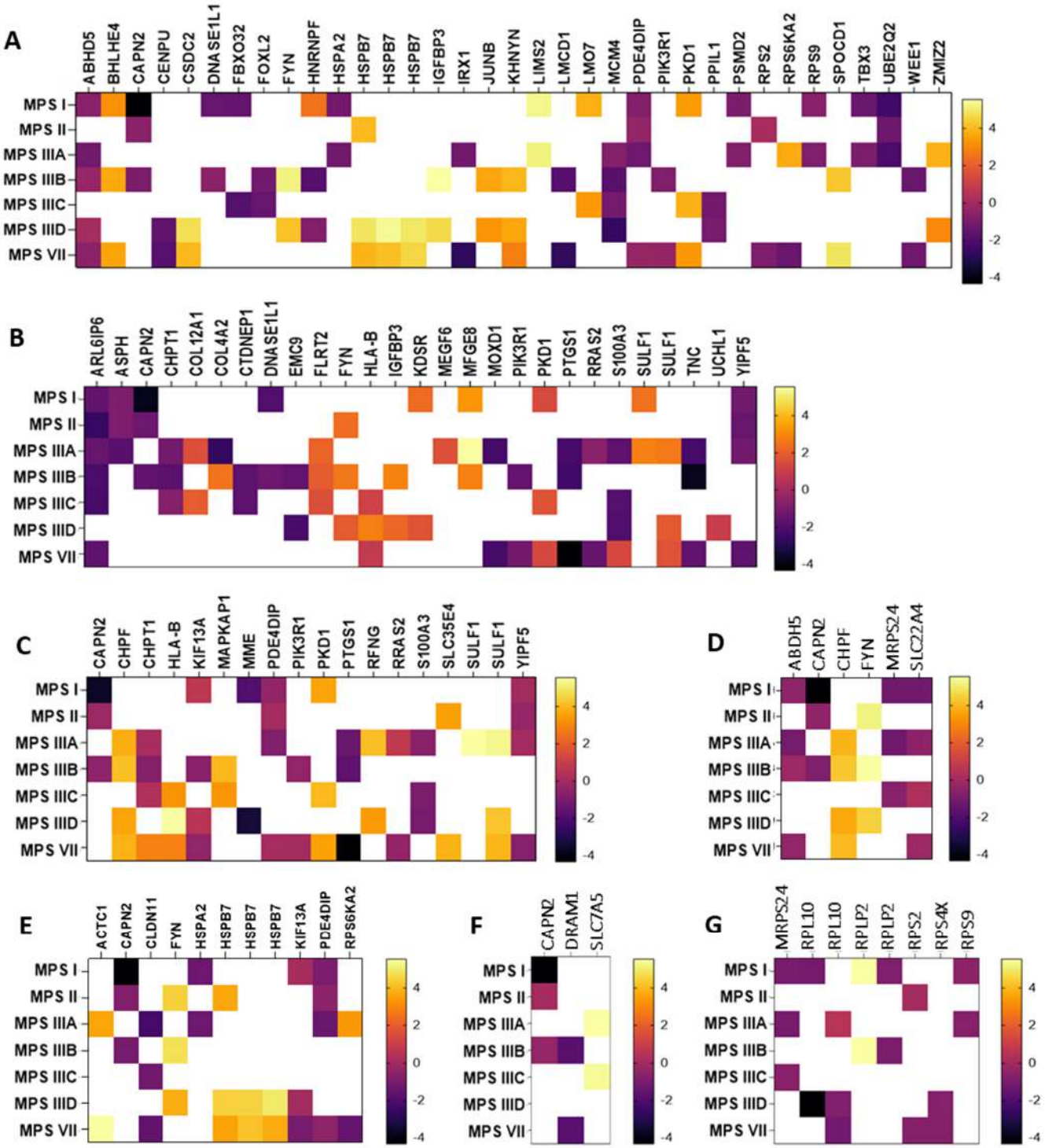

Figure 2. Heat maps presenting transcripts related to the nucleus (**A**), endoplasmic reticulum (**B**), Golgi apparatus (**C**), mitochondria (**D**), cytoskeleton (**E**), lysosome (**F**), and ribosomes (**G**) whose expression undergoes particularly high ($\log_2$FC > 1.5 or $\log_2$FC < −1.5) changes in neuronopathic MPS types/subtypes relative to control cells (HDFa). Abnormal expression levels of these transcripts were not observed in non-neuronopathic MPS types/subtypes.

An example of a severely down-regulated gene in MPS I, II, IIIA, and VII is *PDE4DIP*, encoding phosphodiesterase 4D interacting protein (PDE4DIP), a protein involved in the regulation of intracellular cAMP concentration [44,45]. PDE4DIP interacts with other proteins in cis-Golgi networks, making it an important component of ER-to-Golgi trafficking [46]. Another example of a gene that is particularly down-regulated in MPS I, IIIA, IIIB, IIID, and VII is *ABHD5*, which encodes Alpha/beta hydrolase domain-containing protein 5 (ABDH5 or CGI-58). This highly conserved protein is involved in regulating lipid metabolism and lipid droplet dynamics by activating the triglyceride hydrolase ATGL and other hydrolases [46–48]. The YIPF5 protein (a Yip1 domain family member 5), a product of the *YIPF5* gene, is involved in ER-to-Golgi transport.(. It is located in the ER and Golgi apparatus, as well as in vesicle transporters and down-regulated in some neuronopathic types/subtypes of MPS (I, II, IIIA, and VII). YIPF5-containing protein complexes are thought to play a key role in the transport of COPII-coated vesicles from the ER to the cis-Golgi and vesicle fusion to the Golgi apparatus [47]. The last gene to look at would be *CLDN11*, whose protein product (claudin 11) is a major component of central nervous system myelin and plays an important role in regulating oligodendrocyte proliferation and migration. Claudin 11 ensures rapid nerve conduction, mainly in myelinated axons of small diameter. Mice lacking claudin 11 have been shown to have preserved myelin and axonal architecture, but as much as 60% decreased in conduction. They are also characterized by an increased action potential threshold, severely slowed conduction velocities, and a large juxtaparanode potassium ion (K^+) as a result of changes in the biophysical properties of myelin [48].

3.2. Electron and Fluorescent Microscopy

Since many of the genes whose expression is disrupted in neuronopathic types/subtypes of MPS are related to the function or morphology of the Golgi apparatus and fragmentation of the Golgi apparatus is observed in many different neurodegenerative diseases, we decided to check whether this phenomenon is also present in those types/subtypes of MPS that are characterized by CNS symptoms. Visualization of the Golgi apparatus by electron microscopy and fluorescence microscopy indicated an increase in the number of components of the Golgi apparatus in neuronopathic types/subtypes of MPS compared to control cells and cells taken from patients with non-neuronopathic types/subtypes of MPS (Figures 3 and 4, respectively). This fragmentation affects an average of 20% of the Golgi apparatus structures in MPS IV, VI, and IX, and as much as 80% of the Golgi apparatus structures in MPS I, II, III, and VII (Figure 4).

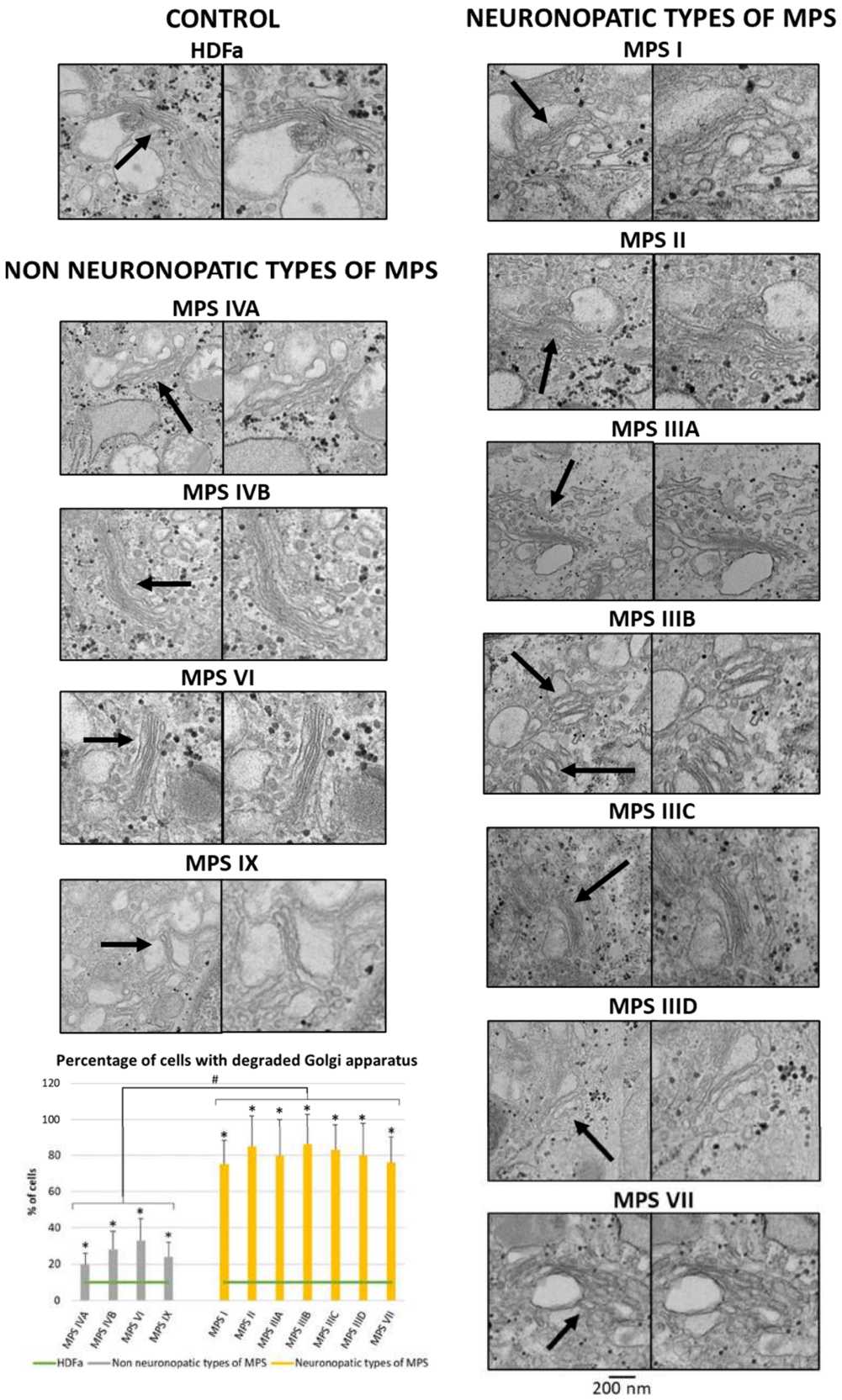

Figure 3. Morphology of the Golgi apparatus in neuronopathic and non-neuronopathic types/subtypes of MPS relative to control cells (HDFa) as studied by electron microscopy. The analysis of the percentage of fragmented Golgi apparatus structures was performed using at least 100 electron micrographs. Mean values ± SD are presented, with (*) representing statistically

significant ($p < 0.05$) differences relative to the HDFa control and (#) representing statistically significant ($p < 0.05$) differences between neuronopathic and non-neuronopathic types/subtypes of MPS. Arrows indicate changes in Gogli apparatus morphology.

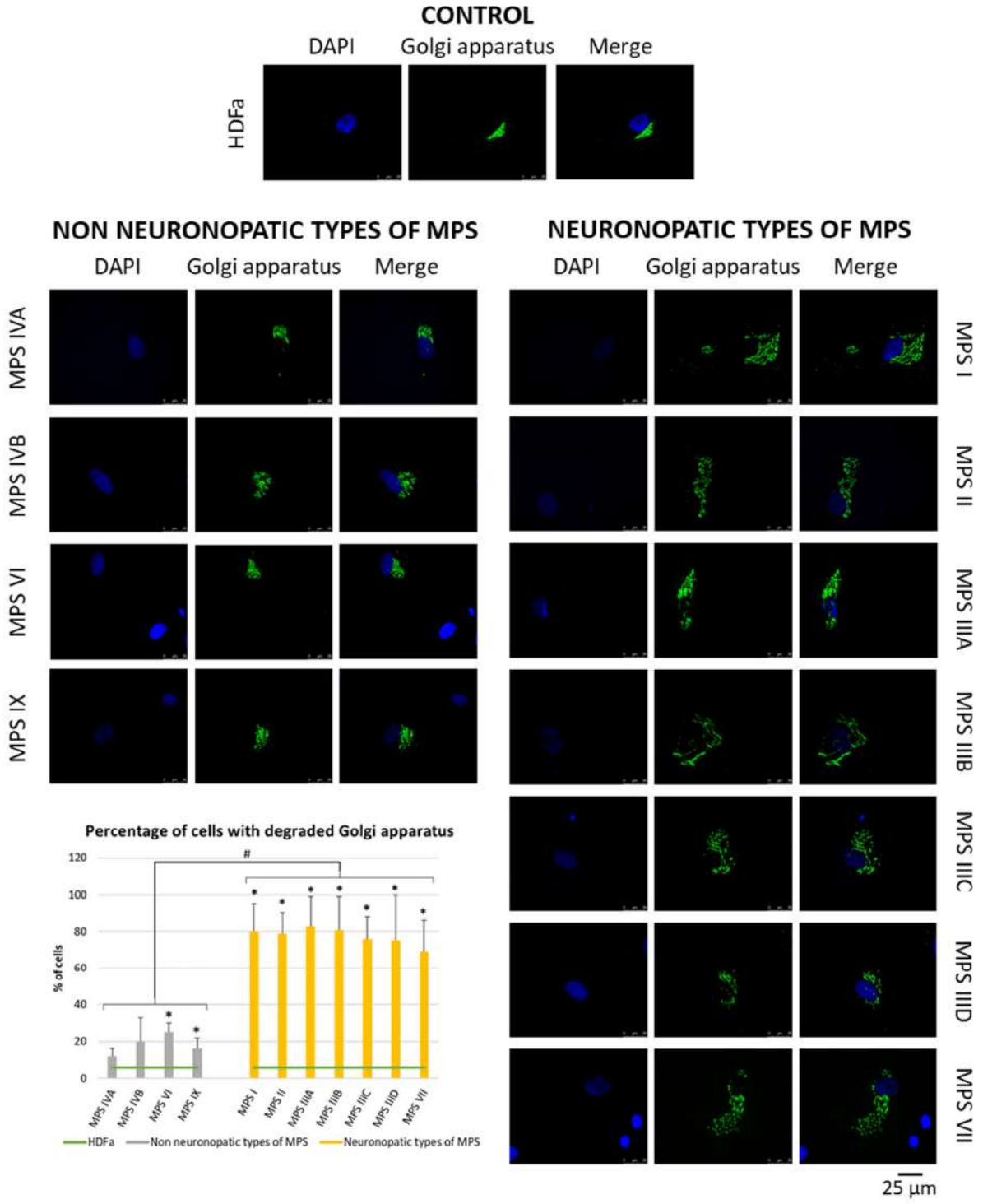

Figure 4. Morphology of the Golgi apparatus in neuronopathic and non-neuronopathic types/subtypes of MPS relative to control cells (HDFa) as studied by fluorescent microscopy using the fluorescent dye CellLight™ Golgi-GFP BacMam 2.0. The analysis of the percentage of fragmented Golgi apparatus structures was performed using at least 30 images. Mean values ± SD are presented, with (*) representing statistically significant ($p < 0.05$) differences relative to the HDFa control and (#) representing statistically significant ($p < 0.05$) differences between neuronopathic and non-neuronopathic types/subtypes of MPS.

4. Discussion

The study of the mechanisms of neurodegeneration is of great interest to neuroscience specialists. Some data report that a component of the degeneration of cells in the nervous system is related to the function or structure of certain cellular organelles. This includes, for example, mitochondria, lysosomes, the cell nucleus, and the Golgi apparatus.

Studies on one of the most common neurological diseases, AD, have pointed to changes in the activities of enzymes involved in oxidative phosphorylation, oxidative damage, and mitochondrial binding of beta-amyloid and its precursor. Many mutations in genes encoding mitochondrial proteins in black matter neurons have been discovered in PD patients. Also noted in amyotrophic lateral sclerosis (ALS) are abnormalities in the efficiency of mitochondrial respiratory chain enzymes and mitochondrial programmed cell death proteins [49]. Mutations in genes encoding lysosomal proteins have been described not only in AD and ALS but also in frontotemporal dementia (FTD) and in lysosomal storage diseases (LSD) [15]. Lo and Zeng identified defective lysosomal acidification as an early indicator of neurodegeneration due to the onset of these disorders even before neurodegeneration occurs [50]. Fragmentation of the Golgi apparatus, as well as disturbances in the metabolism of proteins involved in maintaining its structure, have been described in AD, ALS, and Creutzfeldt-Jacob disease. Moreover, these events occur before clinical signs and other pathological manifestations become apparent [13,51,52].

Disorders of ER structure and function have also been identified in AD, PD, and prion diseases. On top of this, abnormalities of ER-phagy or RE degradation by lysosomes have been observed in AD, PD, Niemann–Pick type C, and schizophrenia [12,53]. Gupta and his team also identified large changes in the expression of genes encoding nuclear transcription factors by analyzing data from 300,000 patients with 30 different diseases of aging [54]. Some researchers emphasize that changes occurring within the structure of the aforementioned organelles or changes in the expression of genes related to their function occur even before the onset of the neurodegenerative process, pointing to these changes as possible markers of neuronal death.

Abnormalities in cellular organelle morphology and changes in the expression of organelle-related genes were also noted in MPS [16]. However, such abnormalities have never been considered markers to differentiate the neurodegenerative component of MPS. Table 4 compares the prevalence of abnormalities of the listed cell organelles in neuronopathic types/subtypes of MPS and other neurodegenerative diseases.

In this work, we identified genes related to the functions or structures of cellular organelles that showed changes in expression levels in neuronopathic types of MPS compared to healthy cells, while not showing these changes in non-neuronopathic types of MPS. Most such genes were identified as being characteristic of the cell nucleus, endoplasmic reticulum, and Golgi apparatus (Figure 1). Identification of these transcripts made it possible to select those that recur most frequently among the different neuronopathic MPS types/subtypes, or those whose changes in expression levels were particularly high (Table 3, Figure 2).

There is evidence that mutations or abnormal expression of the genes presented in this paper are associated with CNS symptoms. Mutations of the *ARL6IP6* gene, undergoing reduced expression in almost all neurodegenerative types/subtypes of MPS, have been found in many other neurodegenerative diseases. Neurological disorders such as abnormal gait, locomotor activation, limb grasping, and abnormal behavior have been indicated in mice with knockout of this gene [55]. *ARL6IP6* loss-of-function mutations have also been identified as a possible cause of cutis marmorata telangiectatica congenita, presenting with major dysmorphism, developmental delay, transient ischemic attacks, and cerebral vascular malformations. Additionally, they are associated with increased susceptibility to ischemic stroke [55,56] and the occurrence of disorders such as spastic paraplegia, diffuse sensorimotor polyneuropathy, and acromutilation [55,57]. Other studies have also indicated changes associated with the *PDIA3* gene in neurological diseases. Analysis performed as part of this work indicated that expression of this gene was elevated in

neuronopathic types/subtypes of MPS. Expression levels of this gene were also elevated in the tissues of patients and mouse models affected by prion diseases, or ALS. *PDIA3* has been proposed as a possible risk factor and marker for the development of ALS [58]. Similarly, the *RPN2* gene encoding ribophorin II has recently been linked to AD. Studies on the mRNA level of the *RPN2* gene in the frontal or intraparietal cortex of AD patients indicated that it was reduced relative to controls [59]. Ribophorin II is a component protein of the subunits of STT3 complexes that catalyze N-glycosylation of emerging or misfolded proteins [60]. Mutations in genes encoding components of the aforementioned complexes cause congenital glycosylation disorders with impaired brain function [61].

Table 4. Abnormalities related to the function or morphology of cell organelles in MPS and other neurological disease/disturbances or mental disorders.

Neurological Disease/Disturbances *	Lysosome	Cytoskeleton	Nucleus	ER	Ribosomes	Golgi	Mitochondria
MPS I, II, III, VII	+	+	+	+	+	+	+
Alzheimer disease	+	+		+	+	+	+
Parkinson disease	+	+		+	+	+	+
ALS	+	+		+	+	+	+
SMA				+	+		+
Cognitive disturbances				+	+		+
Huntington's disease	+				+		+
Stroke		+					+
Epilepsy		+		+	+		+
Friedreich's ataxia							+
Tourette Syndrome							+
Williams Syndrome							+
Type I lissencephaly		+					
Intellectual disabilities		+					
FXTAS					+		
OPMD			+				
Intracerebral hemorrhage					+		+
Christianson syndrome	+						
Ischemia				+			
Mental Disorders *	**Lysosomes**	**Cytoskeleton**	**Nucleus**	**ER**	**Ribosomes**	**Golgi**	**Mitochondria**
ADHD							+
Schizophrenia		+	+	+	+		+
Mood Disorders		+		+			+
ASD	+	+	+	+	+	+	+
Bipolar disorder		+	+	+	+		+
Depression		+		+	+		+
Personality disorders							+
OCD							+
Rett Syndrome			+				
Frontal Dementia			+		+		
PTSD				+	+		
Addictions				+			
FTD	+				+		

* Abbreviations and symbols: ER, endoplasmic reticulum; ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; FXATS, fragile X-associated tremor/ataxia syndrome; OPMD, oculopharyngeal muscular dystrophy; ADHD, attention deficit/hyperactivity disorder; ASD, autism spectrum disorder; OCD, obsessive-compulsive disorder; PTSD, post-traumatic stress disorder; FTD, frontotemporal dementia; +, the occurrence abnormalities in specific organelle or cellular structure.

Increased expression levels of the *MFGE8* gene in MPS cells are consistent with other neurodegenerative disease findings involving this gene. A beneficial role for MFGE8 has been demonstrated in stroke, neurodegenerative diseases (AD and PD), and traumatic brain injury. In stroke, MFGE8 promotes the proliferation of neural stem cells and their migration toward ischemic brain tissues [62]. Many studies point to an important role

of MFGE8 in the process of neurogenesis, as its high levels reduce neuronal cell death through reduced expression of cleaved caspase-3 and IL-1β. This would suggest that the up-regulation of this gene's expression level is a defense mechanism of the nervous system against neuronal cell death. The MFGE8 protein also has the ability to inhibit the production of pro-inflammatory cytokines or down-regulate the expression of apoptotic protein genes in models of cerebral ischemia and subarachnoid hemorrhage [62]. The need to study MFGE8 signaling pathways as a possible drug candidate for the bedside management of neurodegenerative diseases has been highlighted [62]. The *HSPB7* gene, encoding one of the molecular chaperones whose expression is up-regulated in MPS II, IIID, and VII, also appears to have a protective role in neurodegeneration. Studies of its role in diseases associated with polyQ expansion have indicated that it inhibits polyQ aggregation and prevents polyQ-induced toxicity [63]. Moreover, it was found that *HSPB7* overexpression alone did not increase autophagy, one of the mechanisms that may be responsible for the degradation of misfolded proteins. Nevertheless, in *ATG5*$^{-/-}$ cells characterized by defective autophagy, HSPB7 activity against aggregation was significantly reduced. It was concluded that this chaperon prevents polyQ protein toxicity by a mechanism that requires active autophagy machinery [63]. Transcriptomic analyses also indicated up-regulation of *HSPB7* expression levels in dorsal root ganglia and sciatic nerve tissue in a mouse model of diabetic peripheral neuropathy [64]. The up-regulation of the *SULF1* gene, encoding sulfatase 1, observed in MPS I, IIIA, IID, and VII, may also be related to protein aggregation. The role of this sulfatase is to remove specific 6-O-sulfate groups from heparan-sulfate proteoglycans. Recent studies have indicated a role for 6-O sulfation in regulating the internalization of the tau protein, one of the hallmark proteins responsible for AD pathogenesis, in human central nervous system cell lines, iPS-derived neurons, and mouse brain slice cultures [65].

The down-regulation of *ABHD5* expression demonstrated in this work in MPS I, IIIA, IIIB, IIID, and VII was also observed in other CNS disorders. Mutations of this gene have been observed in patients with neutral lipid storage disease with neurological disorders [66] as well as Chanarin-Dorfman syndrome [67]. In both of these conditions, both somatic and neuronal symptoms appear. In addition to hepatic steatosis, skeletal myopathy, cardiomyopathy, and growth retardation, there are bilateral cataracts, ataxia, bilateral sensorineural hearing loss, and intellectual disability [67,68]. PDE4DIP, encoded by the gene of the same name, also appears to be an interesting protein. Its expression level is reduced in MPS I, II, IIIA, and VII. Deletion of a fragment of this gene resulted in behaviors characteristic of autism spectrum disorders, while duplication led to symptoms of psychosis/schizophrenia [69]. Bioinformatic analyses indicated that elevated *PDE4DIP* expression co-occurs with dementia in the course of neurodegenerative diseases such as AD, vascular dementia, frontotemporal dementia, or major depressive disorder [70,71]. A similar situation could be observed with reduced expression levels of *YIPF5* in MPS I, II, IIIA, and VII. Homozygous mutations of this gene have been observed as a genetic cause of congenital microcephaly syndrome, epilepsy, and neonatal/early-onset diabetes. In vitro studies indicated that loss of YIPF5 function resulted in proinsulin retention in the ER, marked ER stress, and β-cell failure. Partial silencing of this gene or the introduction of a mutation increased the sensitivity of β cells to ER stress-induced apoptosis [72]. The last example is *CLDN11*, which encodes claudin-11, which is a component of the myelin sheath of nerve cells [73]. *CLDN11* knockout mice are characterized by impaired auditory processing and reduced anxiety/avoidance. Importantly, these behaviors are associated with increased transmission time along myelinated fibers as well as imbalances of the neurotransmitters glutamate and GABA in the auditory brainstem and amygdala [74]. Abnormal expression of the *CLDN11* gene has also been reported in hypomyelinating leukodystrophy. Riedhammer et al. identified the *CLDN11* mutation in three patients suffering from this disease. The patients developed spastic movement disorder, expressive speech disorder, and eye abnormalities, including hypermetropia, and an MRI scan showed myelin deficits [73]. All of the genes discussed above, whose expression was altered in

MPS, are included in a summary Table 5, indicating the role and function of the proteins they encode as well as the conditions associated with modulation of the expression of a particular gene.

Table 5. Characterization of genes whose expression is altered in MPS types/subtypes, their products, and related diseases.

Gene	Protein	Function	Localization	MPS Type	Regulation	Other Diseases
ARL6IP6	ADP ribosylation factor-like GTPase 6 interacting protein 6	high-curvature ER tubules, regulation of intracellular transport pathways, interaction with proteins involved in membrane vesicle formation	nuclear envelope, RE	I II IIIA IIIB IIIC VII	↓	cutis marmorata telangiectatica congenita, ischemic stroke, spastic paraplegia, diffuse sensorimotor polyneuropathy, acromutilation
PDIA3	protein disulfide isomerase A3	regulation of the folding of newly synthesized glycoproteins, promotion of the re-folding of misfolded proteins	nucleus, RE endoplasm, cytoplasm	I II IIIA IIIB IIIC	↑	prion disease, ALS
RPN2	ribophorin II glycoprotein	mediation of the translocation of secretory proteins, maintainance of the specificity of ER, N-glycosylation proteins	membrane of the ER	I, II IIIA IIIB IIID	↑	AD
MFGE8	milk fat globule-epidermalgrowth factor VIII	alleviation of ER stress, apoptotic cell phagocytosis, anti-inflammatory reactions, tissue regeneration	cytoplasm	I IIIA IIIB	↑	subarachnoid hemorrhage, cerebral ischemia (stroke), AD, PD, traumatic brain injury
HSPB7	heat shock protein family B (small) member 7	chaperone protein	nucleus, cytoplasm	II IIID VII	↑	poliQ-related diseases, diabetic peripheral neuropathy
SULF1	sulfatase 1	removal of specific 6-O-sulfate groups from heparan-sulfate proteoglycans	Golgi apparatus	I, IIIA IIID VII	↑	AD
ABHD5	alpha/beta hydrolase domain-containing protein 5	regulation of lipid metabolism and lipid droplet dynamics	cytoplasm, nucleus, mitochondria	I IIIA IIIB IIID VII	↓	lipid storage disease with neurological disorders, Chanarin-Dorfman syndrome
PDE4DIP	phosphodiesterase 4D-interacting protein	regulation of intracellular cAMP concentration, component of ER-to-Golgi trafficking, maintainance of the structure of Golgi apparatus	Golgi apparatus, centrosome	I II IIIA VII	↓	autism spectrum disorders, psychosis, schizophrenia, AD, vascular dementia, frontotemporal dementia, major depressive disorder
YIPF5	Yip1 domain family member 5	transport of COPII-coated vesicles from the ER to the cis-Golgi and vesicle fusion to the Golgi apparatus	ER, Golgi apparatus, vesicle transporters	I II IIIA VII	↓	congenital microcephaly syndrome, epilepsy, neonatal/early-onset diabetes
CLDN11	claudin-11	component of the myelin sheath of nerve cells, transmission along myelin fibers, maintainance of the balance of neurotransmitters	plasma membrane, cytoskeleton	IIIA IIIC VII	↓	hypomyelinating leukodystrophy

Abbreviations and symbols: ER, endoplasmic reticulum; AD, Alzheimer disease; PD, Parkinson disease; ALS, amyotrophic lateral sclerosis; ↓, down-regulation of gene expression; ↑, up-regulation of gene expression.

In addition, since many of the genes indicated in the transcriptomic analyses relate to the function or morphology of the Golgi apparatus, it was decided to visualize it using electron and fluorescence microscopy. This analysis indicated fragmentation of this organelle in neuronopathic MPS types/subtypes to a greater extent than in non-neuronopathic MPS types/subtypes (Figures 3 and 4). This is consistent with other literature data indicating

that fragmentation and dispersion of the Golgi apparatus precede neuronal cell death. Nakagomi et al. indicated that pharmacological intervention or overproduction of the C-terminal fragment of Grasp65, a protein associated with the Golgi apparatus, inhibited fragmentation and reduced or delayed neuronal death. In addition, inhibition of pathways leading to cell death reduced fragmentation of the Golgi apparatus. The authors indicated that the Golgi apparatus may be a sensor of signals resulting in cell death [75]. Martínez-Menárguez and his team also pointed out that in many neurodegenerative diseases, such as AD, PD, or ALS, the Golgi apparatus did not take the form of a classical ribbon but was usually divided into isolated elements, which occurred as a very early event before clinical symptoms became apparent. However, it is not known whether this phenomenon is caused by mechanisms related to cell death or, conversely, initiates apoptosis [13]. Conducting studies focusing on the search for early pathological events occurring in the nerve cells of people affected by neurodegenerative diseases, Haukedal et al. identified Golgi fragmentation as one of the earliest phenotypes of AD, occurring even before the phenomena of increased Aβ secretion and tau hyperphosphorylation, as well as mitochondrial and synaptic deficits [76].

Turning to MPS, Golgi apparatus-related abnormalities in fibroblasts taken from MPS patients have already been indicated. Changes in the number of Golgi apparatus, which was increased, especially in MPS II, IIIA, IIIB, and IVB, were noted [16]. Detailed studies on the structure of the Golgi apparatus were performed on a mouse model of mucopolysaccharidosis type IIIB. An accumulation of intracellular storage vesicles bearing GM130 (GOLGA2), a Golgi matrix protein that mediates vesicle binding in both pre- and cis-Golgi compartments, was observed. An alteration of Golgi ribbon architecture, which comprised a distended cisterna connected to LAMP1-positive storage vesicles, was also noted. It was pointed out that the accumulation of vesicles and disorganization of the Golgi apparatus caused by changes in the expression of the *GM130* gene can cause neuronal dysfunction and death [77]. Our analysis identified genes that undergo impaired expression in neuronopathic types/subtypes of MPS while not undergoing changes in expression in non-neuronopathic types/subtypes and may be related to the observed changes in the structure of the Golgi apparatus. These include the already-mentioned *GM130* (*GOLGA2*) and *PDE4DIP* genes. *GOLGA2*, encoding golgin A2, undergoes increased expression in MPS I, IIIA, IIIB, IIIC, IIID, and IX. The studies cited above identified this gene as one of the key genes for the adoption of normal structure by the Golgi apparatus in a mouse model of MPS IIIB. These data are consistent with the data indicated in this paper on increased expression of the gene encoding this protein not only in MPS IIIB but also in other MPS types/subtypes. Shamseldin et al. also described a patient with a homozygous *GOLGA2* mutation with a neuromuscular disorder characterized by developmental delay, seizures, progressive microcephaly, and muscular dystrophy, while deletion of this gene in the striped danio also caused severe skeletal muscle disorganization and microcephaly [78]. Another gene that may be related to changes in the structure of the Golgi apparatus is the *PDE4DIP* gene, which encodes a phosphodiesterase 4D-interacting protein that undergoes reduced expression in MPS I, II, IIIA, and VII, which has already been described above. It was demonstrated that PDE4DIP interacts with other proteins in cis-Golgi networks, which affects the stability of both PDE4DIP itself and other proteins [46]. Moreover, disruption of this gene expression not only impairs the efficiency of transport from the ER to the Golgi apparatus but also causes fragmentation of the Golgi apparatus [46]. Experiments on the role of oxidative stress in maintaining the proper structure of the Golgi apparatus have indicated that activation of co-cellular stress elevates intracellular Ca^{2+} and protein kinase Cα (PKCα) activity, which phosphorylates the Golgi stacking protein GRASP55, leading to fragmentation of the Golgi apparatus [79]. A similar situation has been described for AD. A β-amyloid-induced increase in cytosolic Ca^{2+} levels leads to activation of calpain, which in turn activates Cdk5 kinase, leading to phosphorylation of the GRASP65 protein [52,80,81]. Interestingly, recent studies indicated that disruption of the Golgi structure by knocking out GRASP55 and GRASP65 increases HS synthesis and decreases CS synthesis in cells,

as well as changing the sulfation pattern and decreasing the secretion of both of these GAGs. Those studies provided evidence that a structural defect in the Golgi apparatus can significantly alter GAG synthesis and secretion [82].

Taking into account both the above-described abnormalities of gene expression related to the functions/structures of cellular organelles and the fragmentation of the Golgi apparatus in neurodegenerative diseases, it should not be surprising that symptoms occurring in the course of MPS I, II, III, and VII sometimes cause misdiagnosis, indicating either a neurological disease or a psychiatric disorder [83]. Escolar et al. compiled information on the use of drugs affecting behavior and mood in the treatment of MPS, which are typically used for psychiatric disorders. The data presented shows that the use of pharmacotherapy to treat sleep, mood, and other behavioral disorders affects patients with MPS to varying degrees, but the authors pointed to the benefits of using the described drugs as complementary therapy [84].

It may be surprising to use fibroblasts taken from patients to study neurodegenerative diseases. It is worth mentioning, however, that research conducted with a model of fibroblasts that are not nerve cells is nevertheless quite common. On the one hand, this is certainly a matter of the difficulty of collecting and culturing neurons. On the other hand, fibroblasts could be relatively easily obtained from patients and control individuals, making the research material both specific and homogeneous. The use of patient-derived cell lines is also advantageous relative to heavily modified cells, like neuronal cells differentiated under laboratory conditions, where potential off-target effects should be taken into consideration, the problem which is absent in fibroblasts obtained directly from affected individuals. It has already been documented that primary fibroblasts from patients with PD, AD, and spinal–cerebellar ataxia type 2 showed a distinct and unique mRNA expression pattern of key genes for neurodegeneration [85]. This approach was also supported by Olesen et al., who presented data on mitochondrial abnormalities from fibroblasts taken from AD, PD, HD, and ALS patients, and these changes turned out to be similar to those observed in the CNS. The authors pointed to fibroblasts as a reliable model for detecting early abnormalities because of their metabolic and biochemical links to neurons [86]. More importantly, with recent advances in single-cell RNA sequencing, the heterogeneity and diversity of fibroblasts within the CNS have been discovered. Based on their distinct anatomical localization in the meninges, perivascular space, and choroid plexus, as well as their molecular diversity, fibroblasts are said to play an important role in the CNS in both physiological and pathological states [87]. The usefulness of experiments with fibroblasts for studies on neurodegenerative diseases, like AD and other disorders, as well as on the functions of the CNS, was also highlighted recently [88–92]. Therefore, we believe that results obtained with fibroblast models can be reliable in studies on mechanisms of neurodegeneration, especially when gene expression patterns are analyzed.

5. Conclusions

In summary, the scope of this study was to compare the molecular features of neuronopathic types of MPS with those of other neurodegenerative diseases. Indeed, we demonstrated that, besides the fact that patients suffering from neuronopathic types/subtypes of MPS and other neurodegenerative diseases share many clinical similarities, there are also molecular similarities between these diseases. Changes in the expression of genes encoding organelle-related proteins and consequent disruption of the organelle structure or function itself are common factors in many entities associated with CNS damage. Here, we have pointed out that neuronopathic types/subtypes of MPS are also among such diseases. Genes whose expression is affected in neuronopathic MPS are often associated with the structures or functions of the cell nucleus, endoplasmic reticulum, or Golgi apparatus, and disruptions in the structures of these organelles could be confirmed. Among genes with changed expression levels in neuronopathic MPS types were up-regulated ones, like *PDIA3*, *GM130* (*GOLGA2*), and *MFGE8*, and down-regulated ones, like *ARL6IP6*, *ABHD5*, *PDE4DIP*, *YIPF5*, and *CLDN11*. Disorders of the described organelle-related genes and

changes in the morphology of the Golgi apparatus were not noted in non-neuronopathic types/subtypes of this disorder. Taking into account that for neuronopathic types/subtypes of MPS there is currently no treatment, and in the case of MPS types I and II, the neurodegenerative component is, with the current state of knowledge, impossible to predict, we propose to consider the genes presented in this paper as candidates for markers of neurodegeneration in MPS.

Limitations of this study include the use of fibroblasts (rather than neural cells) as models of neurodegenerative diseases and the employment of only one cell line per MPS type. However, it was indicated previously that fibroblasts could be useful models in neurodegenerative diseases, and peripheral markers are also usable when assessing neurological disorders. Moreover, since directions of dysregulation (either up-regulation or down-regulation) of specific genes were in most cases the same among all neurodegenerative MPS types, one might conclude that the results of analyses based on one line per MPS type are reliable. Nevertheless, the proposed hypothesis requires verification in the largest possible group of patients with neuronopathic and non-neuronopathic types of MPS, which may be difficult in rare diseases where the overall number of patients is small. However, only such studies would be able to indicate whether the proposal of abnormal expression of organelle-related genes/abnormal organelle morphology as markers of neurodegeneration in MPS would work in practice. These genes or the proteins they encode could also become potential targets for therapies under development for neurological disorders associated with the disease and markers of the effectiveness of the use of these therapies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46030169/s1. Table S1: Transcripts with severely impaired expression ($\log_2$FC < −1.5 or >1.5) in neuronopathic types/subtypes of MPS associated with organelles.

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Supplementary

Table S1. Transcripts with severely impaired expression ($log_2FC<-1.5$ or >1.5) in neuronopathic types/subtypes of MPS associated with organelles.

Structure or organellum	Transcripts with severely impaired expression ($log_2FC<-1.5$ or >1.5) in specific neuronopathic types/subtypes of MPS associated with organelles							
	Transcript	**MPS I**	**MPS II**	**MPS IIIA**	**MPS IIIB**	**MPS IIIC**	**MPS IIID**	**MPS VII**
Nucleus	*ABHD5*	-	-	-1.75	-	-	-	-
	BHLHE4	-	-	-	1.56	-	-	-
	CAPN2	-3.87	-	-	-1.58	-	-	-
	CENPU	-	-	-	-	-	-1.99	-2.2
	CSDC2	-	-	-	-	-	2.29	1.76
	DNASE1L1	-1.95	-	-	-	-	-	-
	FBXO32	-1.96	-	-	-	-2.21	-	-
	FOXL2	-	-	-	-	-1.73	-1.93	-
	FYN	-	-	-	2.05	-	1.89	-
	HNRNPF	-	-	-	-2.19	-	-	-
	HSPA2	-1.73	-	-1.75	-	-	-	-
	HSPB7	-	1.8	-	-	-	2.70	1.85
	HSPB7	-	-	-	-	-	2.38	1.72
	HSPB7	-	-	-	-	-	2.4	2.2
	IGFBP3	-	-	-	2.08	-	2.21	-
	IRX1	-	-	-1.74	-	-	-	-2.82
	JUNB	-	-	-	1.52	-	-	-
	KHNYN	-	-	-	1.75	-	1.55	-
	LIMS2	2.66	-	2.58	-	-	-	-
	LMCD1	-	-	-2.178	-	-	-	-2.75
	LMO7	1.65	-	-	-	-	-	-
	MCM4	-	-	-	-2.14	-1.69	-2.72	-
	PDE4DIP	-	-	-1.8	-	-	-	-
	PIK3R1	-	-	-1.52	-	-	-	-
	PKD1	-	-	-	-	1.67	-	-
	PPIL1	-	-	-	-	-1.73	-1.64	-
	PSMD2	-1.57	-	-	-	-	-	-
	RPS2	-	-	-	-	-	-	-
	RPS6KA2	-	-	1.59	-	-	-	-
	RPS9	-	-1.52	-	-	-	-	-1.88
	SPOCD1	-	-	-	1.96	-	-	2.39
	TBX3	-1.89	-	-1.56	-	-	-	-
	UBE2Q2	-2.55	-1.82	-2.37	-	-	-	-
	WEE1	-	-	-	-1.88	-	-	-1.77
	ZMIZ2	-	-	1.66	-	-	-	-
Endoplasmic reticulum	*ARL6IP6*	-1.57	-2.61	-1.47	-2.12	-2.19	-	-1.64
	ASPH	-	-	-1.79	-	-	-	-
	CAPN2	-3.87	-	-	-1.58	-	-	-
	CHPT1	-	-	-	-1.75	-	-	-
	COL12A1	-	-	1.53	-	1.91	-	-
	COL4A2	-	-	-2.63	2.51	-	-	-
	CTDNEP1	-	-	-	-1.73	-1.74	-	-
	DNASE1L1	-1.95	-	-	-	-	-	-

	EMC9	-	-	-	-1.54	-	-	-2.18
	FLRT2	-	-	2.10	1.9	1.54	-	-
	FYN	-	2.37	-	2.05	-	1.89	-
	HLA-B	-	-	-	-	-	2.08	-
	IGFBP3	-	-	-	2.08	-	2.21	-
	KDSR	2.03	-	-	-	-	1.64	-
	MEGF6	-	-	1.59	-	-	-	-
	MFGE8	3.03	-	5.05	2.82	-	-	-
	MOXD1	-	-	-2.25	-	-	-	-2.28
	PIK3R1	-	-	-	-1.52	-	-	-
	PKD1	-	-	-	-	1.67	-	-
	PTGS1	-	-	-2.22	-2.38	-	-	-4.32
	RRAS2	-	-	-	-	-	-	-1.52
	S100A3	-	-	-1.65	-	-1.88	-1.98	-1.65
	SULF1	2.04	-	2.80	-	-	-	-
	SULF1	-	-	2.63	-	-	-	-
	TNC	-	-	-2.25	-3.85	-	-	-1.63
	UCHL1	-	-	-	2.42	-	-	-
	YIPF5	-	-	-	-	-	-	-1.71
Golgi apparatus	*CAPN2*	-3.87	-	-	-1.58	-	-	-
	CHPF	-	-	1.52	1.75	-	-	1.58
	CHPT1	-	-	-	-1.75	-	-	-
	HLA-B	-	-	-	-	-	2.08	-
	KIF13A	-	-	-	-1.65	-	-	-1.54
	MAPKAP1	-	-	-	1.65	-	-	-
	MME	-2.61	-	-	-	-	-3.62	-
	PDE4DIP	-	-	-1.8	-	-	-	-
	PIK3R1	-	-	-	-1.52	-	-	-
	PKD1	-	-	-	-	1.67	-	-
	PTGS1	-	-	-2.22	-2.38	-	-	-4.32
	RFNG	-	-	1.74	-	-	-	-
	RRAS2	-	-	-	-	-	-	-1.52
	S100A3	-	-	-1.65	-	-1.88	-1.98	-1.65
	SLC35E4	-	-	-	-	-	-	1.58
	SULF1	2.04	2.80	-	-	-	-	-
	SULF1	-	2.63	-	-	-	1.85	1.62
	YIPF5	-	-	-	-	-	-	-1.71
Mitochondrion	*ABHD5*	-	-	-1.75	-	-	-	-
	CAPN2	-3.87	-	-	-1.58	-	-	-
	CHPF	-	-	1.52	1.76	-	-	1.58
	FYN	-	2.37	-	2.05	-	1.89	-
	MRPS24	-1.84	-	-1.85	-	-	-	-
	SLC22A4	-1.83	-	-	-	-	-	-
Cytoskeleton	*ACTC1*	-	-	1.74	-	-	-	3.15
	CAPN2	-3.87	-	-	-1.58	-	-	-
	CLDN11	-	-	-2.43	-	-1.64	-	-1.91
	FYN	-	2.37	-	2.05	-	1.89	
	HSPA2	-1.73	-	-1.75	-	-	-	-
	HSPB7	-	1.8	-	-	-	2.70	1.85

	HSPB7	-	-	-	-	-	2.38	1.72
	HSPB7	-	-	-	-	-	2.4	2.2
	KIF13A	-	-	-	-	-	-	-1.54
	RPS6KA2	-	-	1.59	-	-	-	-
	PDE4DIP	-	-	-1.8	-	-	-	-
Lysosome	*CAPN2*	-3.87	-1.36	-	-1.58	-	-	-
	DRAM1	-	-	-	-2.41	-	-	-2.4
	SLC7A5	-	-	1.82	-	1.73	-	-
Ribosome	*MRPS24*	-1.84	-	-1.85	-	-	-	-
	RPL10	-1.89	-	-	-	-	-4.61	-
	RPL10	-	-	-	-	-	-1.62	-1.94
	RPLP2	-1.66	-	-	-1.72	-	-	-
	RPLP2	3.80	-	-	3.81	-	-	-
	RPS2	-	-	-	-	-	-	-1.55
	RPS4X	-	-	-	-	-	-1.53	-
	RPS9	-	-	-	-	-	-1.55	-1.56

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polegał na:

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2. przygotowaniu preparatów do mikroskopii elektronowej i fluorescencyjnej
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1. recenzji wewnętrznej manuskryptu
2. udziale w interpretacji wyników
3. asyście w przygotowaniu odpowiedzi na uwagi recenzentów
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1. przygotowaniu pierwotnej wersji manuskryptu
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Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes

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Research paper

Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes

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ABSTRACT

Mucopolysaccharidoses (MPS) are genetic disorders that affect up to 1 in 25,000 births. They are caused by dysfunctions of lysosomal hydrolases that degrade glycosaminoglycans (GAGs) which accumulate in cells, damaging their proper functioning. There are 7 types of MPS, distinguished by the kind of accumulated GAG and the defective enzyme, which differ significantly in the course of the disease. Despite the storage of the same GAGs, two of them (MPS III and IV) are divided into subtypes. While the course of MPS IV A and B is similar, the variability between MPS III A, B, C and D is high. This suggests that there are additional aspects that could influence the course of the disease. Therefore, the aim of this study was to determine differences of patterns of gene expression between all MPS III and IV subtypes. Transcriptomic studies, carried out with dermal fibroblasts from patients with all MPS III and IV subtypes, showed a significant variation in the gene expression pattern between individual MPS III subtypes, in contrast to MPS IV. Detailed analysis of transcripts with altered expression levels between MPS III subtypes indicated that these transcripts are mainly involved in maintaining the proper structure of connective tissue (*COL4A1*, *COL4A2*, *COMP*) and the structure of ribosomes (*RPL10*, *RPL23*, *RPLP2*). The results presented in this study indicate a significant role of genetic factors in the diversified course of MPS III subtypes.

1. Introduction

Mucopolysaccharidosis (MPS) is a group of inherited lysosomal storage diseases in which the main cause of pathogenesis is glycosaminoglycan (GAG) storage (Tomatsu et al., 2018). It is caused by mutations in genes encoding enzymes involved in GAG catabolism. Therefore, GAGs accumulate in lysosomes, leading to impairment of normal functions of cells, tissues, organs, and whole organisms. There are 11 types and subtypes of MPS, depending on the type of the stored GAG and the defective enzyme: MPS type I (Hurler, Scheie and Hurler/Scheie syndromes), II (Hunter syndrome), III A/B/C/D (Sanfilippo syndrome), IV A/B (Morquio syndrome), VI (Maroteaux–Lamy syndrome), VII (Sly syndrome) and IX (Natowicz syndrome) (Fecarotta et al., 2020). Only MPS type II is an X chromosome-linked disease, the rest are inherited in an autosomal recessive manner.

Symptoms of the MPS disease vary greatly depending on its type. They are usually not yet visible at birth but appear and worsen as GAGs accumulate. Common MPS symptoms are coarse facial features, organomegaly, damage to the sensory organs, dysostosis multiplex, impairment of certain motor functions, dysplasia, emerging hernias, joint mobility disorders, and susceptibility to infections. Most patients die in childhood, mainly those with neuronopathy (MPS I, II, III and VII), but persons with mild forms may live to adulthood (Tomatsu et al., 2018; Fecarotta et al., 2020).

Among all MPS types, following two are further divided into subtypes - MPS III (subtypes A, B, C, and D) and IV (subtypes A and B) (Supplementary Table S1). The most common symptoms of Sanfilippo disease are similar and include language delay, abnormal behavior, autism spectrum disorder, epilepsy, and coarse facial features. However, despite cognitive and behavioral abnormalities predominate in all Sanfilippo disease patients, those with different MPS III subtypes differ significantly in the frequency of occurrence of individual symptoms, the age of patients at onset, and life expectancy, as summarized previously (Zelei et al., 2018).

Abbreviations: FC, fold change; FDR, false discovery rate; FPKM, fragments per kilobase million; GAG, glycosaminoglycans; MPS, mucopolysaccharidosis.

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Patients suffering from Morquio disease have usually no cognitive or behavioral disturbances, but skeletal and connective tissue manifestations are particularly severe (Sawamoto et al., 2020; Yuskiv et al., 2020). They include short stature, dysostosis multiplex, spinal deformity, and dental abnormalities. Contrary to MPS III subtypes, MPS IV A and B are generally similar, though there are also some differences. MPS IVB is generally milder than IVA, particularly expressed as lower intensity of skeletal deformity and longer life span. Moreover, contrary to MPS IVA, neuronopathic features may occur in IVB, though only in untypical cases which have intermediate phenotype between MPS IV and GM1 gangliosidosis (these diseases are caused by different mutations in the same gene) (Sawamoto et al., 2020; Yuskiv et al., 2020).

The reasons for such variations within these patient groups, despite the storage of the same GAGs (heparan sulfate in Sanfilippo disease and keratan sulfate in Morquio disease), remain unclear (Fecarotta et al., 2020; Kobayashi, 2019). In addition to environmental factors and different access to emergency treatment and rehabilitation, this phenomenon may be modulated by genetic factors at the molecular level which are indirect consequences of GAG storage and lead to varied clinical pictures of the disease. In the current trend, more and more attention has been focused on disturbed cellular processes as crucial contributors to MPS pathogenesis (Kobayashi, 2019; Gaffke et al., 2021). Therefore, in this work we hypothesized that the reasons for the diversity of symptoms among patients could reflect changes in expression of a battery of genes influencing various cellular functions. We performed analyses indicating different transcriptional profiles within different MPS III and IV subtypes, with particular emphasis on the analysis of cellular processes.

2. Materials and methods

2.1. Cell lines

Lines of dermal fibroblasts obtained from patients with MPS III A, B, C, D and IV A, B, purchased from NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research, are characterized in Table 1. Control fibroblast cell line (HDFa) was used, as described previously (Gaffke et al., 2020). Patients being donors of these cell lines represented typical features of disease severity, age of onset and life expectancy for all disease subtypes. Available clinical descriptions (which are limited for these cell lines) indicate that all patients had severe phenotypes, considering the typical severity spectrum observed within all tested MPS types and subtypes. Fibroblasts were grown under standard conditions in DMEM medium (Dulbecco's Modifies Eagle Medium) supplemented with antibiotics and 10% fetal bovine serum (FBS). Cells between 4th and 15th passages were used for experiments.

2.2. Isolation and purification of RNA, and RNA-seq analysis

The RNA isolation and purification procedure as well as the RNA-seq analysis was performed as described previously (Gaffke et al., 2020). Four biological repetitions were applied for each experiment. Raw RNA-seq data has been deposited into the NCBI Sequence Read Archive (SRA) (accession no. PRJNA562649). For identification of gene functions, GeneCards®: The Human Gene Database (https://www.genecards.org) was used.

2.3. Measurement of GAG levels in cells

GAG levels in fibroblasts, cultured as described in Section 2.1, were measured using the Glycosaminoglycan Assay BlyscanTM (Biocolor Ltd., Carrickfergus, UK), according to manufacturer's instructions. The results were expressed in μg od total GAG per ml.

2.4. Determination of activities of lysosomal enzymes

Activities of enzymes known to be deficient in MPS IIIA, IIIB, IIIC, IIID, IVA, and IVA, i.e. heparan sulfamidase, N-acetylglucosaminidase, heparan acetyl CoA:α-glucosaminide N-acetyltransferase, N-acetylglucosamine 6-sulfatase, galactose-6-sulfate sulfatase, and β-galactosidase, respectively, were measured. Following 4-methylumbelliferyl derivatives were used as substrates in the reactions with lysates of appropriate cells (activities of all tested enzymes were measured in the control line, HDFa, and particular MPS cell lines were tested for residual activities of corresponding deficient enzymes): 4MU-alpha-N-sulpho-D-glucosaminide (Biosynth Carbosynth, cat. no. EM06602), 4-Methylumbelliferyl-N-acetyl-α-D-glucosaminide (Sigma Aldrich cat. no. 474500), 4MU-beta-D-glucosaminide (Biosynth Carbosynth, cat. no. EM31025), 4MU-alpha-N-acetyl-D-glucosaminide-6-sulphate (Biosynth Carbosynth cat. no. EM31027), 4MU-beta-D-galactoside-6-sulphate (Biosynthesis Carbosynth cat. no. EM05134), and 4-Methylumbelliferyl β-D-galactopyranoside (Sigma Aldrich cat. no. M1633). Reaction conditions are presented in Supplementary Table S2. The results are presented as pmol of reaction product per minute per mg of protein.

2.5. DNA sequencing

Sequence of the *HGSNAT* gene was determined using isolated DNA

Table 1
Characteristics of fibroblast lines derived from MPS III and IV patients.

MPS subtype and cell line no.[a]	Race	Sex[b]	Age[c]	Mutated gene	Mutations[d]	GAG level (μg/ml)[e]	Enzyme activity [normal activity] (pmol/min/mg)[f]
IIIA GM00879	Caucasian	F	3	*SGSH*	p.Glu447Lys p.Arg245His	2.50	11.5 [21400.0]
IIIB GM00156	Caucasian	M	7	*NAGLU*	p.Arg626Ter p.Arg626Ter	2.92	10.2 [1974.5]
IIIC GM05157	Unknown	M	8	*HGSNAT*	p.Gly262Arg pArg509Asp	2.19	33.5 [191.3]
IIID GM05093	Indian	M	7	*GNS*	p.Arg355Ter p.Arg355Ter	2.91	<0.1 [18.47]
IVA GM00593	Caucasian	F	7	*GALNS*	p.Arg386Cys p. Phe285Ter	2.90	9.1 [462.5]
IVB GM03251	Caucasian	F	4	*GLB1*	p.Trp273Leu p.Trp509Cys	2.85	21.2 [4578.3]

Abbreviations: (a) catalogue numbers of cell lines from NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research; (b) F – female, M – male; (c) Age at the time of sample collection; (d) Mutations according to Coriell Institute for Medical Research, our results obtained in this work (for MPS IIIC; c.784G > A, c.1526C > A), and from the literature (MPS IVA) (Alméciga-Diaz et al., 2019); (e) GAG level measured in HDFa (control) cells was 0.51 μg/ml); (f) Activities of following enzymes were determined: MPS IIIA - heparan sulfamidase, MPS IIIB - N-acetylglucosaminidase, MPS IIIC - heparan acetyl CoA: alpha-glucosaminide N-acetyltransferase; MPS IIID - N-acetylglucosamine 6-sulfatase, MPS IVA - galactose-6-sulfate sulfatase, MPS IVB - β-galactosidase, while normal activities of these enzymes were measured in HDFa (control) cells.

from MPS IIIC fibroblasts characterized in Table 1. Primers used for DNA amplification by PCR, and conditions of PCR are described in Supplementary Table S3. DNA sequencing was performed commercially by Macrogen Europe (Amsterdam, The Netherlands).

2.6. Statistical analysis

In transcriptomic analyses, the normalization of the expression values was carried out using the FPKM (Fragments Per Kilobase Million) algorithm with the use of the Library-norm-method classic-fpkm parameter of the Cyffmerge program. The one-way ANOVA method was used to estimate statistical significance between groups (with normal continuous distribution) and the Benjamini-Hochberg method was used to asses false discovery rate (FDR). Comparisons between two groups was assessed by post hoc *Student's t-test* with Bonferroni correction. Differences were considered significant when $p < 0.1$ (according to previously published reports (Lamanna et al., 2012; Gaffke et al., 2018; Wiebe et al., 2020; Klauck et al., 2006; Gong et al., 2009; Brooks et al., 2014). All statistical analyses were conducted using R software v. 3.4.3.

3. Results

Experiments were carried out on a model of human dermal fibroblasts, obtained from patients suffering from Sanfilippo (subtypes A, B, C and D) and Morquio (subtypes A and B) diseases (Table 1), and healthy control (HDFa) cell line. Cells were grown under standard laboratory conditions. Then, using samples of RNA isolated from fibroblasts, RNA-seq analysis was performed according to the procedure described in Section 2.2.

3.1. Number of transcripts showing differences in expression levels between disease subtypes within MPS III and IV

The obtained results, depicting the number of transcripts which differ in the level of expression (false discovery rate (FDR) < 0.1; $p < 0.1$) between individual subtypes of the same disease types, indicated a significant variability in MPS III while this feature was considerably less pronounced in MPS IV. The number of transcripts occurring at different levels in certain MPS III subtypes, namely MPS IIIA vs. IIIB, IIIA vs. IIID, IIIB vs. IIID, and IIIC vs. IIID, was as high as > 400. In contrast, the number of transcripts showing differential expression in MPS IV A vs. IVB was below 100. When comparing specific transcripts, in each pair of subtypes, both upregulated and downregulated genes (one subtype vs. another) could be indicated. The results of this analysis are shown in Fig. 1. When comparing each of tested MPS cell line with control (HDFa) fibroblasts, hundreds of genes revealed altered expression, indicating that most of these changes are related to the disease, rather than being normal variations between individuals (Supplementary Figure S1).

Moreover, we determined if transcripts whose expression is changed in multiple MPS III subtypes relative to one selected subtype could be identified. Such an analysis allows to indicate which disease subtype differs the most from the others in terms of changes in gene expression. Venn diagrams illustrating this type of analysis are present in Fig. 2. In relation to both MPS IIIA and MPS IIIB, all the remaining subtypes showed the highest number of altered transcripts, 35 and 34, respectively. Then, these subtypes were followed by MPS IIID (31 transcripts) and MPS IIIC (11 transcripts). Analogous Venn diagrams showing analyses of MPS III and IV subtypes *versus* control (HDFa) cells are presented in Supplementary Figure S2. These results confirmed that in terms of changes in gene expression, control cells are more different from MPS III and MPS IV fibroblasts than MPS III or MPS IV subtypes within these MPS types.

3.2. Transcripts showing differences in expression levels between disease subtypes within MPS III and IV with especially high fold change

In the next step, we conducted a detailed analysis of the number of transcripts with a particularly high fold change (Fig. 3). This examination allowed us to determine what are numbers of genes which expression differs especially strongly between the tested disease subtypes. Such genes might be of particular importance as potentially determining significant differences in cell (and possibly also organism) physiology. Analysis of transcripts revealing especially high differences in expression, i.e. with over 16-fold changes in expression levels ($\log_2FC > 4.0$ or $\log_2FC < -4.0$), between various pairs of MPS III subtypes showed 18 such transcripts. These differences were mainly between MPS IIIA and IIID (6 transcripts), MPS IIIB and IIIC (5 transcripts) as well as MPS IIIB and IIID (6 transcripts).

Then, it was crucial to identify specific genes revealing such especially high differences in expression between subtypes of MPS III and MPS IV. Among described genes, there were those which products are related to ribosome functions (*RPLP2, RPL23, RPL10*), maintaining the proper structure of connective tissue (*COL4A1, COL4A2, COMP*),

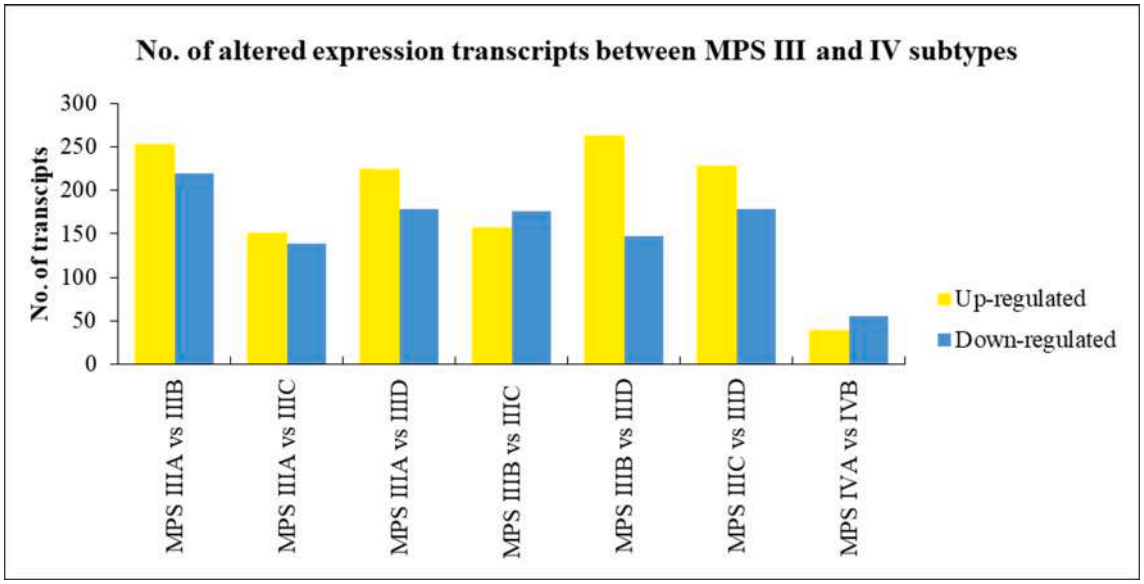

Fig. 1. Number of transcripts with altered expression between all MPS III and IV subtypes.

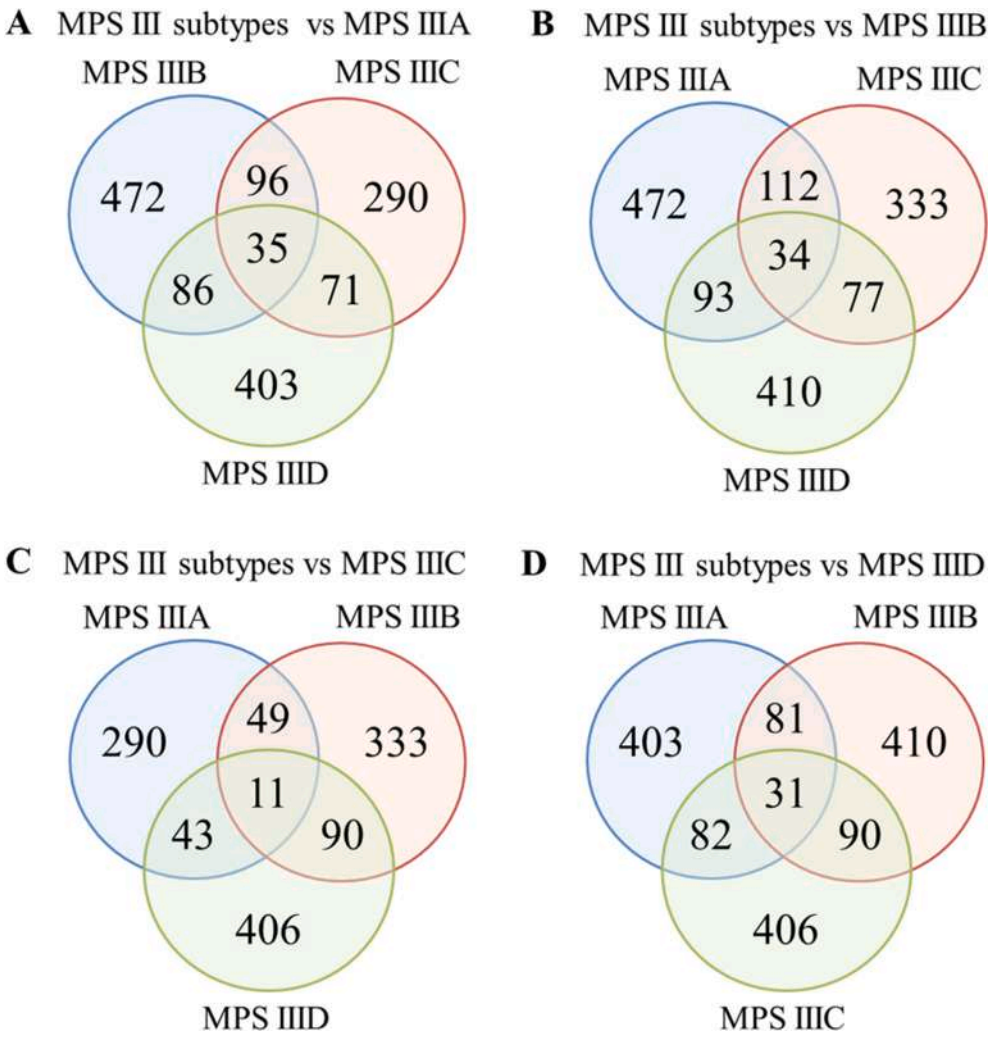

Fig. 2. Number of altered expression of transcripts between MPS IIIA (A), IIIB (B), IIIC (C) and IIID (D) and the rest of subtypes (multiple and separately).

	MPS IIIA vs IIIB	MPS IIIA vs IIIC	MPS IIIA vs IIID	MPS IIIB vs IIIC	MPS IIIB vs IIID	MPS IIIC vs IIID	MPS IVA vs IVB
$log_2FC>5.0$	2	1	-	1	2	-	-
$log_2FC>4.0$	4	1	4	3	3	-	-
$log_2FC>3.0$	14	7	12	5	9	2	-
$log_2FC>2.0$	44	23	45	16	46	20	1
$log_2FC<-2.0$	31	29	27	27	31	12	4
$log_2FC<-3.0$	3	4	8	10	7	4	1
$log_2FC<-4.0$	-	1	2	2	3	4	-
$log_2FC<-5.0$	-	-	1	-	2	2	-

Fig. 3. Number of transcripts with altered expression at particularly high fold change between all MPS III and IV subtypes.

involved in intracellular signaling (*NME2, WISP2, SRFP1*) as well as receptors and various factors (*RARRES2, CRLF1, IGFBP5, TFPI2*). These transcripts are listed in Table 2, and changes in expressions of these genes are illustrated by a heat-map to visualize relationships between their expression in different MPS III and MPS IV subtypes (Fig. 4).

Analogous analyses were performed with comparison of each MPS III and MPS IV subtype to wild-type (HDFa) cells (Supplementary Figure S3 and Table S4). These results confirmed that the observed changes are related to the investigated diseases rather than representing natural variations.

4. Discussion

Differences in the course of the disease between MPS patients are mainly due to the defective enzyme and type of GAG stored. On this basis, the disease is classified into one of 7 types of MPS. In the case of two of them (MPS III and IV), disease subtypes are distinguished based on the kind of defective enzyme which, however, lead to the storage of the same GAG (heparan sulfate in MPS III and keratan sulfate in MPS IV) (Supplementary Table S1) (Fecarotta et al., 2020). Despite this, the variation in the course of the disease in patients suffering from MPS III subtypes is significant while less pronounced in MPS IV (Zelei et al., 2018; Sawamoto et al., 2020; Yuskiv et al., 2020).

In Sanfilippo disease, autism spectrum disorders are present in almost 30% of MPS IIIA patients and only 8% of MPS IIIC patients. Epilepsy occurs in 17% of MPS IIIA patients and 8% of MPS IIIC patients. Likewise, symptoms that are characteristic for most MPS types, i.e. coarse features and hepatomegaly, occur in 94% and 56% of MPS IIIB patients, and 85% and 39% of MPS IIIC patients, respectively, which makes timely diagnosis of the disease difficult. The diagnosis may also be difficult due to the different time of appearance of individual symptoms in different subtypes of MPS III. For example, the mean age of diagnosis of MPS IIIB ranges from 2.5 to 5 years, and MPS IIIC from 4.5 to 19 years. The life expectancy of patients also shows a significant variation, and it is 15–18 years for MPS IIIA, 17–19 years for MPS IIIB, 19–34 years for MPS IIIC (no data available for MPS IIID) (Zelei et al., 2018).

In MPS IV, the variability between typical course of different subtypes of the disease is considerably less pronounced than in MPS III. The symptoms of MPS IVA and MPS IVB are similar, and the only reported difference seems to be relatively milder course of MPS IVB relative to MPS IVA (Sawamoto et al., 2020; Yuskiv et al., 2020.).

Some studies on lysosomal storage diseases indicated possible roles of various factors in symptom diversity. They include (apart from the lack or residual activity of enzymes) oxidative stress, inflammation, infections, performance of some cell organelles, efficiency of autoimmune response, and environmental factors. Such factors may affect the disease course significantly. However, previous studies mostly concerned other lysosomal storage diseases such as Gaucher, Fabry, Pompe diseases or sometimes MPS I and MPS VI (Jakóbkiewicz-Banecka et al., 2014).

In the context of MPS III and IV, only a few such studies have been con-ducted. Recent reports indicated significant variability in the efficiency of GAG synthesis in humans, which does not affect the physiology of a healthy person but may have a significant impact on the development of symptoms in patients with Sanfilippo or Morquio disease. It was indicated that MPS III patients with low efficiency of heparan sulfate synthesis showed milder phenotypes than those producing GAG more efficiently (Jakóbkiewicz-Banecka et al., 2014). This hypothesis is also supported by research on genistein or rhodamine B, which indicated that reduction of heparan sulphate synthesis led to an improvement in biochemical parameters and the physiological state of mice (Malinowska et al., 2010; Roberts et al., 2006; Roberts et al., 2007). Such conclusion could be corroborated by results of studies on mice which were defective in both GAG degradation and synthesis due to combinations of various mutations (Lamanna et al., 2012). Thus, reduced GAG synthesis is currently assessed as the major biochemical factor that, apart from the lack or residual activity of the enzyme, may affect the course of Sanfilippo disease (Jakóbkiewicz-Banecka et al., 2014).

The profile of gene expression in the cell may significantly affect both the efficiency of GAG synthesis and other regulatory mechanisms ensuring the proper functioning of cells. Thus, it is possible that, among many factors, it plays an important role in the diversity of the course of individual MPS III and IV subtypes. This can be mediated by the influence of the expression of specific genes on the efficiency of not only GAG synthesis, but also cell response to the appearance of these molecules in partially degraded forms, the initiation of compensation reactions, metabolic processes, and many others.

Therefore, the aim of this study was to determine changes in gene expression patterns in cells derived from patients with all Sanfilippo and Morquio disease subtypes, which may indicate the involvement of various genetic factors (other than those believed to be causative, i.e. mutated genes encoding defective enzymes involved in GAG degradation) in development of different symptoms in patients. Although mutations of specific genes coding for enzymes involved in GAG degradation are primary genetic causes of MPS, and GAG storage is the primary biochemical cause of this disease, we hypothesized that changes in expression of many different genes, appearing as associated processes in the disease, might significantly modulate further cellular changes, causing variations in the disease course even if the same GAG is stored.

Our analysis showed a significant number of genes with altered expression between individual MPS subtypes, indicating significant variations in the gene expression patterns (Fig. 1 and Fig. 2). Studies with control cells indicated that the observed differences are related to the disease rather than reflecting natural variations between individuals (Supplementary Figures S1, S2, S3, and Table S4). The obtained results do not marginalize the crucial role of GAG accumulation in development of MPS, however, they call into question all the hypotheses about GAG storage as the only cause of MPS. Such a large dysregulation of expression of many genes (even at the level of the transcriptome) between disease subtypes that store the same GAG suggests that these changes do not necessary result directly from GAG storage. This, in turn, indicates a significant role for these genes in the pathogenesis of the disease, putting them in correlation with the previously considered

Table 2
List of transcripts showing differences in expression levels between disease subtypes within MPS III and IV with a particularly high fold change.

Transcript	log_2FC of selected transcripts between different subtypes of MPS III and IV						
	MPS IIIA vs. IIIB	MPS IIIA vs. IIIC	MPS IIIA vs. IIID	MPS IIIB vs. IIIC	MPS IIIB vs. IIID	MPS IIIC vs. IIID	MPS IVA vs. IVB
	up-regulated transcripts						
COL4A1	5.93	-	-	-	-	-	-
COL4A2	5.14	–	–	–	–	–	–
COL4A2	4.65	–	–	–	–	–	–
RPLP2	4.35	–	–	–	–	–	–
NME2	–	6.20	–	5.10	–	–	–
WISP2	–	–	4.79	4.35	5.31	–	–
RARRES2	–	–	4.65	–	5.49	–	–
COMP	–	–	4.25	–	–	–	–
CRLF1	–	–	4.16	–	–	–	–
GAL	–	–	–	4.53	–	–	–
IGFBP5	–	–	–	–	4.54	–	–
	down-regulated transcripts						
RPL23	–	−4.05	–	–	–	–	–
RPL10	–	–	−4.40	–	−4.49	−4.61	–
GNS	–	–	−5.16	–	−5.34	−5.17	–
TFPI2	–	–	–	−4.03	–	–	–
RPLP2	–	–	–	−4.15	–	–	–
SRFP1	–	–	–	–	−5.51	−4.66	–
NME2	–	–	–	–	–	−6.91	–

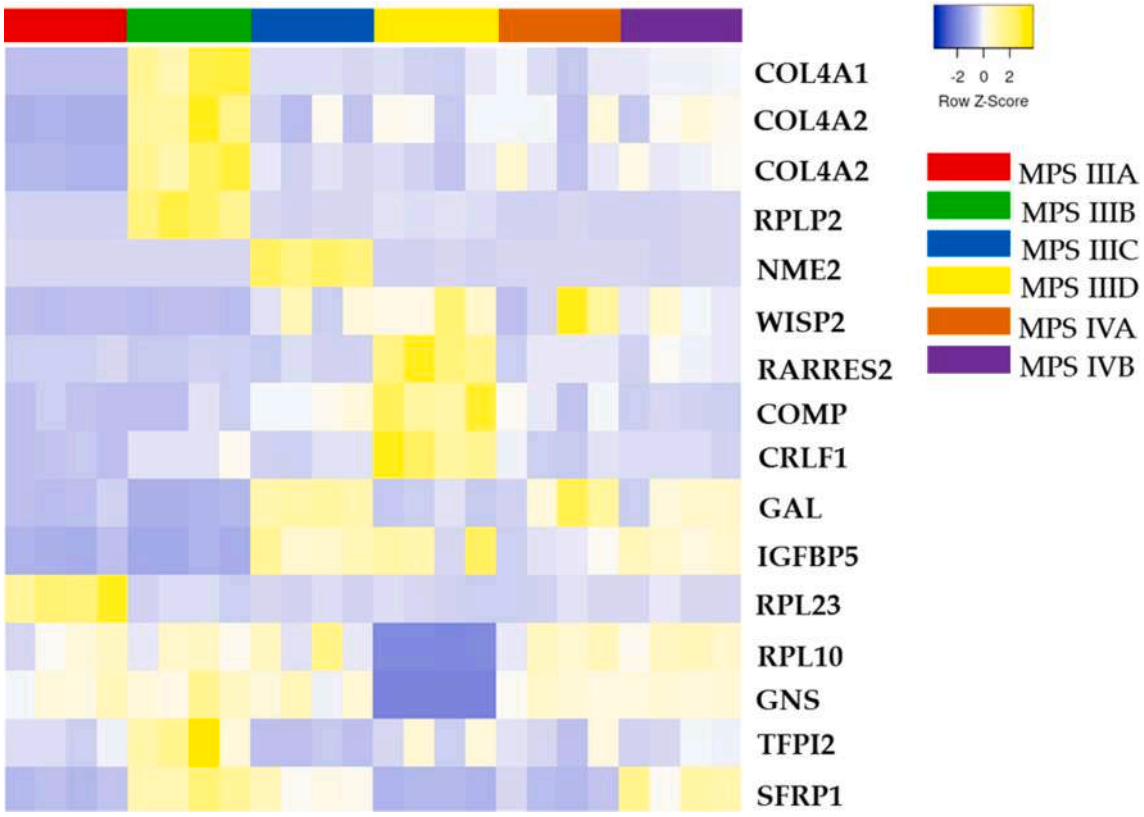

Fig. 4. Heat-map presenting differences in gene expression levels between disease subtypes within MPS III and MPS IV with a particularly high fold change.

primary cause. According to this hypothesis, these results may show the causes of failure of therapies based on lowering GAG levels, especially in the case of neuronopathic types of MPS. It is worth noting that the reasons for the failure of these therapies have been correlated mainly to the fact that recombinant enzymes used in enzyme replacement therapy could not cross the blood–brain barrier efficiently (Gaffke et al., 2018). However, there are also evidences that some recombinant enzymes dedicated to MPS treatment can be insufficiently modified (for example, poor glycosylation was reported), and even trials with therapies which in theory should solve the problem of MPS, like direct infusion of the therapeutic enzyme into the brain or gene therapy, also failed to correct all symptoms of the disease (Tomatsu et al., 2018; Gaffke et al., 2018).

The role of transcripts which expression is characterized by especially high changes in the differentiation of the development of the disease symptoms is probable. Such changes in transcripts' levels were detected between different MPS III subtypes, while not in the case of MPS IV (Fig. 3, Table 2, Fig. 4). While the large variation in the expression of genes involved in the preservation of connective tissue functions is not surprising, because it has already been mentioned in the context of the pathogenesis of MPS, a significant number of such genes involved in ribosome functions is a new discovery. As ribosomes are key organelles involved in gene expression, these results suggest a significant variation in the efficiency of protein synthesis in general, which may be the cause of different course of these diseases (apart from the varied efficiency of GAG synthesis).

Some genes with significant changes in expression are involved in disorders which are present in MPS. One example is autism spectrum disorder that is common in MPS III. Decreased levels of expression on two genes, *RPL10* and *RPL23*, coding for ribosomal proteins, occurred in MPS IIIA relative to MPS IIIC and IIID. Protein synthesis dysfunctions were implicated in autism spectrum disorder (Wiebe et al., 2020). More detailed analyses of the *RPL10* gene indicated the presence of mutations in this gene in autistic patients (Klauck et al., 2006; Gong et al., 2009), as well as those with other psychic disorders, like X-linked microcephaly (Brooks et al., 2014) or X-linked syndromic intellectual disability (Thevenon et al., 2015). It is, therefore, possible that decreased levels of expression on these genes, detected in MPS IIIA relative to MPS IIIC and IIID, is responsible for more frequently occurring autistic behavior of children with the former Sanfilippo disease subtype than in other subtypes. Mutations in the *RPL10* gene, leading to ribosomopathy, were also reported to be connected with syndromic intellectual disability and epilepsy (Bourque et al., 2018). Thus, lower levels of expression of this gene in MPS IIIA relative to other MPS III subtypes might also explain, to some extent, more severe epileptic events observed in this subtype. Another example of genes connected with epilepsy in *RARRES2*, coding for chemerin, which is detected in blood of children with idiopathic epilepsy as a prognostic agent (Elhady et al., 2018). In fact, expression of this gene is elevated in MPS IIIA relative to IIID, and epilepsy is more frequent in the former subtype than in the latter one. Intriguingly, significant changes in expression on genes coding for collagens and other proteins involved in maintenance of the connective tissue occurred in MPS III (Table 2, Supplementary Table S4) which is known as a neurodegenerative disease. However, it is worth reminding that apart from the brain disorder, Sanfilippo disease patients also suffer from somatic dysfunctions, including connective tissue abnormalities (Tomatsu et al., 2018; Zelei et al., 2018). Therefore, our results may strengthen the importance of these somatic disorders in MPS III.

It is worth noting that contrary to MPS III, no such drastic differences in the gene expression patterns (Fig. 1, Fig. 2) and in genes revealing the most pronounced expression variations (Fig. 3, Table 2, Fig. 4) were observed between subtypes of MPS IV. In fact, phenotypic differences between patients suffering from various subtypes of MPS IV are significantly lower than those between various MPS III subtypes. Therefore, we suggest that the less differentiated pattern of expression on genes between MPS IVA and IVB is partially responsible for this phenomenon.

The major limitation of this work is that only one cell line of each

MPS subtype was investigated. Definitely, extension of this work in the future will require studies on separate MPS types/subtypes with more patients and more genetic variants in each type/subtype. Nevertheless, analyses performed in previously published articles indicated that transcriptomic analyses with the use of single cellular lines from each MPS type/subtype, but involving all types/subtypes, are informative, giving statistically significant results (Gaffke et al., 2020; Pierzynowska et al., 2020; Rintz et al., 2020; Pierzynowska et al., 2020; Pierzynowska et al., 2020; Brokowska et al., 2021; Gaffke et al., 2021). Moreover, the results of our analyses have been strengthened by performing four biological repeats of each experiment (as stated in Section 2.2), thus, four series of transcriptomic results were considered in bioinformatic analyses to obtain reliable results.

Another limitation is that we have used patient-derived fibroblasts, while MPS III is known to be a severe brain disease, and MPS IV is primarily a bone and cartilage disease (Tomatsu et al., 2018; Fecarotta et al., 2020; Zelei et al., 2018; Sawamoto et al., 2020; Yuskiv et al., 2020). However, obtaining patient-derived cells from these organs is unlikely. On the other hand, it was demonstrated previously that careful transcriptomic analyses might provide reliable results even if other types of cells, which can be obtained from patients, were used. For example, even behavioral disorders could be correlated to changes in expression of genes in cells derived from patient somatic tissue (Pierzynowska et al., 2020).

5. Conclusions

Transcriptomic analyses presented in this report indicated significant differences in patterns of gene expression between subtypes of MPS III, and most of these differentially expressed genes are involved in cellular processes. Analogous differences between MPS IVA and IVB were considerably less pronounced. Since differences in symptoms are higher between subtypes of Sanfilippo disease than between subtypes of Morquio disease, we suggest that changes in gene expression patterns may be involved in diversity of the disease course among patients suffering from various subtypes of MPS III. To our knowledge, this is the first report indicating a possibility of the correlation between global expression patterns of genes with development of different symptoms in patients suffering from various MPS III subtypes.

CRediT authorship contribution statement

Karolina Wiśniewska: . **Lidia Gaffke:** Methodology, Validation, Investigation. **Karolina Krzelowska:** . **Grzegorz Węgrzyn:** Conceptualization, Methodology, Project administration, Supervision, Funding acquisition. **Karolina Pierzynowska:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and material statement

Raw RNA-seq data has been deposited into the NCBI Sequence Read Archive (SRA) (accession no. PRJNA562649).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.146090.

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Supplementary

Supplementary data

Table S1. Characteristics of MPS III and MPS IV

MPS type	subtype	stored GAG	defective enzyme
MPS III	A	heparan sulfate	heparan sulfamidase
	B	heparan sulfate	N-acetylglucosaminidase
	C	heparan sulfate	heparan acetyl CoA:α-glucosaminide N-acetyltransferase
	D	heparan sulfate	N-acetylglucosamine 6-sulfatase
MPS IV	A	keratan sulfate chondroitin 6-sulfate	galactose-6-sulfate sulfatase
	B	keratan sulfate	β-galactosidase

Table S2. Incubation conditions for enzyme activity tests

MPS TYPE	SAMPLE	INCUBATION TIME	INCUBATION I	INCUBATION II	STOP BUFFER	REFERENCE
MPS IIIA	cell lysate	Inc I: 17h Inc II: 24h	cell lysate containing 15µg of protein; 20µl 10mM MU-αGlcNS in Michaelis' barbital sodium acetate buffer pH 6.5	6 µl twice-concentrated Mctlvain's buffer, pH 6.7 containing 0.02% sodium azide and 10,ul (0.1 U) yeast a-glucosidase in water	200 µl 0.5 M Na_2CO_3/$NaHCO_3$pH 10.7	Karpova et al., 1995
MPS IIIB	cell lysate	Inc I: 17h	cell lysate containing 15µg of protein; 50µl of 2mM MUG in 0.2 M Na-acetate buffer pH 4.5	-	150 ml 0.2 M glycine buffer pH 10.8	Mauri et al., 2013
MPS IIIC	cell lysate	Inc I: 17h	cell lysate containing 20µg of protein; 10µl 6 mM acetyl-CoA in water;10µl 3mM MU-βGlcN in in McIlvain's phosphate/citrate buffer pH 5.7	-	200 µl 0.5 M Na_2CO_3/$NaHCO_3$pH 10.7	Karpova et al., 1993a
MPS IIID	cell lysate	Inc I: 17h Inc II: 6h	cell lysate containing 20 µg of protein; 5µl 10mM MU-GlcNAc-6S in 0.2mM sodium acetate buffer, pH 5.6	20µl twice-concentrated Mctlvain's buffer, pH 6.7	200 µl 0.5 M Na_2CO_3/$NaHCO_3$pH 10.7	Karpova et al., 1993b

MPS IVA	cell lysate	Inc I: 17h Inc II: 2h	cell lysate containing 15 µg of protein; 40µl of 10mM MU-Gal-6S in 0.1M sodium acetic acid buffer with 0.002% sodium azide pH 4.3	10µl of 0.9M sodium phosphate buffer pH 4.3; 0.002% sodium azide; β-galactosidase galactohydrolase 10U	400 µl 0.5 M Na_2CO_3/$NaHCO_3$pH 10.7	Camelier et al., 2011
MPS IVB	cell lysate	Inc I: 3h	cell lysate containing 15 µg of protein; 40 µl 0.8mM MU-βGal in 100 mM citrate-phosphate buffer, pH 4.4	-	200 µl 0.5 M Na_2CO_3/$NaHCO_3$pH 10.7	Cozma et al., 2015

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Table S3. Primers and PCR amplification conditions for sequencing of the *HGSNAT* gene

Oligonucleotide	Forward sequence	Reverse sequence	Reaction conditions
Ex1al	GCGGTGACTCAGGCGGCGGTGACG	CGCGGCCTCTGTTAGACGGAAGG	1
Exon2	TAACACATCTTTCGGGTGCC	GATCATATCAAACAAGCCCCA	5
Exon3	GGATATTTATGTCCTCCAGCA	TGGGTACGGACCAGAAAAGA	6
Exon4	TCTTTTCTGGTCCGTACCCA	GGACTGCTTTGCTTTACAAAC	6
Exon5	TGATGTGATGTCTTTGATGTTC	ATCATCGAAGAATATGGCCAC	2
Exon6	CTTAGTAATATAGAATATGAGCT	CTACAACCTGGCCTTCCTC	3
Exon7	CACAGAGCGAGACTCTGTC	AAGTCCTTAAAAGCTAAAAGTGT	4
Exon8	GCATGATTGCACCTTGTGAG	TCCTGCCCTGAAGCTCTGT	2
Exon9	CAGTGTGTAATGAAGTCCACA	AGGCTCCAGCATCATCTGAA	2
Exon10	TGGTTGCTTCTCTTTTATAGCA	CACCTGAGATGGAGGAATTG	3
Ex11al	TCCCTGACTGACCCTCCCTCTATT	CCTGTATCAACAACAACAAGAACA	7
Exon12	GAAATGAGTCACCGGGAATTT	GAGAAGGAAGAGGGAAAGAG	3
Ex12al	ATGTGCTTAGTTCCCTTCTATTTG	TCATCAGTTCTCAGTGTGTTTTCT	6
Exon14	TTGTATTTGGTCTAGGAGCTG	GCCCATAGCACAAGAGAGAA	2
Exon15	TCTTTGTCAGGTAGTTAAGACA	AATGGCTAGCTCAGTGTTGC	2

Exon16/17	TCAGCCCTCTCTACGTGATT	CAGTGGCTCAAGACCCCAG	2
Exon18	TTTCATTGAACTGGTTTCAAGAA	ACACGCAGCCAGTCAATTTC	6

1. 95°C, 30s; 35 x (95°C, 30 s; 55°C, 30 s; 72°C, 90 s); 72°C, 5 min.
2. 94°C, 7 min; 35 x (94°C, 1 min; 60°C,1 min; 72°C,1 min); 72°C, 7 min.
3. 95°C, 30s; 35 x (95°C, 30 s; 51.5°C, 30 s; 72°C, 90 s); 72°C, 5 min.
4. 95°C, 30s; 35 x (95°C, 30 s; 58.3°C, 30 s; 72°C, 90 s); 72°C, 5 min.
5. 98°C, 30s; 30 x (98°C, 10 s; 50°C, 30 s; 72°C, 90 s); 72°C, 10 min.
6. 98°C, 30s; 30 x (98°C, 10 s; 53.4°C, 30 s; 72°C, 90 s); 72°C, 10 min.
7. 98°C, 30s; 30 x (98°C, 10 s; 55.7°C, 30 s; 72°C, 90 s); 72°C, 10 min.

Table S4. List of transcripts showing differences in expression levels between MPS III/IV subtypes and HDFa with especially high fold change.

transcript	log_2FC of selected transcripts between different MPS III/IV subtypes and HDFa					
	MPS IIIA vs. HDFa	MPS IIIB vs. HDFa	MPS IIIC vs. HDFa	MPS IIID vs. HDFa	MPS IVA vs. HDFa	MPS IVB vs. HDFa
	up-regulated transcripts					
AC004556.1	-	3.52	-	-	-	-
AC011611.3	-	3.64	-	-	-	-
ADAMTSL1	-	-	-	-	3.55	
CAPG	4.71		4.27		4.17	4.14
CD9	3.75	4.10	4.00	3.96	-	4.86
CLU	-	-	4.13	-	-	3.60
COL4A1	-	-	-	-	-	3.84
COL8A2	3.88	-	-	4.36	-	-
COMP	-	-	-	4.75	-	-
CRIP1	4.02	3.90	-	-	-	-
CRLF1	-	-	-	4.38	-	-

ENPP1	-	-	3.57	-	-	-
FAM167A	-	-	3.97	-	-	-
IGFBP5	-	-	-	4.12	-	3.77
KRT19	-	-	-	-	4.20	-
MFAP5	6.14	5.00	4.51	3.75	3.93	4.11
MFGE8	5.05	-	-	-	-	-
MFGE8	3.90	-	-	-	-	-
MFGE8	3.88	-	-	-	-	-
MFGE8	3.70	-	-	-	-	-
MKNK2	-	-	3.70	-	-	-
NME2	-	3.66		-	-	-
NOTCH3	-	-	5.14	-	-	-
OXTR	3.77	5.20	6.43	4.21	-	6.07
PFN1	3.51	3.64	3.73	3.53	3.62	3.67
POSTN	5,27	7,26	5,42	5,01	-	5,69
POSTN	5.14	7.75	5.63	4.98	-	5.84
POSTN	4.68	5.63	3.67	4.16	-	4.16

RPLPA	-	3.81	-	-	-	-
TFPIA	-	3.60	-	-	-	-
UCHL1	-	3.64	-	-	-	-
down-regulated transcripts						
APOE	-6.11	-	-	-	-	-
CAV1	-	-	-	-	-	-4.74
CXCL8	-5.08	-	-	-	-	-
EMP1	-	-3.67	-4.71	-	-4.09	-3.74
EPDR1	-	-	-	-	-	-4.69
ERH	-	-	-	-	-3.69	-
GNS	-	-	-	-5.16	-	-
HOXB6	-	-	-3.96	-	-	-
IGFBP3	-	-	-	-	-5.17	-
LY6K	-	-	-3.61	-5.34	-	-4.28
LY6K	-	-	-	-4.79	-	-3.95
LY6K	-	-	-	-3.59	-	-
MME	-	-	-	-3.62	-	-

MMP12	-4.20	-5.30	-4.49	-	-	-
PKM	-	-	-	-	-3.58	-
PTGDS	-6.16	-4.79	-4.28	-	-	-4.01
RARRES2	-4.08	-4.91	-	-	-	-
RND3	-	-3.71	-	-	-	-
RPL10	-	-	-	-4.61	-	-
RPL23	-	-	-4.24	-4.16	-4.09	-3.72
SERPINB2	-	-	-	-	-	-3.56
SNX3	-	-	-4.14	-	-	-
SPON2	-4.15	-3.77	-	-	-	-
THBS1	-	-	-	-	-4.08	-
TRPV2	-	-3.77	-	-	-	-
WISP2	-	-4.03	-	-	-	-
WISP3	-6.71	-7.23	-	-	-	-

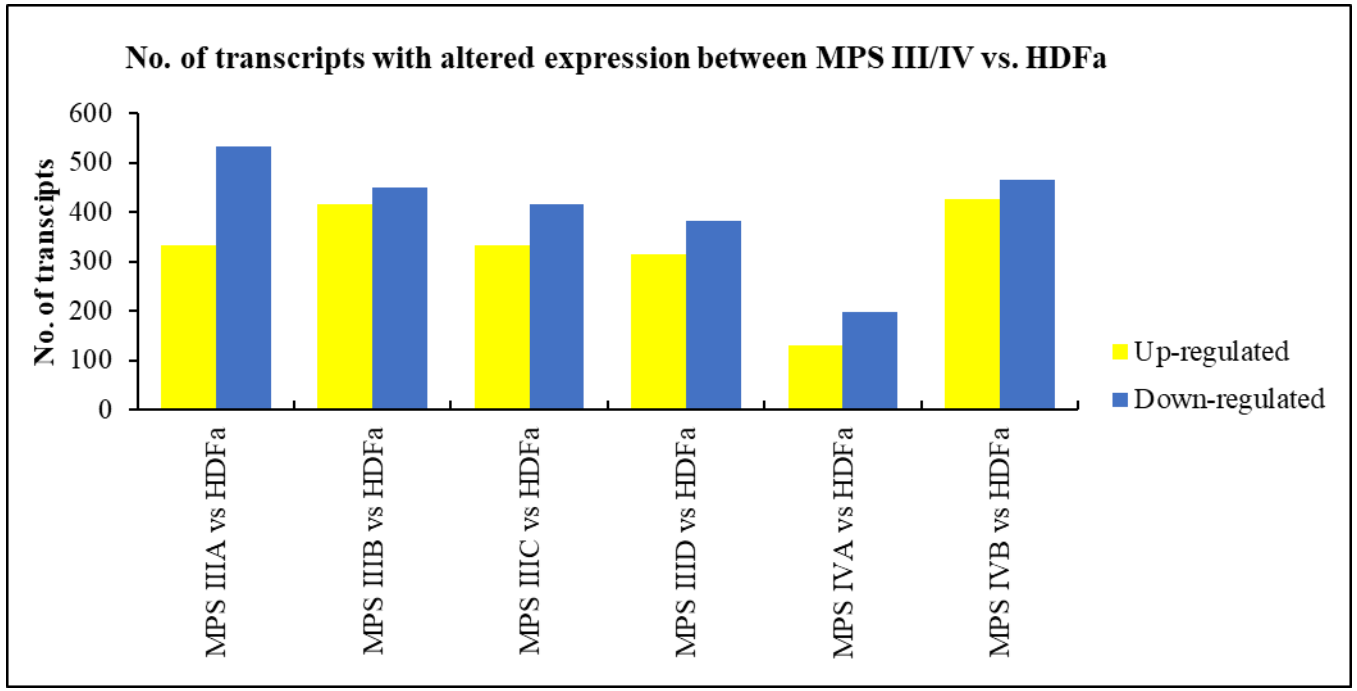


Figure S1. Number of transcripts with altered expression between MPS III/IV subtypes and HDFa.

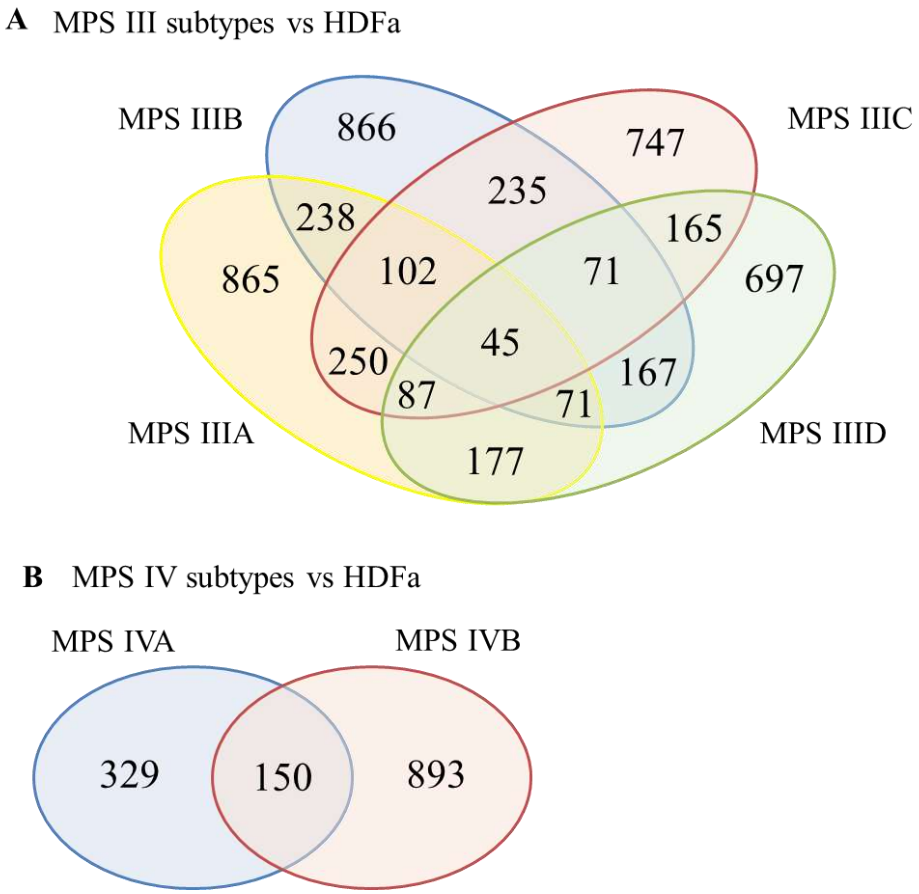
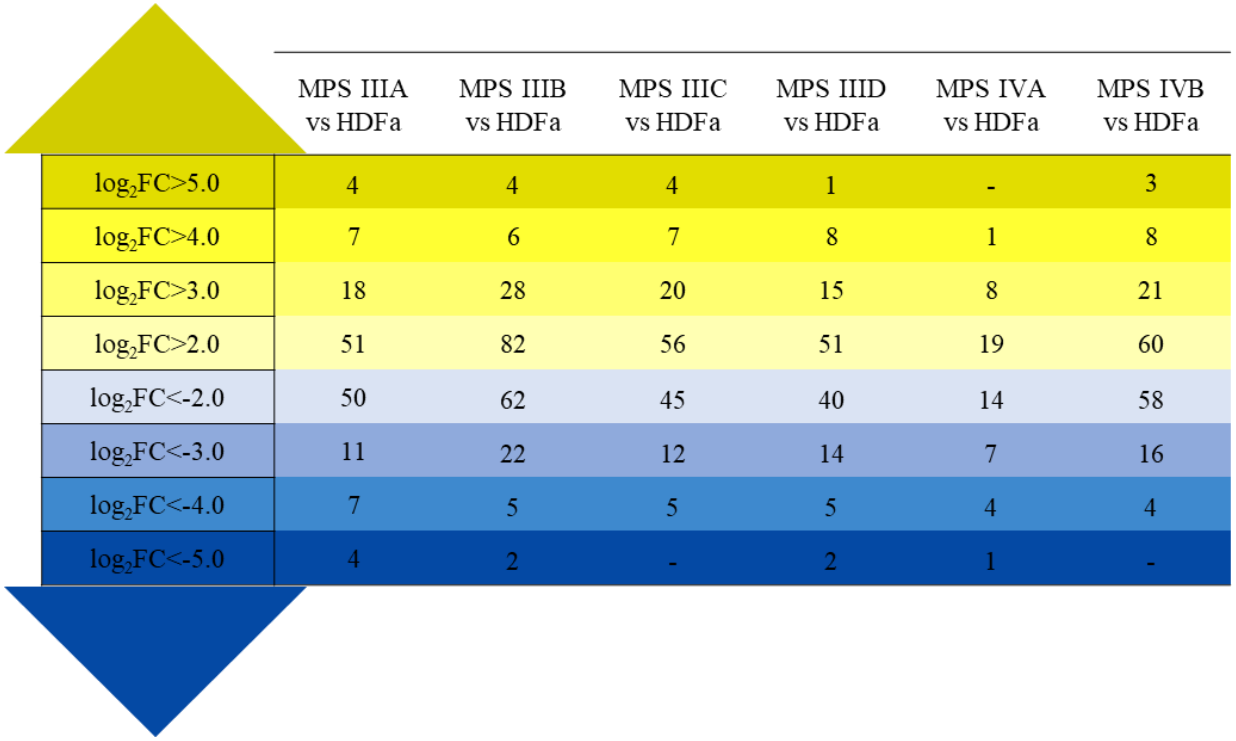


Figure S2. Number of transcripts with altered expression between MPS III (A) and IV (B) subtypes and HDFa (multiple and separately).



	MPS IIIA vs HDFa	MPS IIIB vs HDFa	MPS IIIC vs HDFa	MPS IIID vs HDFa	MPS IVA vs HDFa	MPS IVB vs HDFa
$log_2FC>5.0$	4	4	4	1	-	3
$log_2FC>4.0$	7	6	7	8	1	8
$log_2FC>3.0$	18	28	20	15	8	21
$log_2FC>2.0$	51	82	56	51	19	60
$log_2FC<-2.0$	50	62	45	40	14	58
$log_2FC<-3.0$	11	22	12	14	7	16
$log_2FC<-4.0$	7	5	5	5	4	4
$log_2FC<-5.0$	4	2	-	2	1	-

Figure S3. Number of transcripts with altered expression at especially high fold change between all MPS III/IV subtypes and HDFa.

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polegał na:

1. przeprowadzeniu doświadczeń
2. analizie i interpretacji wyników
3. przygotowaniu figur i tabeli
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polegał na:

1. recenzji wewnętrznej manuskryptu
2. udziale w interpretacji wyników
3. asyście w przygotowaniu odpowiedzi na uwagi recenzentów
4. pozyskaniu finansowania w postaci otrzymania grantu

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polegał na:

1. zaproponowaniu tematyki pracy
2. przygotowaniu pierwotnej wersji manuskryptu
3. interpretacji i analizie wyników
4. rewizji manuskryptu po uwagach recenzentów
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Shared Gene Expression Dysregulation Across Subtypes of Sanfilippo and Morquio Diseases: The Role of PFN1 in Regulating Glycosaminoglycan Levels

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Original Research

Shared Gene Expression Dysregulation Across Subtypes of Sanfilippo and Morquio Diseases: The Role of PFN1 in Regulating Glycosaminoglycan Levels

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Abstract

Background: Mucopolysaccharidosis (MPS) is a class of hereditary metabolic diseases that demonstrate itself by accumulating incompletely degraded glycosaminoglycans (GAGs). MPS are classified according to the kind(s) of stored GAG(s) and specific genetic/enzymatic defects. Despite the accumulation of the same type of GAG, two MPS diseases, Sanfilippo (MPS III) and Morquio (MPS IV), are further distinguished into subclasses based on different enzymes that are deficient. Although genetic defects in MPS are known, molecular mechanisms of particular MPS types are still incomplete. This work aimed to investigate gene expression patterns in MPS III and MPS IV subtypes to identify dysregulated genes that could indicate unidentified molecular mechanisms of the diseases. **Methods**: Transcriptomic analyses were conducted to assess gene expression patterns in MPS and control cells. Western blotting and immunohistochemistry determined selected protein levels (products of the most significantly dysregulated genes). Effects of decreased levels of gene expression were investigated using small interferring RNA (siRNA)-mediated gene silencing. **Results**: Transcriptomic analyses indicated 45 commonly dysregulated genes among all MPS III subtypes and as many as 150 commonly dysregulated genes among both MPS IV subtypes. A few genes revealed particularly high levels of dysregulation, including *PFN1*, *MFAP5*, and *MMP12*. Intriguingly, elevated levels of profilin-1 (product of the *PFN1* gene) could be reduced by decreasing GAG levels in genistein-treated MPS III and MPS IV cells, while silencing of *PFN1* caused a significant decrease in GAG accumulation in these cells, indicating an interdependent correlation between profilin-1 and GAG levels. **Conclusions**: A plethora of commonly dysregulated genes were identified in MPS subtypes III and IV. Some of these genes, like *PFN1*, *MFAP5*, and *MMP12*, revealed highly pronounced changes in expression relative to control cells. An interdependent correlation between GAG levels and the expression of the *PFN1* gene was identified. Thus, *PFN1* could be suggested as a potential new therapeutic target for MPS III and IV.

Keywords: Mucopolysaccharidoses; profilin-1; *PFN1*; glycosaminoglycans; Sanfilippo disease; Morquio disease

1. Introduction

Mucopolysaccharidoses (MPS) are a group of rare, genetically inherited lysosomal storage disorders caused by a deficiency of enzymes responsible for the degradation of glycosaminoglycans (GAGs). The inability to break down these complex molecules leads to their accumulation in the lysosomes, resulting in progressive damage to cells, tissues, and organs. There are several types of MPS, each differing clinically and biochemically depending on the specific enzyme that is deficient (MPS types I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, IX, X) [1,2]. Some sources also report the existence of MPS IIIE; however, its classification is still uncertain. It was demonstrated to occur in artificially constructed mouse models but not yet in humans [3]. Among all types of MPS, two have their subtypes: MPS III (Sanfilippo syndrome) and MPS IV (Morquio syndrome). These subtypes of MPS III and IV types are characterized by the accumulation of the same GAG(s) (heparan sulfate (HS) in MPS III, and keratan sulfate (KS) in MPS IV), but they differ in the dysfunction of various lysosomal enzymes [1,2] (Table 1).

In Sanfilippo syndrome, the accumulation of HS leads to severe neurological symptoms, including developmental delay, regression of acquired skills, behavioral problems, and eventually severe mental impairment. Unlike other types of MPS, MPS III has less pronounced somatic symptoms, which often delay the diagnosis of the disease [4]. MPS III is divided into four subtypes: IIIA, IIIB, IIIC, and IIID. MPS IIIA often has the fastest and most aggressive course. Patients with this subtype exhibit early neurological symptoms that progress rapidly. MPS IIIB has a somewhat milder course than MPS IIIA, with a slower progression of neurological symptoms. MPS IIIC and IIID are rarer and usually have a less aggressive course than MPS IIIA and IIIB, with slower progression of neurological symptoms.

Table 1. Characteristics of classical/conventional types of MPS.

MPS type	Defective gene	Deficient enzyme	Stored GAG[a]
MPS I	*IDUA*	α-L-iduronidase	HS, DS
MPS II	*IDS*	Iduronidase-2-sulfatase	HS, DS
MPS IIIA	*SGSH*	Heparan-N-sulfatase	HS
MPS IIIB	*NAGLU*	α-Nacetylglucosaminidase	
MPS IIIC	*HGSNAT*	Heparan α-Glucosaminide N-acetyltransferase	
MPS IIID	*GNS*	N-Acetylglucosamine-6-sulfatase	
MPS IVA	*GLANS*	N-Acetylglucosamine-6-sulfate sulfatase	C6S, KS
MPS IVB	*GLB1*	β-Galactosidase	KS
MPS VI	*ARSB*	N-acetylglucosamine-4-sulfatase (arylsulfatase B)	DS, C4S
MPS VII	*GUSB*	β-Glucuronidase	HS, DS, C4S, C6S
MPS IX	*HYAL1*	Hyaluronidase	Hyaluronan
MPS X	*ARSK*	Arylsulfatase K	DS

MPS, Mucopolysaccharidoses. [a]Glycosaminoglycan (GAG) names: C4S, chondroitin 4,6-sulfate; C6S, chondroitin 6-sulfate; DS, dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate.

However, all subtypes of MPS III predominantly exhibit neurological symptoms over somatic ones [5].

Subtypes also characterize Morquio syndrome (MPS IV): MPS IVA and IVB. In MPS IVA, the storage of KS leads to severe somatic symptoms such as skeletal dysplasia, short stature, spinal deformities, and breathing problems. MPS IVB has similar symptoms but may be milder than MPS IVA. Overall, both subtypes of MPS IV are characterized by significant somatic dysfunctions, particularly related to the musculoskeletal system, without neurological symptoms or to a minimal extent [2].

Despite having precise knowledge about the etiology of MPS, our understanding of the pathomechanisms is still not insufficient to develop therapies for all types of this group of diseases. The enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT) are available for only some types (ERT for types I, II, IVA, VI, VII; HSCT for types I, II, IVA, VI, VII), and allow for only alleviation of symptoms having significant biological, medical, and economic limitations [5,6]. The main limitation of ERT is the inefficiency of crossing the blood-brain barrier by the recombinant enzyme. This is a significant problem for MPS disorders primarily characterized by central nervous system abnormalities. Moreover, ERT proves to be unsatisfactory in cases of pathological changes in the skeletal system. Despite showing slightly better results, HSCT is also an insufficiently effective form of the therapy, and any efficacy can be observed only when administered early in life (before the age of 2–3 years while MPS diagnosis is often made considerably later) [5]. No treatment methods have been registered so far for types IIIA, IIIB, IIIC, IIID, IVB, IX, and X [4]. Gene therapy aimed at delivering a healthy copy of the gene responsible for producing the missing enzyme directly into the patient's cells is currently under intensive research, yet it is still unavailable in clinical practice [7]. Symptomatic treatment is focused on alleviating neurological and behavioral symptoms, utilizing antiepileptic drugs, antipsychotic medications, behavioral therapies, and rehabilitation. Patients with MPS IV often require surgical interventions due to severe skeletal problems. These interventions may include spinal corrections, joint replacement surgeries, and various orthopedic operations [8]. Although the underlying cause of the disease has been known for many years, the molecular mechanisms of this group of diseases remain unclear.

In this light, finding an effective treatment for MPS III and MPS IV is challenging due to the difficulty of delivering drugs across the blood-brain barrier and into bone tissues. This makes treating the neurological symptoms in MPS III and the somatic, movement-related symptoms in MPS IV particularly difficult. Additionally, the wide variability in clinical presentation among patients with MPS III and MPS IV complicates the development of therapies that would be effective for all individuals. Each subtype of the disease may require a specific therapeutic approach.

The possibility of the influence of other factors such as gene expression dysregulation, organelle dysfunctions, or secondary accumulation of other substances, on the development and progression of the disease is being considered [9]. Transcriptomic study, which analyze global gene expression patterns, hold immense potential in understanding the biology of MPS. Such study can answer critical questions about these diseases' pathogenesis by identifying common and specific changes in gene expression levels across all subtypes of MPS III and MPS IV. Understanding these changes may reveal previously unknown molecular mechanisms responsible for neurological and somatic symptoms and help identify potential therapeutic targets [10].

Transcriptomic research conducted so far with the MPS models have indicated disruptions in the expression of over 800 genes, related, for example, to behavior, proteasome, receptors and chaperons [10]. Despite the significant importance of such study, relatively few reports detail

the similarities and differences in the progression of individual MPS III and MPS IV subtypes at the transcriptomic level. Conducting a comprehensive gene expression analysis across all MPS III and MPS IV subtypes could provide new insights into common molecular pathways involved in these diseases and specific mechanisms leading to clinical differences between the subtypes [10]. The only transcriptomic study that has focused on comparing gene expression between MPS III and IV subtypes have indicated significant differences in the expression of genes mainly involved in maintaining the proper structure of connective tissue (*COL4A1*, *COL4A2*, *COMP*) and the structure of ribosomes (*RPL10*, *RPL23*, *RPLP2*) [11].

A recent study indicated that both intact and partially degraded GAGs can interact with various proteins. Such interactions have already been observed with oxytocin receptor (OXTR) and G-protein coupled estrogen receptor 1 (GPER1) receptors, which interacted with high concentrations of HS [12]. Another work in this field suggested that aberrantly accumulated HS in patients with MPS closely binds to cathepsin V and inhibits its elastolytic activity. An HS antagonist was able to restore this activity [13]. Therefore, one might suggest that these findings encourage the exploration of new approaches to treat MPS and related disorders based on the molecular interactions between GAGs and proteins.

In the case of MPS III and IV, no studies have yet identified genes with expression changes occurring similarly across all disease subtypes. Identifying such genes could help determine whether GAGs regulate changes in gene expression or if the products of these genes interact with GAGs at varying levels of degradation, contributing to the development of specific symptoms. Therefore, this study aimed to identify genes and their products that exhibit similar expression level disturbances across all MPS III and IV subtypes compared to control cells. The conclusions from these studies could not only deepen our understanding of the pathogenesis of MPS III and MPS IV but also contribute to the development of more precise diagnostic and therapeutic strategies. In the long term, such research may also provide valuable insights into potential treatment targets for other neurodegenerative and somatic diseases that share common pathogenic pathways with MPS III and MPS IV.

2. Materials and Methods

2.1 Cell Lines, Cell Culture and Transcriptomic Analysis

All human dermal fibroblast cell lines used in this study were purchased from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ, USA) and are summarised in Table 2. The HDFa fibroblast cell line was used as a control. Selected cell lines originated from patients exhibiting typical MPS IIIA, IIIB, IIIC, IIID, IVA and IVB severity, age of onset, and life expectancy for each MPS subtype. All patients were reported to exhibit severe phenotypes consistent with the typical severity spectrum observed across all tested MPS types and subtypes. Cell lines used in this work were validated (by Short Tandem Repeat profiling) and tested for the absence of mycoplasma (by microscopic analyses and Polymerase Chain Reaction (PCR), giving negative results for mycoplasma-specific signals).

Fibroblasts were routinely cultured in 6 cm diameter dishes, followed by 15 cm diameter round cell culture dishes, in Gibbco Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA; #A5256701) and Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA; # 15240062) containing penicillin, streptomycin and Amphotericin B at 37 °C with 5% CO_2.

2.1.1 Compounds Used for Cell Treatment

The active compound, genistein (4′,5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4 H–1-benzopyran-4-one), 99% purity, purchased from the Pharmaceutical Research Institute (Warsaw, Poland; #446-72-0), was prepared as a 50 mM stock solution in dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA; #036480.K2) and stored at –20 °C prior experimental use.

2.1.2 Transcriptomic Studies

Transcriptomic analysis was performed as described earlier [10]. Briefly, fibroblasts (5×10^5 cells) were seeded on 10 cm diameter plates, and adherence was allowed overnight. Cells were washed with phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA USA; #10010023), trypsinized (Thermo Fisher Scientific, Waltham, MA USA; # 27250018), and re-washed with PBS before freezing. The following day, cells were lysed and homogenized using a QIA shredder (Qiagen, Hilden, Germany), according to the manufacturer's description. Further, RNA was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany). The extracted nucleic acids were treated with Turbo DNase (Life Technologies, Carlsbad, CA, USA) for 30 min at 37 °C to remove possible DNA contamination. Before further transcriptomic analysis, the RNA extracts were analyzed using a dedicated Nano Chips RNA kit (Agilent Technologies, Santa Clara, CA, USA) on the Agilent 2100 Bioanalyzer System to confirm the samples' quality, integrity and concentration. RNA was isolated from each cell line in four independent replicates.

Further, the Illumina TruSeq Stranded mRNA Library Prep Kit was used to prepare the mRNA libraries according to the manufacturer's description. With reverse transcription, complementary DNA (cDNA) libraries were created to reflect the gene expression profile of the RNA samples. The sequencing was performed on a high-throughput sequencer HiSeq4000 platform (Illumina, San Diego, CA,

Table 2. Cell lines used in this work.

MPS type	Defective gene	Defective enzyme	Storage GAG(s)*	Mutation type	Catalog number of the cell line**
MPS IIIA	*SGSH*	N-sulfoglucosamine sulfhydrolase	HS	p.Glu447Lys/p.Arg245His	GM00879
MPS IIIB	*NAGLU*	α-N-acetylglucosaminidase		p.Arg626Ter/p.Arg626Ter	GM00156
MPS IIIC	*HGSNAT*	Acetyl-CoA: α-glycosaminide acetyltransferas		p.Gly262Arg/pArg509Asp	GM05157
MPS IIID	*GNS*	N-acetylglucosamine 6-sulfatase		p.Arg355Ter/p.Arg355Ter	GM05093
MPS IVA	*GLANS*	N-Acetyloglucosaminase-6-sulfate sulfatase	KS, CS	p.Arg386Cys/p.Phe285Ter	GM00593
MPS IVB	*GLB1*	β-Galactosidase		p.Trp273Leu/p.Trp509Cys	GM03251
HDFa	None	None	None	None	GM00121

* Abbreviations: CS, chondroitin sulfate; HS, heparan sulfate; KS, keratan sulfate; N/A, not applicable.

** Catalog numbers according to the Coriell Institute description.

USA) with PE150 (150 bp fragments sequenced from both ends). At least 40 million raw reads were obtained, resulting in raw data of 12 GB per sample.

The obtained data were analyzed in reference to the widely used assembly GRCh38 of the human genome deposited in the Ensemble database (file: Homo_sapiens.GRCh38.94.gtf, https://www.ensembl.org/index.html; December 5, 2021). The quality of the data was evaluated with FastQC (version v0.11.7; Babraham Bioinformatics, Cambridge, UK). The Hisat2 (version 2.1.0; UT Southwestern, Dallas, TX, USA) software was used to align and annotate the readings based on the GRCh38. Transcript levels were quantified with Cuffquant (version 2.2.1, Trapnell Lab, Seattle, WA, USA) and Cuffmerge (version 2.2.1, Trapnell Lab, Seattle, WA, USA). Cuffquant allowed calculations of the transcript expression levels by estimating the abundance of reads mapped to each transcript. Cuffmerge was run to normalize expression values using the FPKM algorithm (fragments per kilobase of transcripts per million mapped reads method).

Statistical analysis was performed using the R software (version 3.4.3; The R Foundation, Vienna, Austria). Statistical significance between two normally distributed groups, with $\log_2(1 + x)$ values, was determined using one-way Analysis of Variance (ANOVA) and post hoc Student's *t*-test with Bonferroni correction. The false discovery rate (FDR) was calculated using the Benjamini-Hochberg method. Raw results were deposited in the NCBI Sequence Read Archive (SRA) (accession no. PRJNA562649).

2.2 Immunoblotting

The MPS and healthy HDFa fibroblasts for immunoblotting were seeded (6×10^5 cells) on 10 cm diameter dishes and allowed to adhere overnight. The cells were then subjected to 50 µM genistein (Pharmaceutical Research Institute, Warsaw, Poland; #446-72-0) treatment for 24 h. Two control dishes were grown for each tested cell line, untreated and treated with 0.1% DMSO (genistein solvent). After treatment, cells were washed with PBS, trypsinized, and washed again with PBS before freezing. Cells were then lysed for 30 min on ice with brief vortexing every 10 min in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA 1% Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA; #A16046.AE) supplemented with cOmplete™ - an EDTA-free protease inhibitor cocktail (Roche, Vienna, Austria; #04693132001). Cell lysates were centrifuged at 3000 $\times$g for 10 min, and the supernatant for each tested cell line was collected. Protein separation and immunological detection were performed using a capillary electrophoresis-based WES system (Bio-Techne, Minneapolis, MN, USA). This instrument provides rapid (approximately 2–3 h) electrophoretic separation of proteins, followed by immunological detection of the desired protein in a single capillary, without using classical SDS-PAGE, transfer of proteins to a membrane, and incubating such a membrane with antibodies to detect a protein-specific signal. It provides uniformity of protein bands and high reproducibility of the results. Proteins on the WES system were separated with a 12–230 kDa Separation Module followed by immunological detection with either the Anti-Mouse or Anti-Rabbit Detection Module, according to the manufacturer's instructions. The following primary antibodies (all from Thermo Fisher Scientific, Waltham, MA USA) were used for detection in a 1:50 ratio: anti-profilin 1 (PFN1) (#PA5-17444), anti-Microfibril Associated Protein 5 (MFAP5) (#PA5-115573), and anti-Matrix metalloproteinase-12 (MMP12) (#PA5-13181). In addition, total protein levels were determined using the Total Protein Detection Module for Chemiluminescence, which served as the loading control. The band intensity was analyzed with an instrument's Simple Western™ provided software, Compass for Simple Western (Bio-Techne, Minneapolis, MN, USA).

2.3 Fluorescence Microscopy

The MPS and healthy HDFa fibroblasts were seeded (4×10^4 cells) on coverslips in 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA; #140675) for fluorescence microscopy (Leica DM4000B fluorescence microscope; Leica Microsystems, Mannheim, Germany). The

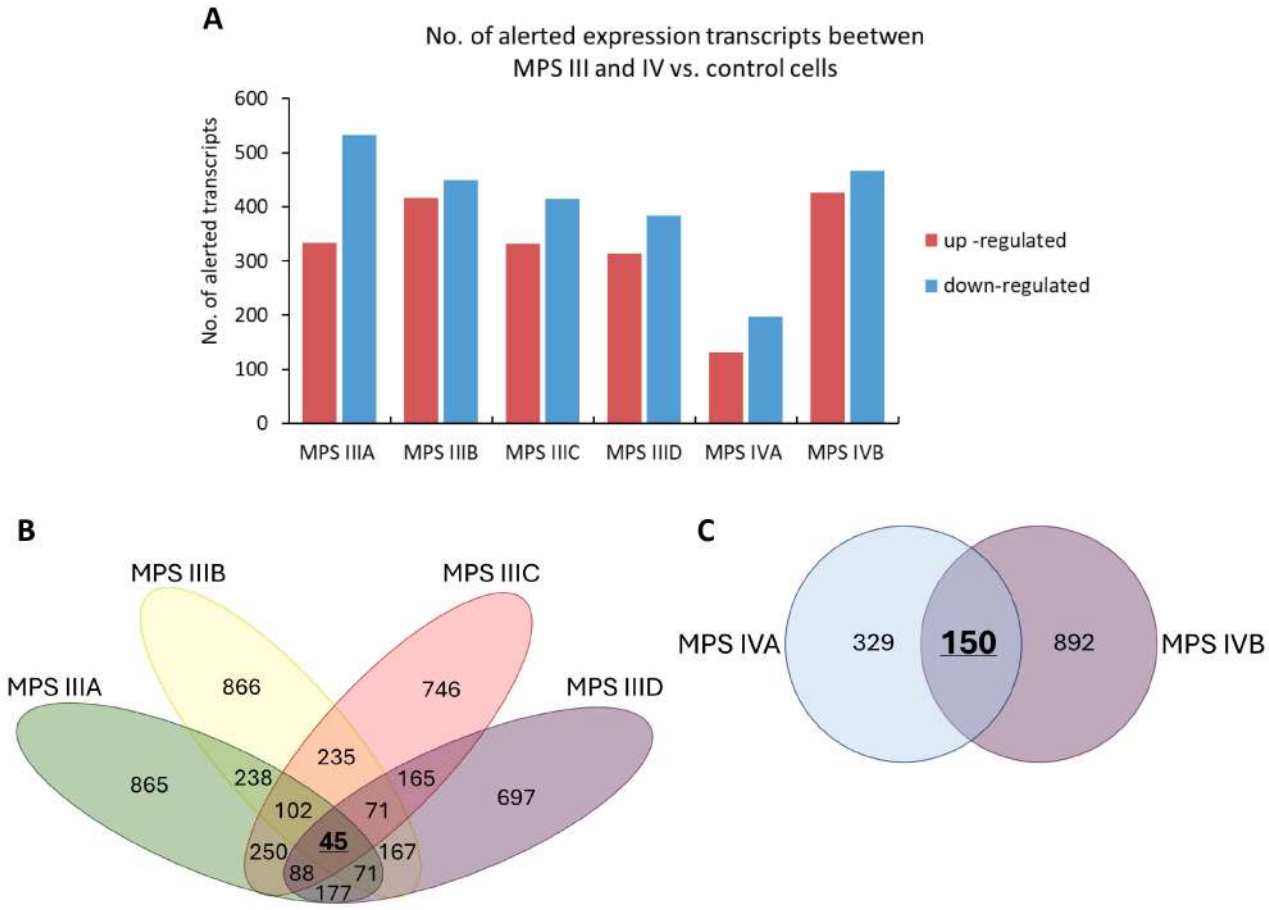
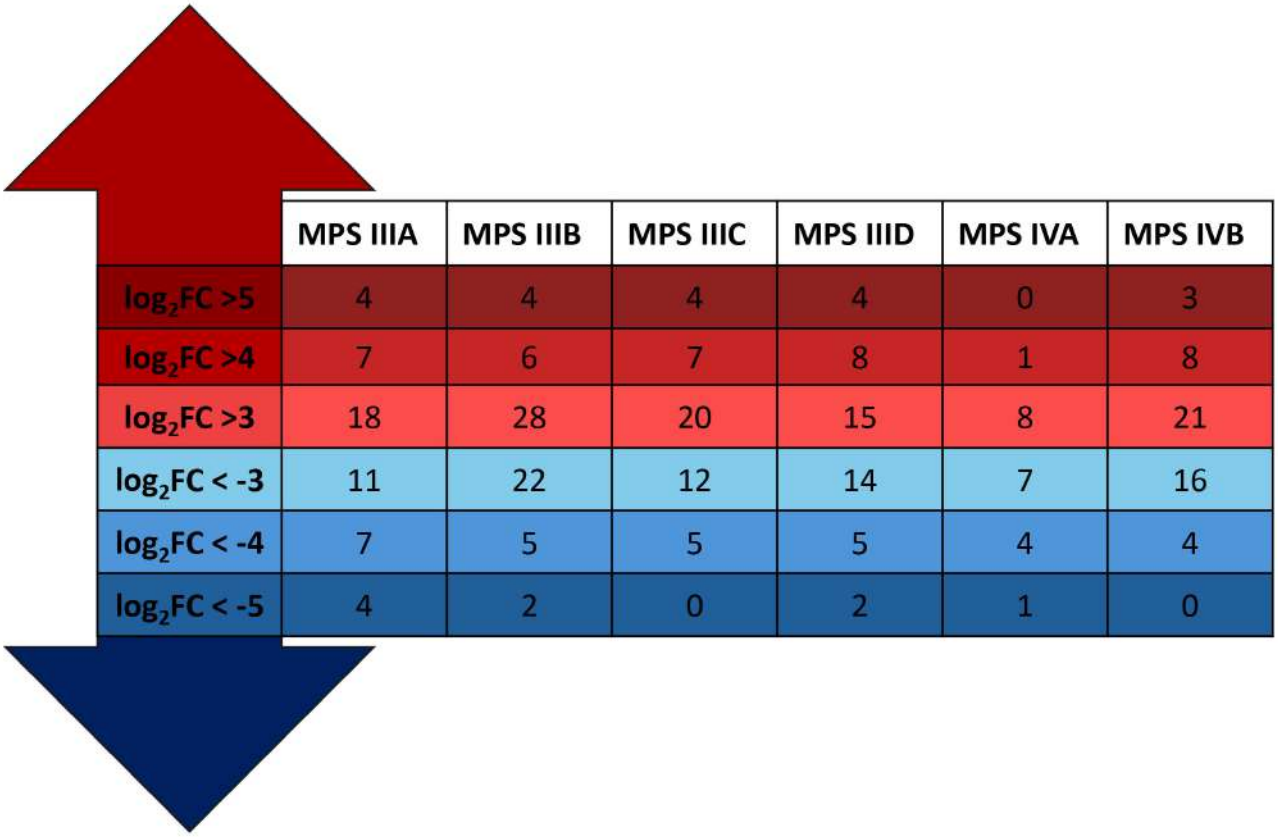


Fig. 1. Alterations in gene expression in MPS III and IV fibroblasts. The number of genes with altered expression in MPS III and IV cells compared to control cells (taken from healthy individuals) (A), and the number of genes with similarly altered expression compared to control cells in all subtypes of MPS III (B) and MPS IV (C).

genistein treatment was performed the same as for the immunoblotting: the fibroblasts were exposed to 50 µM genistein for 24 h, and two control coverslips were prepared for each tested cell line, untreated and treated with 0.1% DMSO. After the genistein treatment, the cells were prepared for microscopy by washing in PBS, followed by fixation with 2% paraformaldehyde in PBS (Thermo Fisher Scientific, Waltham, MA, USA; #J61899.AK) and washing with 0.1% Triton X-100 in PBS. The coverslips were further blocked with 5% BSA (Thermo Fisher Scientific, Waltham, MA, USA; #B14) and 1.5% glycine (Thermo Fisher Scientific, Waltham, MA, USA; #A13816.36) in PBS for 1 h. The fibroblasts were exposed overnight at 4 °C to primary antibodies in PBS: anti-PFN1 (1:1000), anti-MFAP5 (1:250) and anti-MMP12 (1:25), followed by three washes with PBS and 2-h incubation at 4 °C in darkness with secondary anti-rabbit, Alexa Fluor™ 555-conjugated antibody (Thermo Fisher Scientific, Waltham, MA, USA; #A-21428 (1:4000)). The cells were washed five times in PBS. The coverslips were mounted onto glass slides using a ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA; #P36934) with DNA stain DAPI (2-[4-(aminoiminomethyl)phenyl]-1*H*-indole-6-carboximidamide hydrochloride) (Merck, Rahway, NJ, USA; #D9542) mounting medium. The following day, fibroblast fluorescence was observed under DMI4000B inverted fluorescence microscopy (Leica Microsystems, Mannheim, Germany) using filters appropriate for DAPI and Alexa Fluor™ 555.

2.4 Silencing of the Expression of the PFN1 Gene

For *PFN1* silencing, the Profilin 1 (PFN1) Human small interferring RNA (siRNA) Oligo Duplex kit (OriGene, Newark, DE, USA; #SR303470) was used according to the manufacturer's instructions. Subsequent steps followed the methods described for immunofluorescence and immunoblotting.



	MPS IIIA	MPS IIIB	MPS IIIC	MPS IIID	MPS IVA	MPS IVB
$log_2FC > 5$	4	4	4	4	0	3
$log_2FC > 4$	7	6	7	8	1	8
$log_2FC > 3$	18	28	20	15	8	21
$log_2FC < -3$	11	22	12	14	7	16
$log_2FC < -4$	7	5	5	5	4	4
$log_2FC < -5$	4	2	0	2	1	0

Fig. 2. Particularly high changes in gene expression levels in MPS III and IV fibroblasts. Number of transcripts with altered expression (at false discovery rate (FDR) <0.1; $p < 0.1$ in one-way ANOVA and post hoc Student's *t*-test with Bonferroni correction) depending on the level of fold-change (FC) value in different subtypes of MPS III and IV relative to control cells.

3. Results

An analysis of gene expression dysregulation common to all subtypes of MPS types, where the same GAGs accumulate, has never been conducted before. Examples of such MPS types include MPS III and MPS IV, where the accumulating GAGs are HS and KS, respectively. Given that GAGs can influence the expression of many genes and interact with various proteins, we deemed it worthwhile to conduct such analysis to identify gene expression changes shared across all subtypes of a particular disease entity.

The results of the transcriptomic analyses indicated dysregulations in the expression of numerous genes in MPS III and IV cells compared to control cells (from healthy individuals). The number of altered transcripts exceeded 800 for MPS IIIA, IIIB, and IVB, and over 300 for MPS IVA (Fig. 1A). By searching for transcripts with similar expression changes across all disease subtypes, 45 commonly regulated genes were identified for all MPS III subtypes (Fig. 1B) and as many as 150 commonly regulated genes for both MPS IV subtypes (Fig. 1C).

In the study's next step, we assessed the number of commonly regulated transcripts across all types/subtypes of MPS III and IV relative to the logarithmic fold change values (log_2FC). We observed that the vast majority of strongly altered transcripts were up-regulated. The number of transcripts with an 8-fold increase or decrease in expression ($log_2FC > 3$ or $log_2FC < -3$) was over 25 (Fig. 2). Table 3 presents the list of these transcripts, along with their fold change values compared to control cells.

Two up-regulated transcripts exhibited high levels of expression changes across all subtypes of the diseases studied. These are the *PFN1* gene, which encodes profilin, a protein involved in actin binding and cytoskeleton dynamics, and the *MFAP5* gene, which encodes microfibril-associated protein 5, a microfibril-associated glycoprotein that is a component of the extracellular matrix microfibrils. The down-regulated *MMP12* gene, which encodes matrix metalloproteinase 12, also showed high fold change values in expression levels in MPS III cells compared to the control (Table 3).

The levels of the proteins (final gene products) encoded by *PFN1*, *MFAP5*, and *MMP12* genes were determined using antibodies specific to these proteins. The results of immunodetection by western blotting indicated a 3- to 5-fold increase in PFN1 protein levels and a 3- to 9-fold increase in MFAP5 protein levels in MPS III and IV cells compared to control cells, as well as above 5-fold decrease

Table 3. Specific transcripts with particularly high changes in levels in MPS III and IV fibroblasts.*

Transcript	Transcript ID	log_2FC value of selected transcripts between different types/subtypes of MPS III and IV vs control					
		IIIA	IIIB	IIIC	IIID	IVA	IVB
		up-regulated transcripts					
AC004556.1	ENST00000612848	X	-	-	-	3.55	
ADAMTSL1	ENST00000380548	-	3.64	-	-	-	-
CAPG	ENST00000263867	4.71		4.27		4.17	4.14
CD9	ENST00000009180	3.75	4.09	3.9	3.96	X	4.86
CLU	ENST00000521770	X	X	-	X	X	3.84
COL4A1	ENST00000375820	-	3.9	-	X	X	-
COL8A2	ENST00000397799	3.88	X	4.13	4.36	-	3.6
COMP	ENST00000222271	X	X	-	4.75	X	-
CRIP1	ENST00000392531	4.02	X	X	X	X	-
CRLF1	ENST00000392386	X	-	X	4.38	X	X
ENPP1	ENST00000647893	X	X	3.57	X	X	X
FAM167A	ENST00000284486	-	-	-	-	-	3.77
IGFBP5	ENST00000233813	X	X	3.97	4.11	X	X
KRT19	ENST00000361566	X	-	X	X	X	4.19
MFAP5	ENST00000359478	6.14	4.99	4.51	3.75	3.93	4.1
MFGE8	ENST00000560937	5.05	-	X	X	X	X
MFGE8	ENST00000268150	3.9	-	-	X	X	-
MFGE8	ENST00000558018	3.88	-	X	X	X	-
MFGE8	ENST00000268151	3.7	-	X	X	X	-
MKNK2	ENST00000589534	X	X	3.69	X	X	X
NME2	ENST00000503064	X	X	5.14	X	X	X
NOTCH3	ENST00000263388	-	3.66	-	-	X	-
OXTR	ENST00000316793	3.76	5.2	6.43	4.2	X	6.07
PFN1	ENST00000572383	3.51	3.64	3.73	3.53	3.62	3.67
POSTN	ENST00000379742	5.27	7.26	5.42	5.01	X	5.68
POSTN	ENST00000541179	5.14	7.75	5.63	4.98	X	5.83
POSTN	ENST00000379743	4.68	5.63	3.67	4.16	X	4.16
RPLP2	ENST00000530797	X	3.81	X	X	X	X
TFPI2	ENST00000649730	-	3.6	X	-	X	-
UCHL1	ENST00000381760	X	3.64	X	-	X	X
		down-regulated transcripts					
APOE	ENST00000252486	–6.11	X	X	X	X	X
CAV1	ENST00000405348	X	X	X	X	X	–4.74
CXCL8	ENST00000307407	–5.08	X	X	X	X	X
EMP1	ENST00000537612	-	–3.67	–4.71	-	–4.09	–3.74
EPDR1	ENST00000199448	X	X	-	-	X	–4.69
ERH	ENST00000216520	X	X	X	X	–3.689	X
GNS	ENST00000258145	X	X	X	–5.15	X	X
HOXB6	ENST00000225648	X	X	–3.96	X	X	-
IGFBP3	ENST00000381086	X	X	X	X	–5.17	X
LY6K	ENST00000292430	-	-	–3.61	–5.34	X	–4.28
LY6K	ENST00000519387	-	X	-	–4.79	X	–3.95
LY6K	ENST00000518841	X	X	-	–3.59	X	X
MME	ENST00000615825	X	X	X	–3.62	X	X
MMP12	ENST00000571244	–4.2	–5.3	–4.49	-	X	-
PKM	ENST00000563275	X	X	X	-	–3.57	X
PTGDS	ENST00000371625	–6.16	–4.78	–4.28	X	X	–4
RARRES2	ENST00000223271	–4.07	–4.91	X	X	X	X
RND3	ENST00000375734	-	–3.7	X	X	-	X

Table 3. Continued.

Transcript	Transcript ID	log_2FC value of selected transcripts between different types/subtypes of MPS III and IV vs control					
		IIIA	IIIB	IIIC	IIID	IVA	IVB
RPL10	ENST00000406022	X	X	X	–4.61	X	X
RPL23	ENST00000245857	X	X	–4.23	–4.16	–4.09	–3.72
SERPINB2	ENST00000299502	-	X	X	X	X	–3.56
SNX3	ENST00000426155	X	X	–4.13	X	-	X
SPON2	ENST00000290902	–4.15	–3.77	-	X	X	-
THBS1	ENST00000559746	X	X	X	X	–4.07	X
TRPV2	ENST00000338560	-	–3.86	-	X	X	-
WISP2	ENST00000190983	–6.71	–7.23	X	X	X	-
WISP2	ENST00000372868	-	–4.03	X	-	X	X

* Transcripts with significantly changed expression (at FDR <0.1; $p < 0.1$ in one-way ANOVA and post hoc Student's *t*-test with Bonferroni correction) and with particularly high values of the fold change (log_2FC >3.5 or <–3.5) in specific MPS III and IV subtypes relative to control cells are shown. Abbreviations: -, value below the range log_2FC >3.5 or <–3.5; X, no significant change.

in MMP12 protein levels in MPS III cells compared to controls (depending on the type/subtype of the disease) (Fig. 3, **Supplementary material**). Fluorescence microscopy detection also showed an increase in fluorescence intensity, above 4-fold for PFN1 (Fig. 4) and 3- to 5-fold for MFAP5 (Fig. 5) in MPS III and IV cells compared to control, as well as a 5-fold decrease in fluorescence intensity for MMP12 (only in MPS III cells) (Fig. 6).

To address whether the changes in these proteins' levels depend on high GAG concentrations, we reduced GAG levels using a known EGFR inhibitor, genistein. Genistein binds to EGFR, inhibiting its autophosphorylation, which halts the downstream kinase cascade, ultimately suppressing the transcription of genes coding for enzymes responsible for GAG synthesis [14,15]. Interestingly, the incubation of MPS cells in the presence of genistein decreased GAG levels. Under these conditions, a decrease in PFN1 protein levels in MPS cell lines below the level of control cells was also observed, as demonstrated by western blotting. However, there was no observed effect of GAG reduction by genistein on MFAP5 levels. The reduced level of MMP12 protein observed in MPS III cells increased after GAG reduction, but did not reach the control level (Fig. 7, **Supplementary material**). Fluorescence microscopy analysis confirmed these results, showing a decrease in PFN1 fluorescence intensity (Fig. 8), no change in MFAP5 fluorescence intensity (Fig. 9), and a slight increase in MMP12 fluorescence intensity in the cell lines where it was reduced (Fig. 10).

Given that our results indicated that profilin levels strongly depend on GAG levels in the cell, we decided to silence *PFN1* expression using siRNA to investigate this relationship further and determine whether it affects GAG levels in MPS cells. Two representative cell lines, MPS IIIC and IVB, and a control line were selected for the experiments. Treatment with siRNA significantly reduced profilin levels, as measured by western blotting and fluorescence microscopy, indicating an efficient *PFN1* expression silencing. Intriguingly, the silencing of *PFN1* expression caused a significant decrease in GAG levels in MPS IIIC and MPS IVB cells (Fig. 11, **Supplementary material**).

4. Discussion

Treating rare diseases has long been a significant challenge for society, not only because of complicated pathomechanisms, but also due to the high costs of clinical trials and drug production and the small number of patients who would potentially benefit from the therapies that have been developed. MPS, as a group of rare genetic metabolic disorders caused by the deficiency of enzymes responsible for the breakdown of GAGs, is not an exception to this rule. The incidence of MPS is estimated at 1 in 25,000 live births. The number of patients varies by region, with the highest recorded numbers in Japan (467), Germany (474), Poland (392), Portugal (353), the Netherlands (331), and Brazil (1652) [16]. Unfortunately, the therapies used in treating MPS, such as ERT and HSCT, while improving certain symptoms, are not effective in all cases and do not fully prevent disease progression, particularly in the context of neurodegeneration or bone and joint deformities [4–6]. Therefore, there is an ongoing urgent need to investigate the precise molecular mechanisms leading to symptom development and, consequently, to develop new therapeutic strategies to treat the various types of MPS more effectively.

The pathogenic mechanisms of the various subtypes of MPS III (A, B, C, and D) and MPS IV (A and B) remain a mystery. These subtypes involve the accumulation of the same GAGs, yet they differ in the occurrence and severity of specific symptoms. Some transcriptomic analyses have indicated disruptions in numerous cellular processes that differentiate MPS III and IV subtypes at the molecular level, particularly concerning ribosomal function and connective tissue structure [11]. However, MPS III and IV subtypes also exhibit many similarities, with neurodegeneration prominent in MPS III and skeletal system disorders

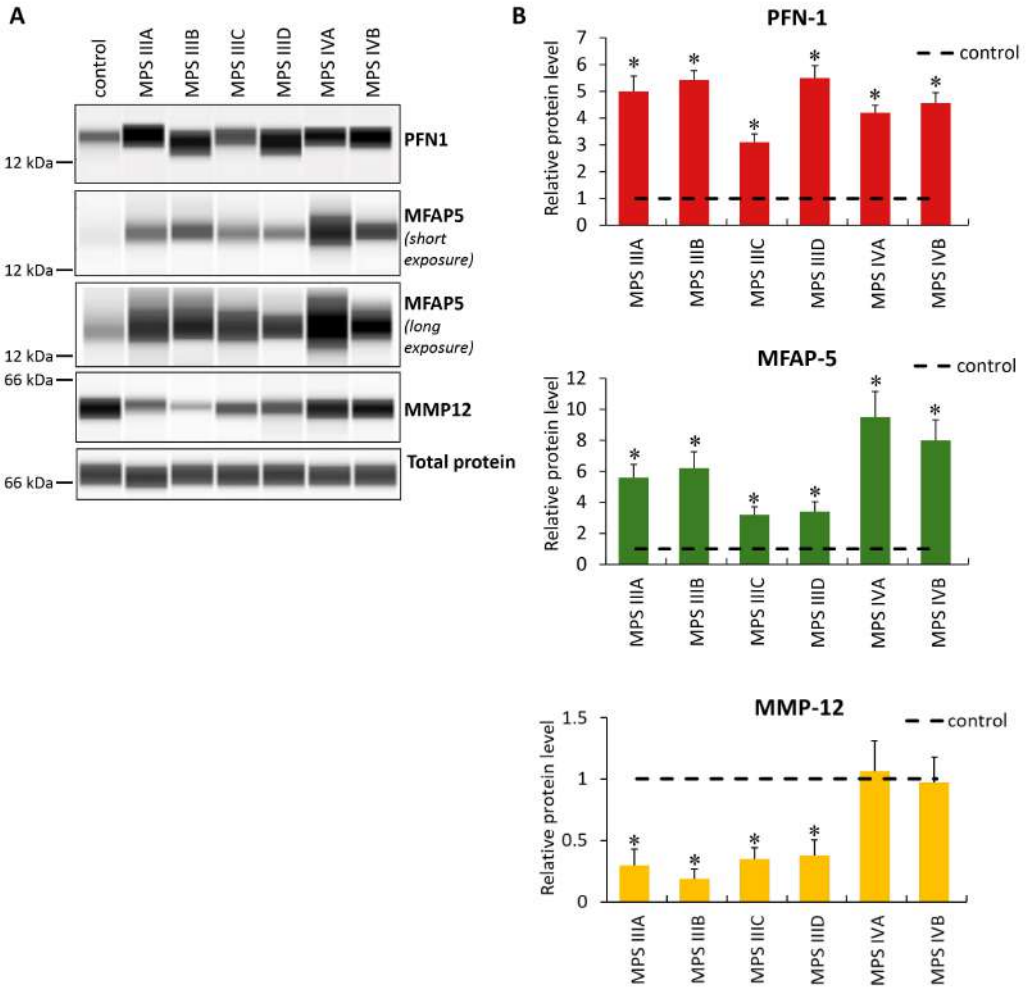


Fig. 3. Levels of PFN1, MFAP-5 and MMP12 in different subtypes of MPS III and IV cells relative to control cells. Representative Western blots (A) and quantitative analyses (B) from 3 independent experiments, with error bars representing SD are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line). Full size blots are shown in **Supplementary material**.

in MPS IV. So far, no studies have been conducted on the similarities in gene expression profiles across different subtypes of the same MPS type. Such studies could potentially identify common gene expression disturbances in MPS III or IV that may contribute to the occurrence of specific, similar symptoms within these subtypes.

Therefore, the aim of this study was to identify genes that undergo common dysregulation of expression across all subtypes of MPS III and IV. Our transcriptomic analyses revealed a large number of genes with similarly altered expression levels in MPS III and IV compared to control cells (Fig. 1), among which 25 genes exhibited high fold-change values (over 8-fold) (Fig. 2, Table 3). These included upregulated genes, such as *PFN1*, encoding profilin 1, *MFAP5*, encoding microfibril-associated protein 5, as well as downregulated *MMP12*, encoding matrix metalloproteinase. Studies on the levels of these proteins in the cells confirmed an increase (PFN1, MFAP5) or decrease (MMP12) in their levels (Figs. 3,4,5,6). Moreover, the impact of reduced GAG levels on these proteins was determined (Figs. 7,8,9,10). The level of one of the proteins studied, profilin, was found to be dependent on the GAG levels in the cell, as reducing GAG levels decreased PFN1 levels (Figs. 7,8). Intriguingly, this correlation is interdependent, as silencing *PFN1* expression using siRNA significantly reduced GAG levels (Fig. 11).

Profilins are small proteins interacting with the cell cytoskeleton by promoting and influencing actin polymerization. They also participate in membrane transport, cell signaling, transcription, autophagy, and many other processes [17–19]. The cytoskeleton plays an important role in synaptogenesis or neurotransmission, and it is also involved in the development of the nervous system and the plasticity of the mature brain [20]. Cytoskeletal disorders

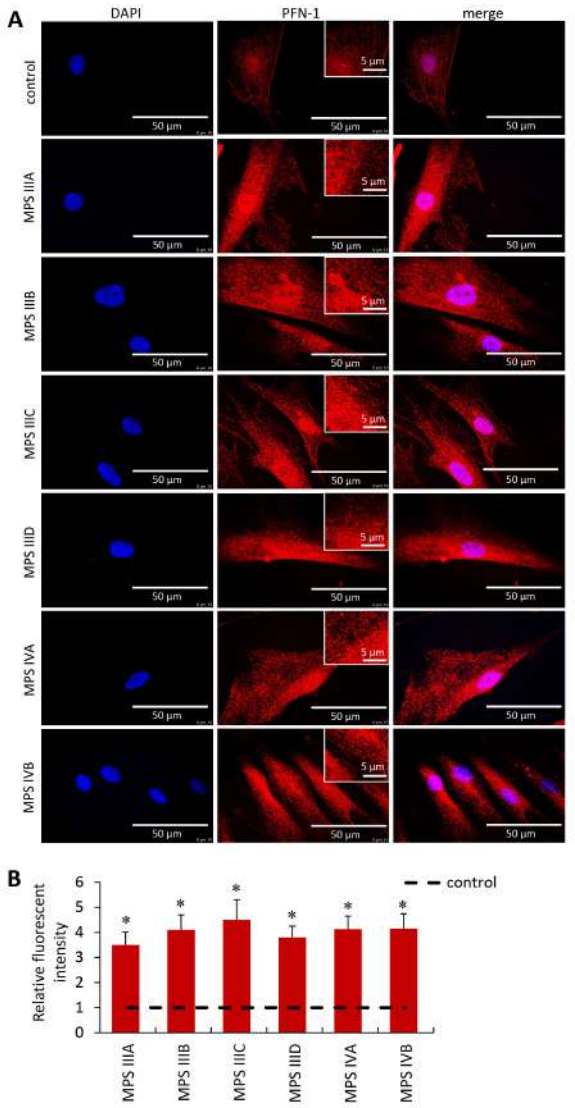


Fig. 4. Levels of the PFN1 protein (Profilin 1) in different subtypes of MPS III and IV cells relative to control cells. Representative fluorescent microscopic images (scale bars represent 50 µm (mail panels) or 5 µm (inserted panels)) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA d post an hoc Tukey's test) relative to control cells (horizontal dashed line). DPAI, cells stain with DAPI; PFN1, cells stained with PFN1-specific antibody.

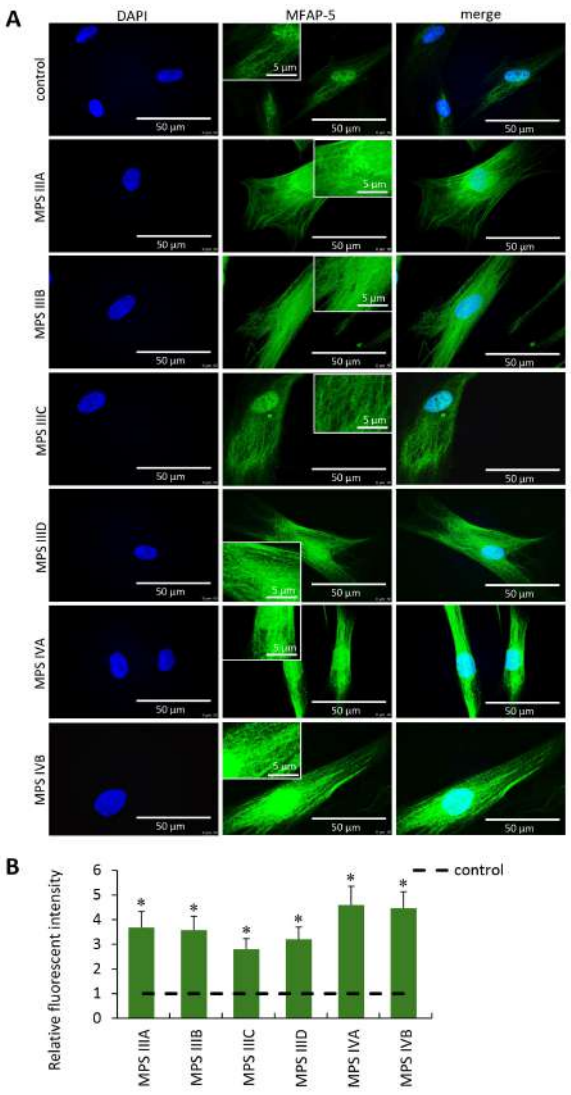


Fig. 5. Levels of the MFAP5 protein (Microfibrillar Associated Protein 5) in different subtypes of MPS III and IV cells relative to control cells. Representative fluorescent microscopic images (scale bars represent 50 µm (mail panels) or 5 µm (inserted panels)) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line). DPAI, cells stain with DAPI; MFAR-5, cells stained with MFAP5-specific antibody.

are also observed in certain psychiatric disorders such as schizophrenia, bipolar disorder, autism, or major depression [21]. This is significant because behavioral problems are one of the neuropathic symptoms of MPS. Moreover, patients with MPS III are sometimes misdiagnosed as individuals with autism spectrum disorders [4]. Therefore, the significance of profilin-1 for the disorders observed in MPS becomes an interesting issue.

Mutations or dysregulation of the *PFN1* gene have been implicated in various human diseases. Prominent among these are neurodegenerative conditions, such as amyotrophic lateral sclerosis (ALS), Fragile X syndrome (FXS), spinal muscular atrophy (SMA), Huntington's disease (HD), Parkinson's disease (PD), and adrenoleukodystrophy.

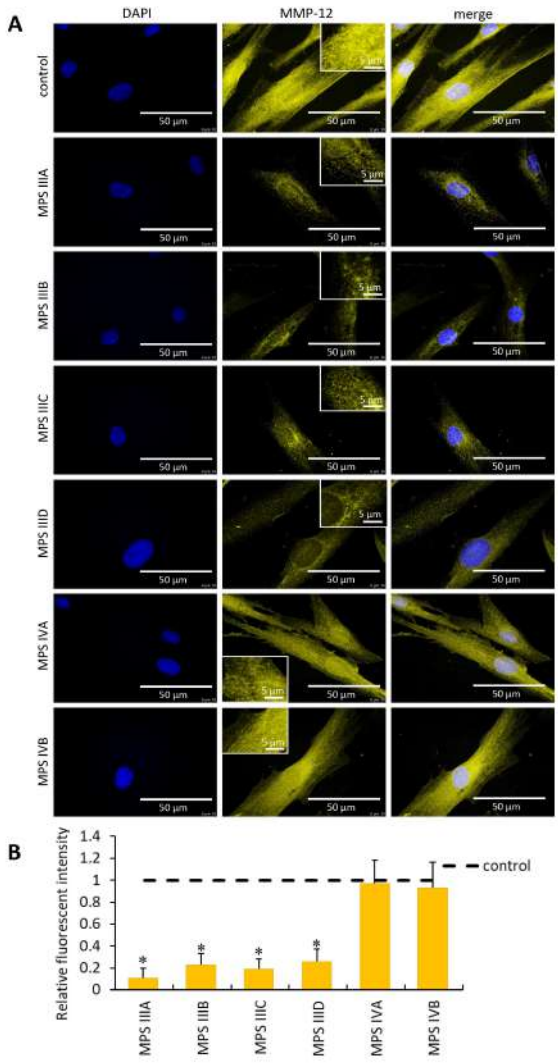


Fig. 6. Levels of the MMP12 protein (Matrix Metallopeptidase 12) in different subtypes of MPS III and IV cells relative to control cells. Representative fluorescent microscopic images (scale bars represent 50 µm (mail panels) or 5 µm (inserted panels)) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line). DPAI, cells stain with DAPI; MMP12, cells stained with MMP12-specific antibody.

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the degeneration of motor neurons in the brain and spinal cord. This results in a gradual loss of muscle control, leading to paralysis. The disease is currently incurable and typically results in death within a few years of diagnosis [22]. As many as eight different mutations in the *PFN1* gene have been identified in patients with both sporadic and familial forms of ALS. A study on the role of profilin in ALS have revealed significant cytoskeletal defects and alterations in neuronal morphology, such as a reduced F-/G-actin ratio, shorter dendrites, and axons with impaired integrity, which progressively undergo Wallerian degeneration in cells expressing ALS-PFN1 [22]. Additionally, numerous reports indicate that *PFN1* mutants associated with ALS produce profilin molecules prone to aggregation. They form insoluble, ubiquitinated aggregates and protein inclusions in cells, similar to other proteins involved in ALS pathogenesis, such as Superoxide Dismutase 1 (SOD1), TAR DNA Binding Protein 43 (TDP43), and Fused in Sarcoma protein (FUS) [23]. Indeed, TDP43 is one of the proteins most commonly found to aggregate with PFN1 in ALS [24]. It has been indicated that some mutant PFN1 proteins exhibit prion-like properties and act as factors that trigger the conversion of endogenous TDP43 into toxic conformational states when taken up by wild-type cells [25]. Using the *Drosophila melanogaster* model, it has been demonstrated that ectopic expression of mutant *PFN1* leads to increased degeneration of retinal photoreceptor neurons through co-expression of *TDP43* [26]. That study suggest that PFN1 aggregation exacerbates ALS progression by enhancing the toxicity of TDP43. Other research conducted in mouse models indicated an accelerated degeneration of motor neurons in mice expressing the mutant *PFN1* variant, similar to what has been observed in patients with ALS [27]. The investigation into the causes of this phenomenon has led to two possible explanations. The first suggests disruptions in microtubule dynamics due to the fact that mutant PFN1 does not promote microtubule growth as wild-type PFN1 does, resulting in impaired axonal integrity [28]. The second hypothesis concerns changes in the activity of actin-binding proteins associated with PFN1. Mutant PFN1 binds with higher affinity to formins, the proteins involved in actin polymerization, by associating with the rapidly growing ends of actin filaments. This interaction results in a significantly increased rate of actin assembly [29].

Another example of a disorder associated with profilin levels is Fragile X syndrome (FXS), one of the most common neurodevelopmental disorders. The main symptoms of this condition include significant intellectual disability, hyperactivity, and repetitive behaviors. Patients often exhibit clinical signs of autism, such as reduced social interactions and speech impairments. This X-linked disorder is caused by mutations in the *FMR1* gene, which affect the expression and/or function of the gene encoding FMRP (Fragile X mental retardation protein). As an RNA-binding protein, FMRP regulates the localization, stability, and translation of many transcripts, including key regulators involved in neuronal development, morphology, and synaptic plasticity [30]. Preliminary findings linking FXS to profilin were obtained in a *D. melanogaster* model, where FMRP directly binds to the profilin mRNA. Flies with the *FMRP* mutation exhibited significantly elevated levels of profilin [31]. Other results obtained from a mouse model of FXS show

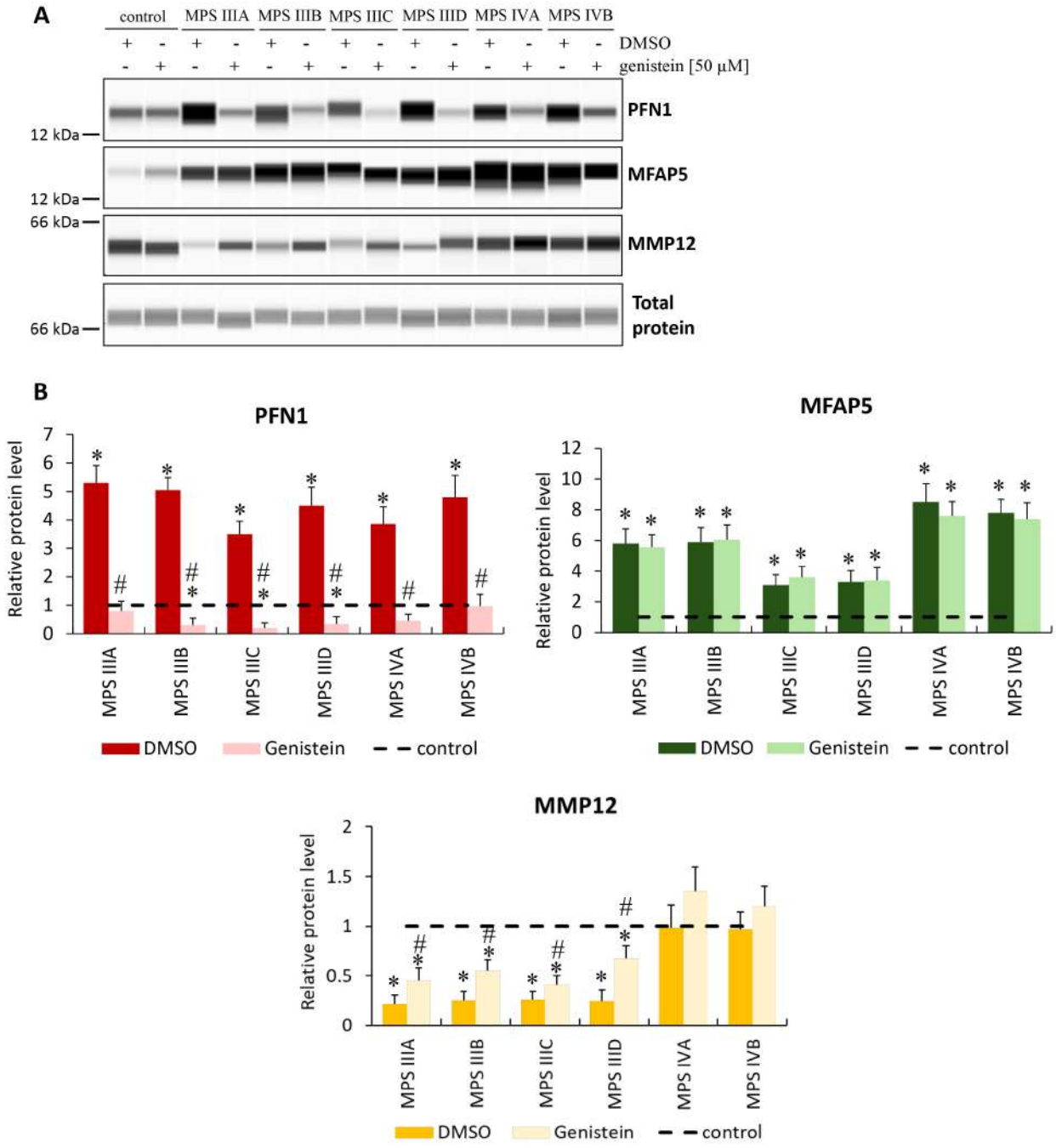


Fig. 7. Levels of PFN1, MFAP-5 and MMP12 in different subtypes of MPS III and IV as well as control cells treated with DMSO (dimethyl sulfoxide; vehicle) or genistein (50 µM) for 24 h. Representative Western blots (A) and quantitative analyses (B) from 3 independent experiments, with error bars representing SD are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line) and hashtags (#) show significant differences relative to non-treated cells of the same subtype of MPS. Full size blots are shown in **Supplementary material**.

that PFN1 protein levels are reduced in *FMR1*-mutant mice, leading to impaired dendritic spine formation in hippocampal neurons. However, overexpression of *PFN1* can correct developmental defects during corticogenesis in these mice [19,32,33].

The survival motor neuron 1 (SMN1) protein interacts with profilin and is associated with spinal muscular atrophy (SMA). SMN1 plays a crucial role in RNA splicing, but it is also involved in RNA metabolism, mRNA transport and localisation, cellular signaling, and cytoskeletal

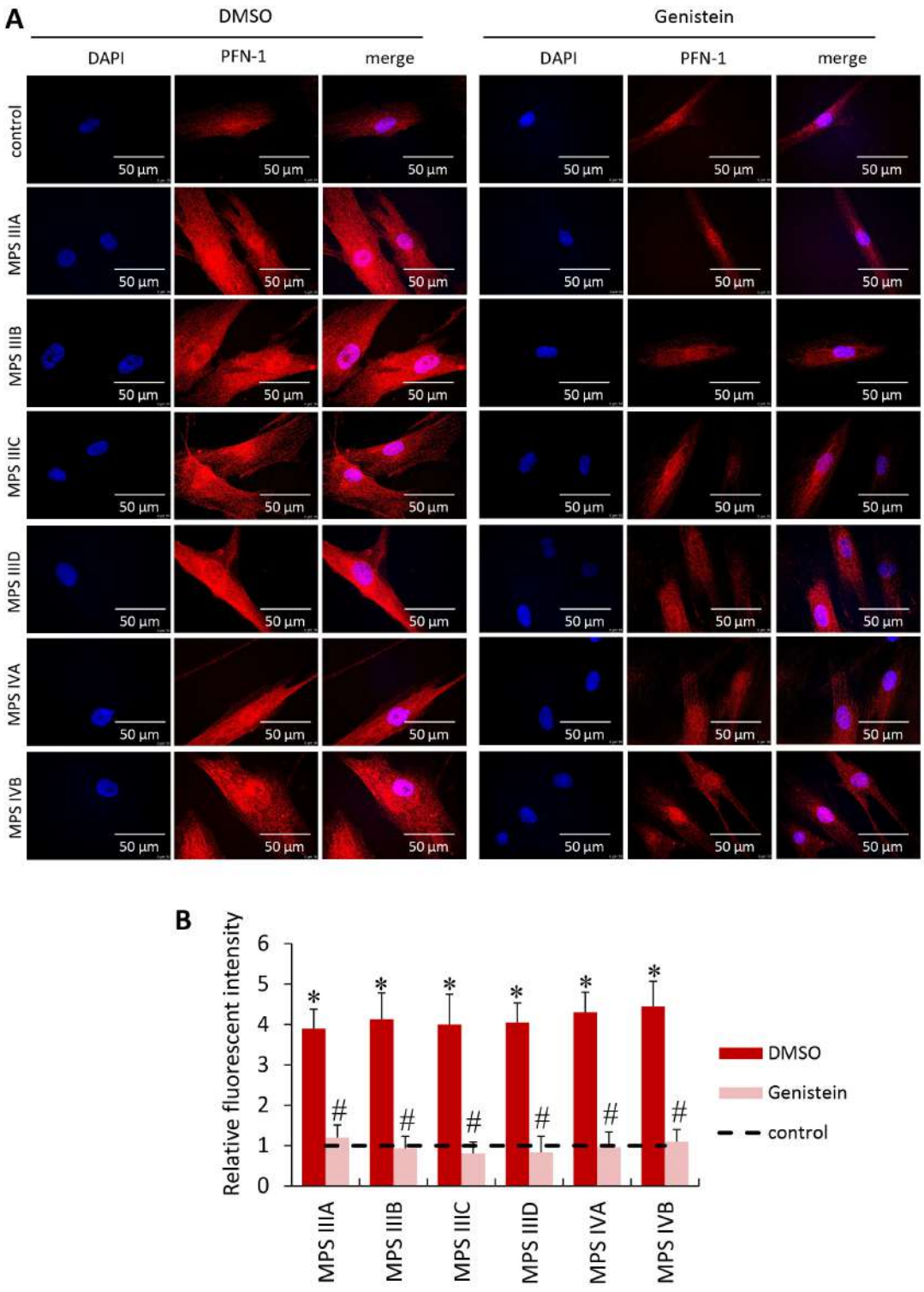


Fig. 8. Levels of the PFN1 protein in different subtypes of MPS III and IV cells as well as control cells treated with DMSO (dimethyl sulfoxide; vehicle) or genistein (50 µM) for 24 h. Representative fluorescent microscopic images (scale bars represent 50 µm) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line) and hashtags (#) show significant differences relative to non-treated cells of the same subtype of MPS.

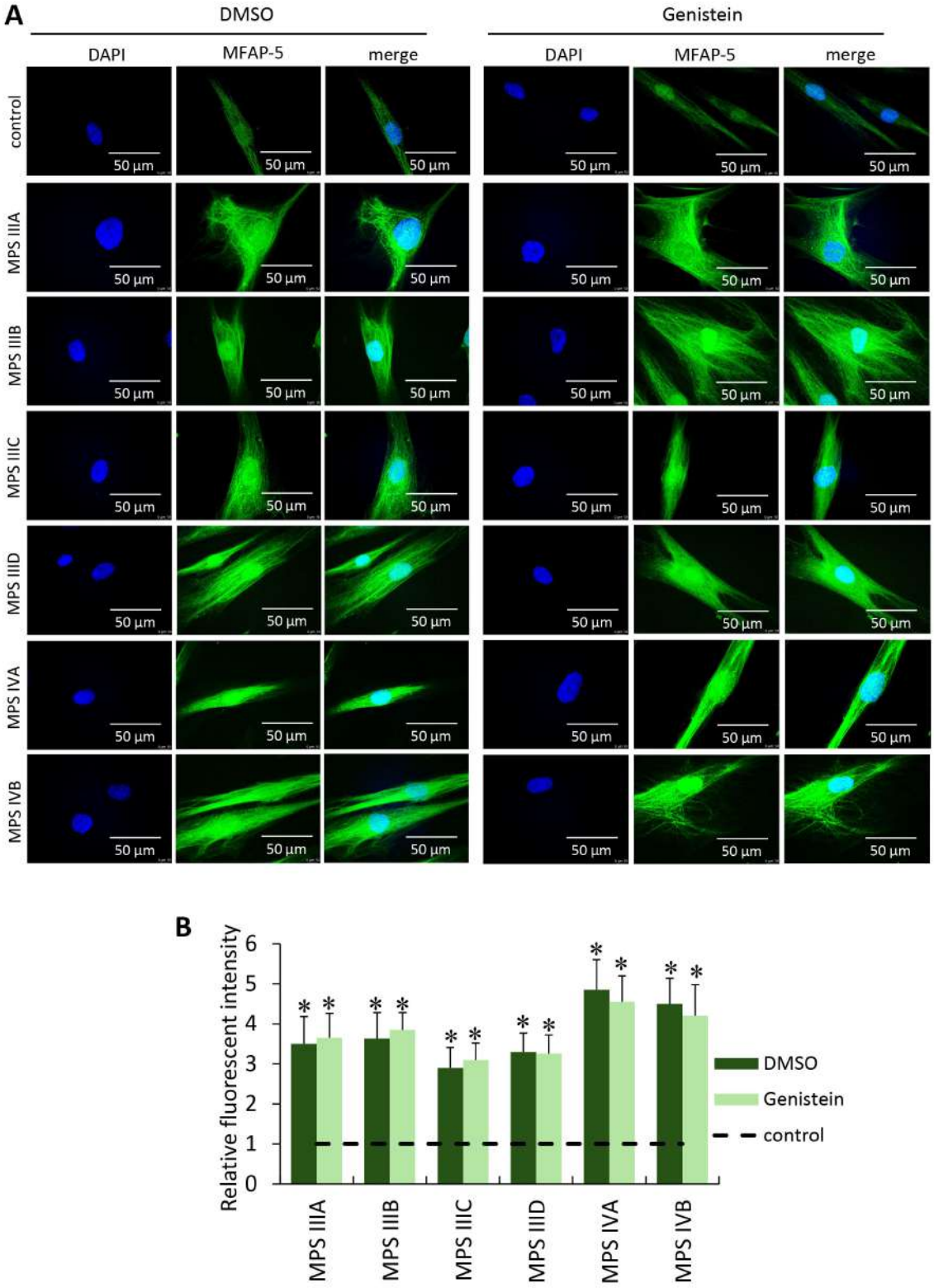


Fig. 9. Levels of the MFAP5 protein in different subtypes of MPS III and IV cells as well as control cells treated with DMSO (dimethyl sulfoxide; vehicle) or genistein (50 µM) for 24 h. Representative fluorescent microscopic images (scale bars represent 50 µm) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line).

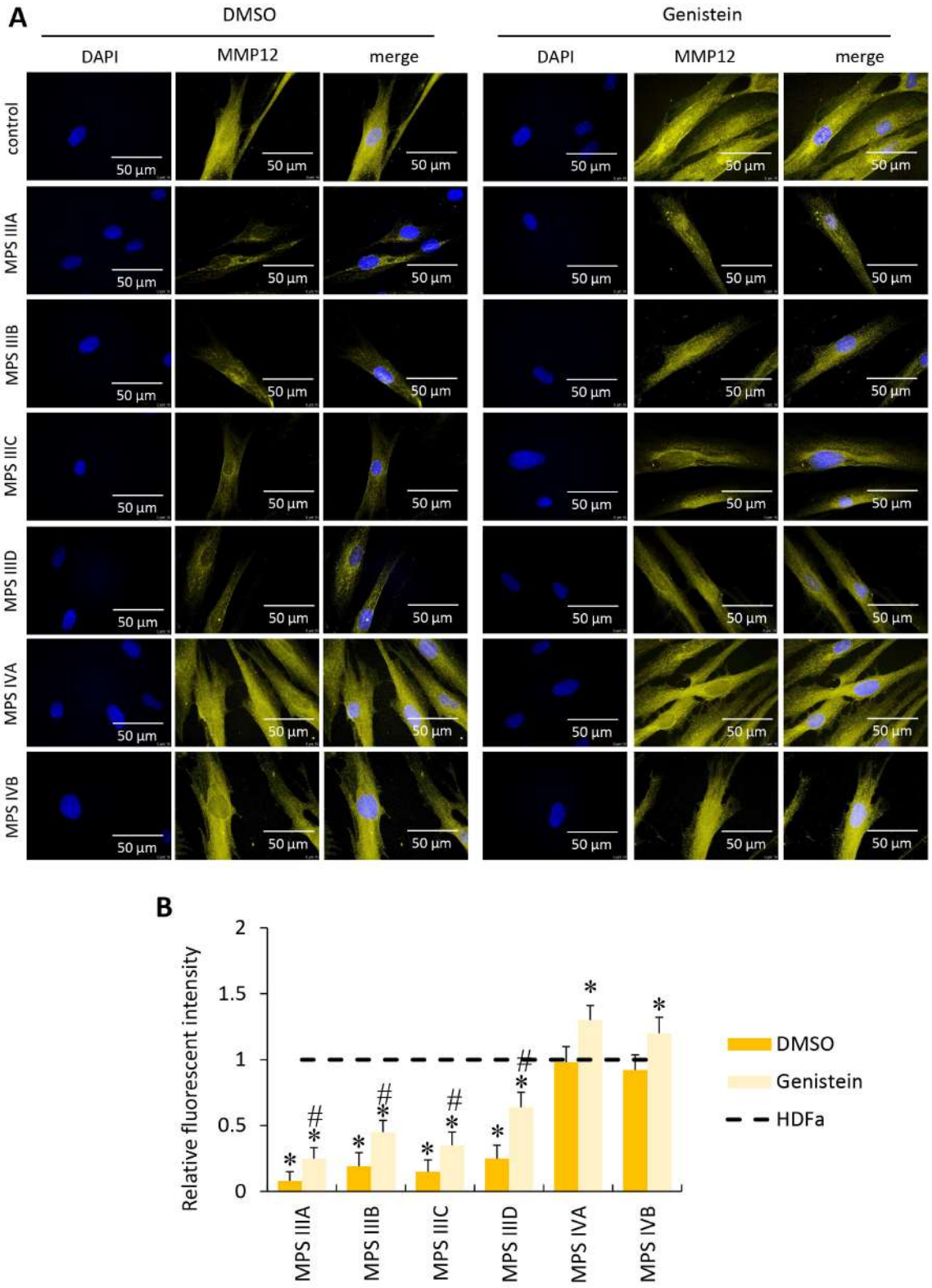


Fig. 10. Levels of the MMP12 protein in different subtypes of MPS III and IV cells as well as control cells treated with DMSO (dimethyl sulfoxide; vehicle) or genistein (50 µM) for 24 h. Representative fluorescent microscopic images (scale bars represent 50 µm) (A) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (B) are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells (horizontal dashed line) and hashtags (#) show significant differences relative to non-treated cells of the same subtype of MPS.

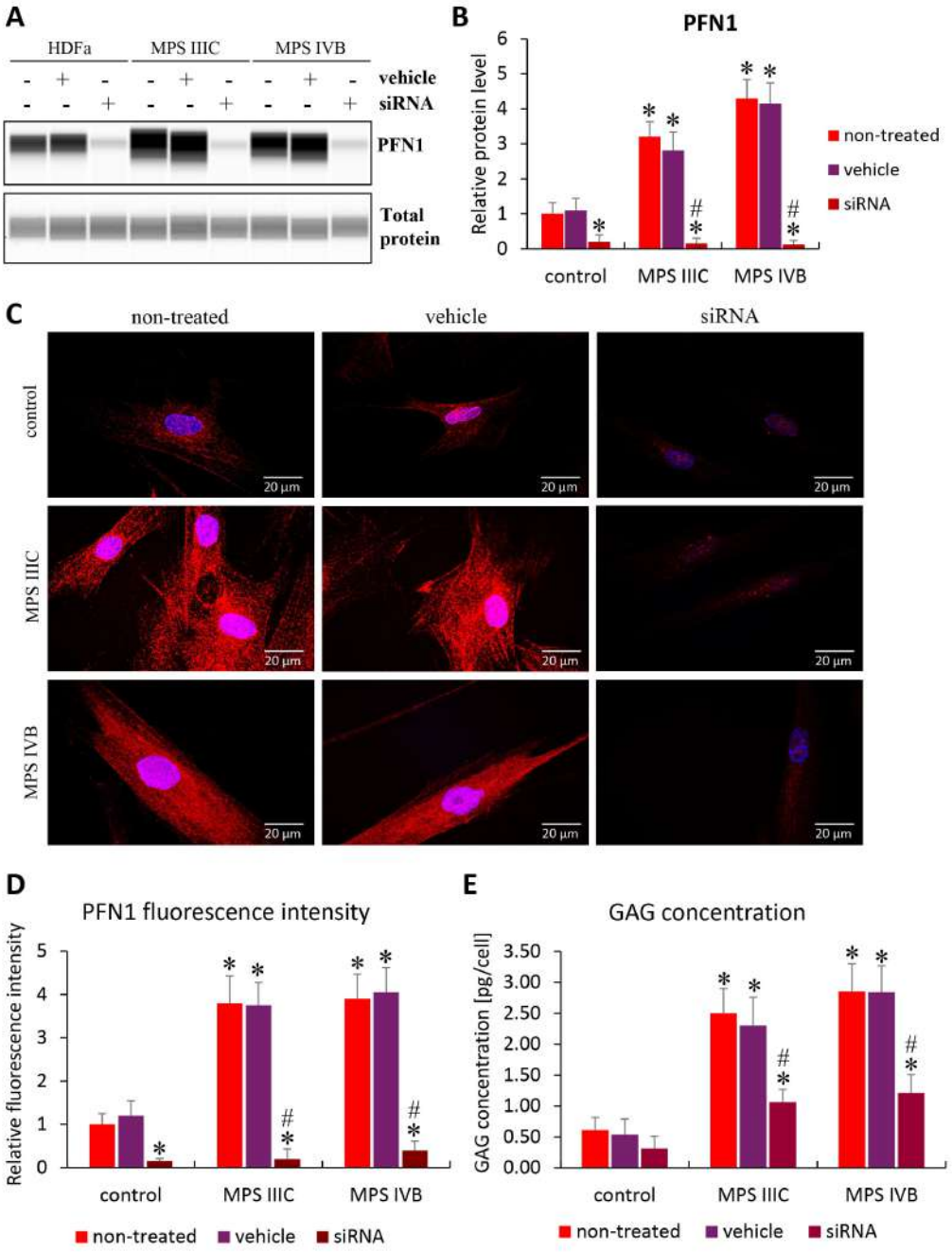


Fig. 11. Levels of the PFN1 protein and GAG concentrations in MPS IIIC, IVB and control cells treated with PFN1 siRNA or vehicle. Representative Western blot (A) with quantitative analyses (B) from 3 independent experiments, with error bars representing SD; representative fluorescent microscopic images (scale bars represent 20 µm) (C) with quantitative analyses (relative fluorescence intensity) from 100 randomly chosen cells of each variant of the experiment (D); as well as glycosaminoglycan (GAG) concentrations (E), mean values from 3 independent experiments with error bars representing SD, are shown. Asterisks (*) indicate statistically significant differences (at $p < 0.05$ in two-way ANOVA and post hoc Tukey's test) relative to control cells, and hashtags (#) show significant differences relative to non-treated cells of the same subtype of MPS. Full size blots are shown in **Supplementary material**. HDFa, Human Dermal Fibroblasts adult; siRNA, short interfering RNA; GAG, glycosaminoglycans.

dynamics. Deletions or point mutations in the *SMN1* gene lead to SMA, causing progressive loss of motor neurons and muscle atrophy [34]. The interaction of SMN with cytoskeletal proteins suggests that disturbances in cytoskeletal dynamics may play a role in SMA [35]. SMN1 interacts with PFN1 and PFN2a [36]. Mutations in SMN1 disrupt

interactions with profilins, affecting profilin's role in actin dynamics [37]. In SMN-deficient cells, PFN2a levels increase and profilin becomes hyperphosphorylated, impacting Rho kinases (ROCK) and other targets, leading to disturbances in cytoskeletal dynamics [38]. These changes are associated with spinal muscular atrophy (SMA) pathogenesis, impairing cytokinesis and endocytosis. The absence of SMN-PFN2a complexes causes unbalanced actions of PFN2a, myosin, and cofilin in SMN-deficient cells, likely leading to disrupted cytoskeletal dynamics, consistent with observed changes in the F/G-actin ratio and stress-related actin filament formation [39,40]. Irregular levels and activity of profilin correlate with SMA pathogenesis, but further research is needed to elucidate the underlying molecular mechanisms.

Profilin's role has also been studied in the context of Huntington's disease (HD). HD is an autosomal dominant, progressive neurodegenerative disorder caused by an expanded CAG triplet sequence of more than 35 repeats in the huntingtin (*HTT*) gene. Wild-type huntingtin protein is involved in various cellular processes, primarily vesicular transport, although its full functions are not yet completely understood. The mutant huntingtin protein forms toxic aggregates that impair neuronal functions, leading to neurodegeneration, particularly in the striatum. Symptoms of HD include progressive motor dysfunction, personality changes, emotional and psychiatric disturbances, and cognitive decline [41]. Huntingtin contains two proline-rich domains (PRD) that bind PFN1 and PFN2. Interaction with profilins significantly reduces the aggregation and toxicity of huntingtin by keeping it in a soluble state and delaying aggregate formation. Conversely, PFN1 binds to the PRD of huntingtin, preventing its tetramerization and stabilizing monomers and dimers, which do not further oligomerize [42,43]. Reduced levels of PFN1 are correlated with increased disease progression in patients [44].

A final example is childhood adrenoleukodystrophy (cALD), a manifestation of adrenoleukodystrophy (ALD) characterized by demyelination of neurons, brain inflammation, and disruption of the blood-brain barrier, with features of an autoimmune disease. A study of plasma samples from 94 boys before and after the onset of cALD revealed that 48 of them (51%) had anti-PFN1 antibodies, while only 2 out of 29 boys with ALD without brain involvement and none of 30 healthy controls had these antibodies. Analyses of cerebrospinal fluid from individuals with cALD showed higher levels of the PFN1 protein compared to samples without cALD. Boys with anti-PFN1 antibodies also exhibited a significant increase in gadolinium signal in MRI scans, likely indicating enhanced blood-brain barrier disruption. These antibodies were also associated with higher levels of very long-chain fatty acids and increased BAFF concentrations in plasma. The authors suggested that anti-PFN1 antibodies could serve as a new biomarker indicating the development of cALD in boys with ALD [45].

The detailed mechanisms involving profilin in human neurodegenerative diseases have been compiled by Murk *et al.* [46].

Another important aspect of the significance of normal profilin-1 levels in the organism is its relevance to skeletal disorders. PFN1 plays a crucial role in skeletal development and bone homeostasis. Available literature indicates that a decrease in profilin-1 levels is associated with skeletal abnormalities such as dwarfism, craniofacial deformities, altered bone structure, and size [47]. Heterozygous deletion in the *PFN1* gene has been identified in patients with Paget's disease of bone (PDB), which is a late onset chronic progressive bone disease characterized by abnormal activation of osteoclasts leading to bone pain, deformities, and fractures [48]. This raises the question of how an excess of profilin-1 affects the skeletal system if a decrease in profilin-1 levels is one of the causes of skeletal abnormalities. There is no available information regarding the association of an increased expression of the *PFN1* gene with skeletal disorders. However, patients with MPS types I, II, IV, and VI are characterized by short stature, excessive joint stiffness (except for MPS IV, where joint laxity is typical), and numerous bone/skeletal deformities [4].

Over the past 20 years, profilin has garnered increased attention as a potential therapeutic target in the fight against various types of cancer. A recent study using a cellular model of breast cancer, specifically the MDA-231 cell line, which stably expresses either control or Pfn1-shRNA, have demonstrated that reduced *PFN1* expression in these cells correlates with lower tumor proliferation and prolonged relapse-free survival in breast cancer patients. This phenomenon is partly attributed to the upregulation of the protein SMAD3 [49].

Research conducted in the MDA-MB 231 human breast cancer cell line also revealed that overexpression of *PFN1* induces changes in the expression of several proteins involved in cell proliferation, motility, and survival [50]. Additionally, decreased PFN1 levels have been observed in clinical pancreatic adenocarcinoma samples compared to surrounding benign tissues, with low PFN1 expression significantly correlated with shorter patient survival. Restoring PFN1 in pancreatic cancer cells with low endogenous *PFN1* expression resulted in a non-tumorigenic phenotype, suggesting that PFN1 may act as a negative regulator of pancreatic cancer progression. Orthotopic mouse xenograft models showed that overexpression of *PFN1* reduced tumor volume through the regulation of the SIRT3-HIF1α axis [51]. Reduced *PFN1* expression has also been noted in lung, colorectal, bladder, esophageal, and thyroid cancers [52].

Interestingly, PFN1 has also been proposed as a potential therapeutic target for psoriasis. Elevated *PFN1* expression has been observed in psoriasis patients' skin and serum. *PFN1* was prominently expressed in the epidermis of psoriatic lesions, and its expression positively cor-

related with the severity of the disease. Furthermore, treatment of keratinocytes with IL-17A increased *PFN1* expression, while TNF-α induced both expression of *PFN1* and secretion of the PFN1 protein. Conversely, reducing *PFN1* expression using shRNA led to suppressing inflammatory markers associated with psoriasis and reducing IκBζ, a key factor in the immunological response in psoriasis [53].

Considering that polymorphisms or varied expression of the profilin gene are ambiguously associated with neurodegeneration and skeletal disorders, and that in this study elevated levels of profilin were observed in all subtypes of both MPS III and IV, it seems justified to determine its precise role in the regulation of GAG levels, something that has never been proposed before. The only existing study suggesting a correlation between elevated *PFN1* levels and GAG levels were conducted on a model of diabetic nephropathy kidney cells. Transcriptomic analysis of these cells indicated that high *PFN1* expression was negatively associated with heparin sulfate biosynthesis [54]. However, this study was not further pursued. Nevertheless, considering both these data and the results presented in this work, it seems justified to explore the potential of profilin-lowering agents as potential drugs, which could not only improve the integrity of the cellular cytoskeleton but also reduce GAG levels. Such compounds include temozolomide and butyrate. Both of these compounds have been demonstrated to downregulate PFN1 expression in cancer therapy studies [55,56].

5. Conclusions

In conclusion, we have demonstrated many commonly dysregulated genes in all subtypes of MPS III and MPS IV. Some of these genes, like *PFN1*, *MFAP5*, and *MMP12*, revealed particularly highly pronounced changes in expression relative to control cells. Furthermore, this study highlights the elevated levels of profilin and the interdependent correlation between GAG levels and the expression of the *PFN1* gene. Given that disturbances in *PFN1* expression levels have also been observed in various other neurodegenerative and musculoskeletal diseases, the intriguing question of the role of GAGs in the development of these symptoms remains open. These compounds have typically been associated with MPS, although it is difficult to ignore that the role of GAGs in the development of general neurodegeneration has already been suggested [57–61]. Finally, the data presented here suggests *PFN1* as a potential new therapeutic target for MPS III and/or IV.

Availability of Data and Materials

Transcriptomic raw data are available in the NCBI Sequence Read Archive (SRA), under accession no. PRJNA562649. Specific materials and raw data from other experiments are available from corresponding authors on request.

Author Contributions

KP and GW designed the research study. KW, MŻ, LG, and AS performed the research. BMW provided help and advice on experimental approaches and data analysis, and contributed to preparing the revised manuscript. KW, LG, and KP analyzed the data. KW, KP, and GW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

Given his role as the Guest Editor member, Grzegorz Wegrzyn had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Ricardo Jorge Pinto Araujo. The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2912415.

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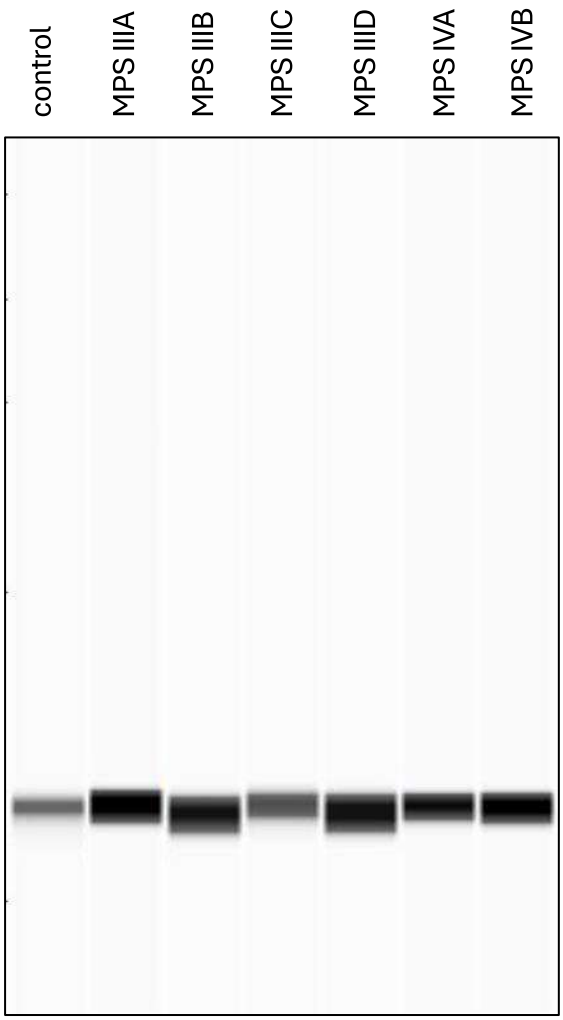
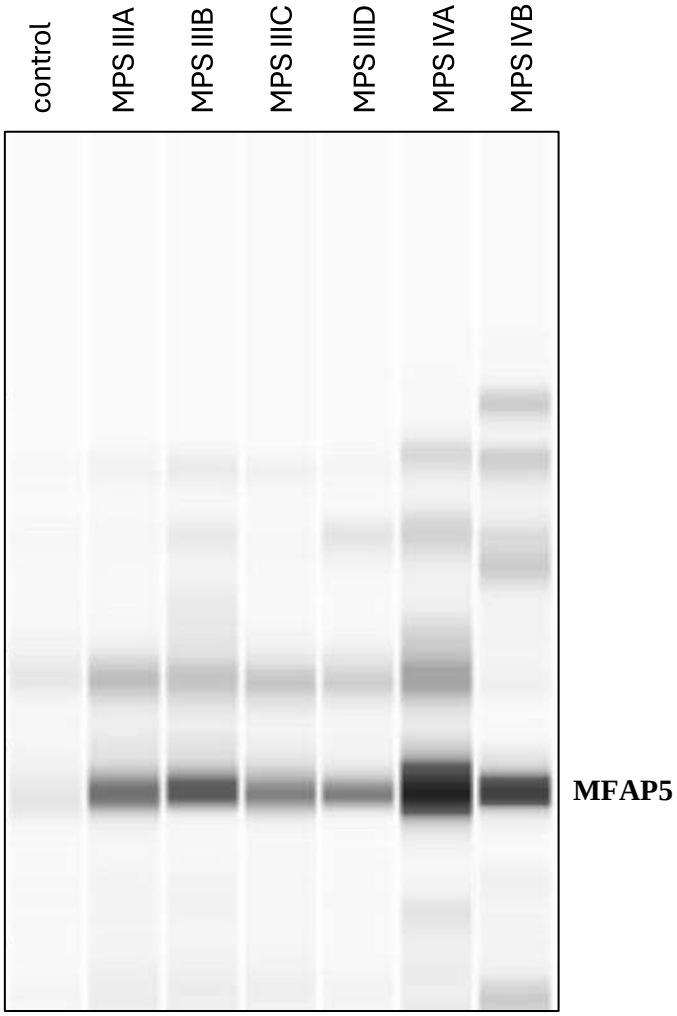
Supplementary

Figure 3 – Full-size blots





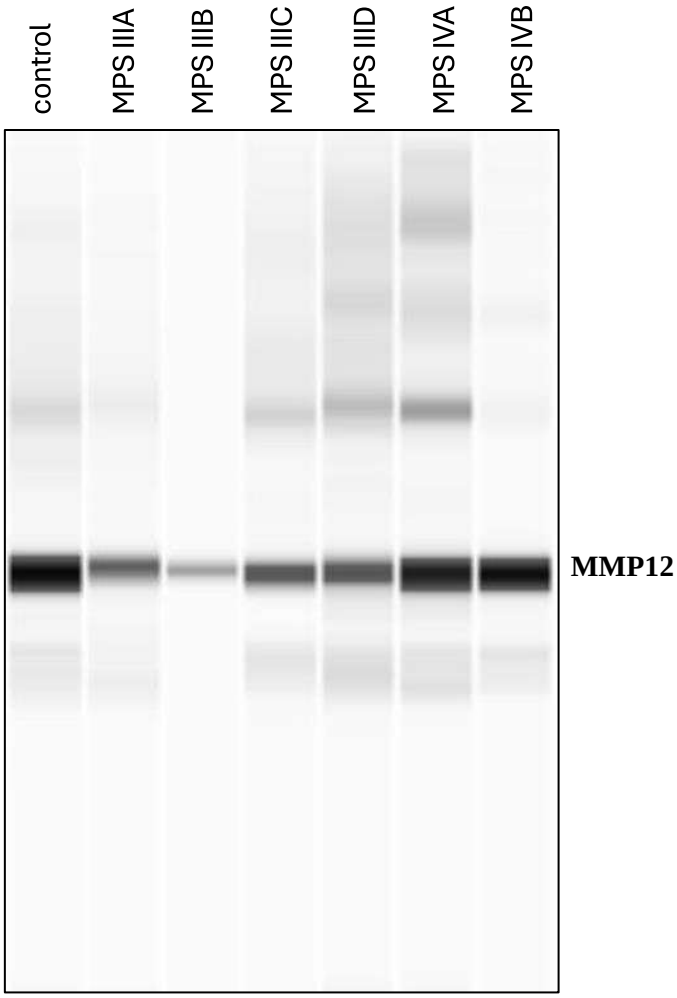


Figure 7 – Full-size blots

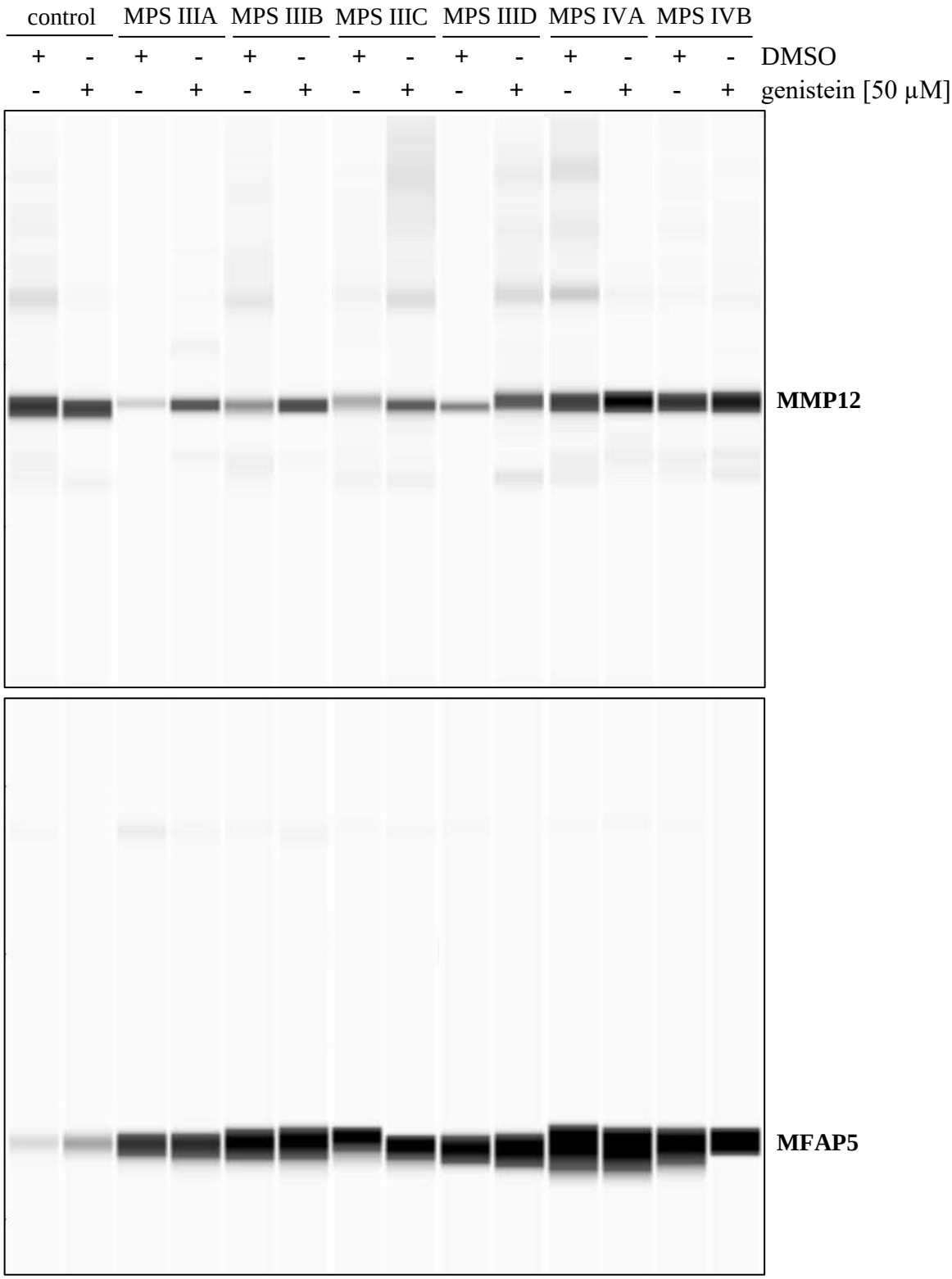


Figure 7 – Full-size blots (continued)

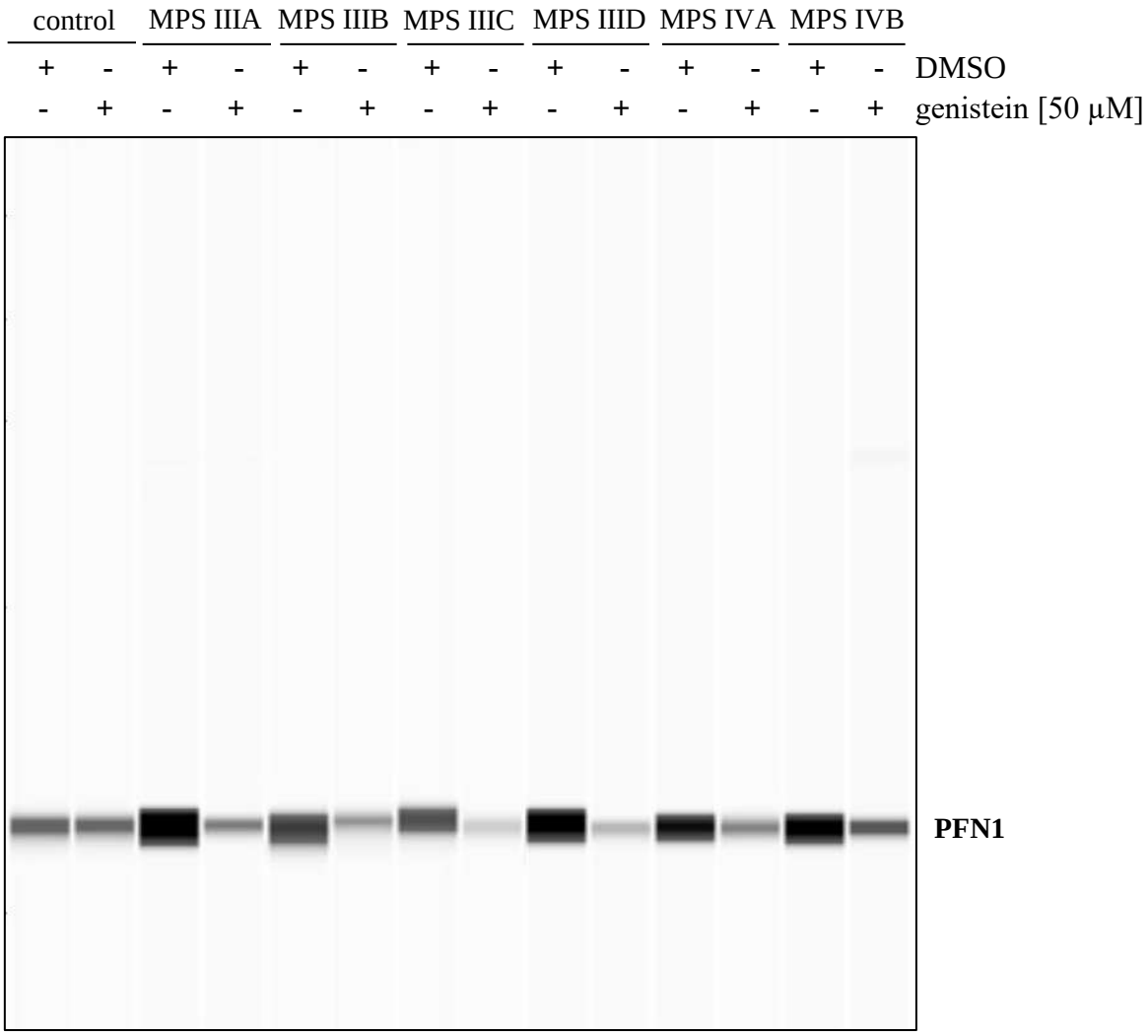
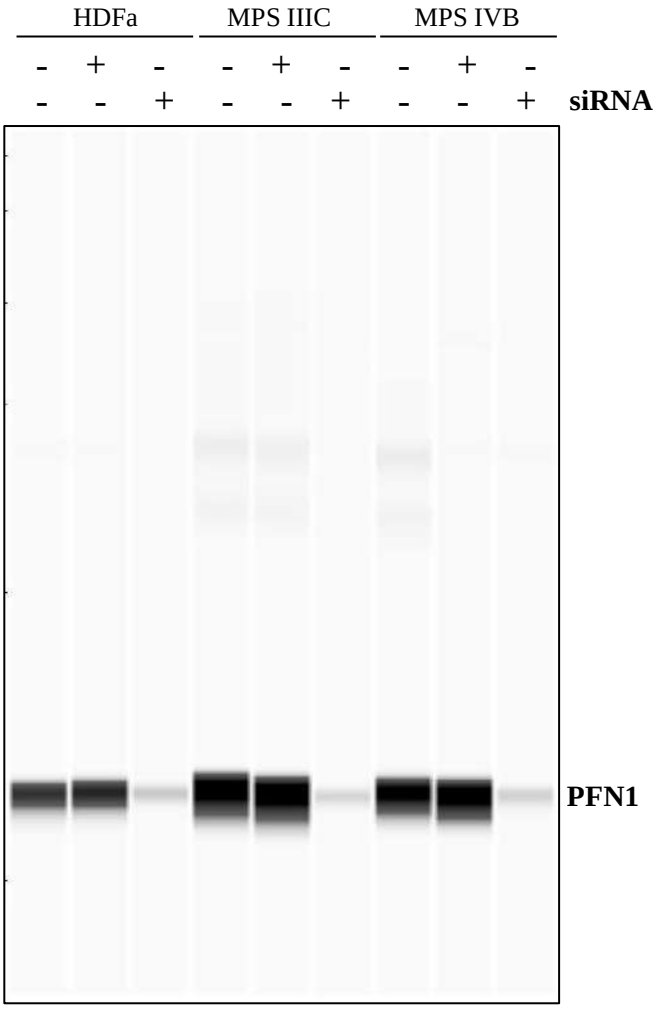


Figure 11 – Full-size blots



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4. asyście w przygotowaniu odpowiedzi na uwagi recenzentów

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polegał na:

1. recenzji wewnętrznej manuskryptu
2. udziale w interpretacji wyników
3. asyście w przygotowaniu odpowiedzi na uwagi recenzentów

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1. zaproponowaniu tematyki pracy
2. przygotowaniu pierwotnej wersji manuskryptu
3. interpretacji i analizie wyników
4. rewizji manuskryptu po uwagach recenzentów
5. przygotowaniu odpowiedzi na uwagi recenzentów
6. pozyskaniu finansowania w postaci otrzymania grantu

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Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients

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Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients

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ABSTRACT

Sanfilippo disease is a lysosomal storage disorder from the group of mucopolysaccharidoses (MPS), characterized by storage of glycosaminoglycans (GAGs); thus, it is also called MPS type III. The syndrome is divided into 4 subtypes (MPS III A, B, C and D). Despite the storage of the same GAG, heparan sulfate (HS), the course of these subtypes can vary considerably. Here, we comprehensively evaluated the levels of protein aggregates (APP, β-amyloid, p-tau, α-synuclein, TDP43) in fibroblasts derived from patients with all MPS III subtypes, and tested whether lowering GAG levels results in a decrease in the levels of the investigated proteins and the number of aggregates they form. Elevated levels of APP, β-amyloid, tau, and TDP43 proteins were evident in all MPS III subtypes, and elevated levels of p-tau and α-synuclein were demonstrated in all subtypes except MPS IIIC. These findings were confirmed in the neural tissue of MPS IIIB mice. Fluorescence microscopy studies also indicated a high number of protein aggregates formed by β-amyloid and tau in all cell lines tested, and a high number of aggregates of p-tau, TDP43, and α-synuclein in all lines except MPS IIIC. Reduction of GAG levels by genistein led to the decrease of levels of all tested proteins and their aggregates except α-synuclein, indicating a relationship between GAG levels and those of some protein aggregates. This work describes for the first time the problem of deposited protein aggregates in all subtypes of Sanfilippo disease and suggests that GAGs are partly responsible for the formation of protein aggregates.

1. Introduction

Mucopolysaccharidoses (MPS) are a group of rare metabolic diseases characterized by abnormal metabolism of glycosaminoglycans (GAGs). The causes of these disorders are mutations in genes encoding enzymes that carry out GAG degradation reactions. As a result, these compounds accumulate in lysosomes, leading to damage to cells, tissues and the entire organism [1]. This results in a set of severe symptoms that include short stature, facial dysmorphism, chronic joint pain, organomegaly, or sensory problems [2]. To date, 13 types/subtypes of MPS have been distinguished, differentiated by type(s) of GAG accumulation and the kind of non-functional lysosomal enzyme. The life expectancy of patients with MPS varies depending on the type/subtype and the severity of symptoms, ranging from 2 to 20 years [2–4].

Some types/subtypes of MPS are characterized by extensive neurodegeneration resulting in neurological disorders such as loss of cognitive-motor skills, aggression, hearing loss, diurnal rhythm changes, speech difficulties and personality changes [5,6]. An example of this type of the disease is MPS type III (Sanfilippo disease) divided into 4 subtypes (MPS III A, B, C and D), which are characterized by the primary heparan sulfate (HS) storage, accompanied by the secondary dermatan sulfate (DS) accumulation. These subtypes are shown in Table 1.

Among the symptoms of MPS III, disorders flowing from the nervous system are mainly observed. However, despite the primary storage of the same GAG, HS, the course of MPS III A, B, C and D can vary considerably. Both somatic symptoms (coarse features and hepatomegaly) and neurological symptoms (language delay, abnormal behavior, autistic spectrum disorder, dementia and epilepsy) occur with varying frequency, age and severity depending on the subtype of the disease [7,8]. For example, abnormal behavior occurs in 75 % of MPS IIIA patients, 69 % of MPS IIIB patients and 77 % of IIIC patients, and language delay occurs in 93 % of MPS IIIA cases, compared to 88 % and 92 % for MPS IIIB and IIIC, respectively. Epilepsy appears in MPS IIIA patients at an average age of 7 years, compared to 12.5 years for MPS IIIB patients, and

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Table 1
Characteristics of the different subtypes of MPS III.

MPS type	Defective gene	Defective enzyme	Stored GAGs
MPS IIIA	*SGSH*	N-sulfoglucosamine sulfhydrolase	Heparan sulfate (primary storage) and dermatan sulfate (secondary storage)
MPS IIIB	*NAGLU*	α-N-acetylglucosaminidase	
MPS IIIC	*HGSNAT*	Acetyl-CoA:α-glycosaminide acetyltransferas	
MPS IIID	*GNS*	N-acetylglucosamine 6-sulfatase	

the first signs of dementia appear before age of 6 years in 83 % of type A patients, compared to only 24 % and 33 % of type B and C patients, respectively.

Other factors that may influence the varying severity and timing of symptoms from different subtypes of Sanfilippo disease remain a question mark. Many of these symptoms are neurological manifestations. One group of factors known for their role in the process of neurodegeneration are abnormally folded proteins, which form protein aggregates that impair normal cell function, and thus, neurotransmission. Such aggregates are thought to be a major cause of much more common neurological diseases, such as Alzheimer's disease (AD) (β-amyloid and hyperphosphorylated form of tau protein (p-tau)), Parkinson's disease (PD) (α-synuclein), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD) (TDP43) [9,10]. There are generally two major types of protein aggregates, described as amorphous aggregates and amyloid fibrils [11]. The former type consist of aggregates which are shaped irregularly and lack higher-ordered structures, while the latter type is characterized by highly ordered structures that are formed from soluble monomers of proteins. The process of formation of such aggregates proceeds through the oligomerization which can be caused by changes in the protein conformation, chemical modification, and other factors [11]. Then, depending on specificity of proteins, the aggregates may form characteristic structures like senile plaques (β-amyloid) or neurofibrillary tangles (p-tau).

In the case of MPS III, the presence of aggregates of β-amyloid, α-synuclein, p-tau, and prion protein was identified in mice with MPS IIIA and IIIB [12–16]. Clusters of α-synuclein were also found in cortical neurons postmortem in two patients with MPS IIIA [17] and a patient with MPS IIIB [18]. However, in the case of the latter one, no p-tau protein or β-amyloid was observed, in contrast to the previously mentioned studies with mouse models. Another study was also conducted post-mortem with the brains of MPS III patients (without indication of a specific subtype) and the brains of a goat model of MPS IIID [19]. Neither neurofibrillary tangles formed by p-tau nor senile plaques formed by β-amyloid were detected in any of the MPS brains studied. However, a 3-fold increase in the number of short β-amyloid peptides (especially β1-40) was detected relative to controls in both models studied [19]. To summarize, reports on the occurrence of protein aggregates show a great contradiction in the results obtained by different research groups on different models, and most often apply only to MPS IIIA and IIIB. Most probably, the differences between the results arose from using different disease models and different sources of the biological material (mouse and goat models of Sanfilippo disease, post-mortem samples of human brains), and performing separate studies usually with only one or two subtype(s) of the disease in one work.

In light of the above described uncertainty, the aim of this work was to comprehensively assess the levels of protein aggregates (β-amyloid, p-tau, α-synuclein, TDP43) in fibroblasts derived from patients with all subtypes of MPS III (A, B, C and D), and to test if lowering the GAG levels in the cells may result in a decrease in the levels of the above-mentioned proteins and the number of aggregates they form. Such an assessment has not yet been performed simultaneously in all subtypes of Sanfilippo syndrome, and has not been correlated with the severity of neurological symptoms present. We suspected that such studies, though allowing better understanding the molecular mechanisms of the disease, might also indicate novel, potential therapeutical targets in this as yet uncurable disease.

Table 2
Characterization of the MPS III cell lines used in the study.

MPS III subtype	Stored GAGs[a]	Defective enzyme	Mutation type	Catalog number of the cell line[b]
IIIA	HS (primary storage) and DS	N-sulfoglucosamine sulfhydrolase	p. Glu447Lys/ p.Arg245His	GM00879
IIIB	(secondary storage)	α-N-acetylglucosaminidase	p. Arg626Ter/ p.Arg626Ter	GM00156
IIIC		Acetyl-CoA:α-glycosaminide acetyltransferase	p. Gly262Arg/ pArg509Asp	GM05157
IIID		N-acetylglucosamine 6-sulfatase	p. Arg355Ter/ p.Arg355Ter	GM05093

[a] Abbreviations: DS, dermatan sulfate; HS, heparan sulfate.
[b] Catalog numbers are according to cell line description in Coriell Institute.

2. Materials and methods

2.1. Cell lines and cell cultures

Dermal fibroblast lines were collected from patients with MPS III A, B, C, D. They were purchased from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research (Table 2). The donor patients for these cell lines were characterized by typical features of disease severity, age of onset and life expectancy for each disease subtype. The HDFa cell line was used as a control. Fibroblasts were cultured under standard laboratory conditions in Dulbecco's Modifies Eagle Medium (DMEM), supplemented with antibiotics and 10 % fetal bovine serum (FBS). The cell cultures used in the experiments were between 7th and 11th passages.

2.2. Genistein

Genistein (4′,5,7-Trihydroxyisoflavone or 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), purchased from Pharmaceutical Research Institute (Warsaw, Poland; #021014GI) was used at a final concentration of 50 μM in DMSO. DMSO (D8418-1 L, Sigma Aldrich) was employed as a control.

2.3. Transcriptomic analyses

Transcriptomic analyzes were performed using the RNAseq method, exactly as described previously [20]. The raw data of these analyses have been deposited in the NCBI Sequence Read Archive (SRA), under accession no. PRJNA562649.

To classify transcripts, the KEGG database was used for the following processes: pathways of neurodegeneration - multiple diseases; Alzheimer disease; Parkinson disease, and Amyotrophic lateral sclerosis (https://www.genome.jp/kegg/kegg1.html; accessed on 18 February 2024).

2.4. Mouse model of MPS IIIB

The animal studies were conducted with a mouse model of MPS IIIB (B6·129S6-Naglu^{tm1Efn}/J; Strain #:003827) *Naglu*$^{-/-}$ from The Jackson Laboratory (Sacramento, CA, USA) and the *Naglu*$^{+/+}$ mouse line

(control). Both males and females were used. Because of the lack of significant differences between males and females in all tested parameters within both MPS IIIB and control groups, the results were merged, and thus, each analyzed group consisted of 8 animals (4 males and 4 females).

The experiments were carried out according to the guidelines of the European Communities Council Directive (2010/63/UE) and approved by the Local Ethics Committee for Animal Experiments in Bydgoszcz (application approval no. BUD13/2020). The animals were housed in polycarbonate cages, under conditions of regular light/dark cycles (12 h day/12 h night; lights on/off at 06:00 a.m./p.m.), constant temperature (22 °C), 50–55 % humidity, and free access to food (standard pellets) and water. Morbital (2 ml/kg) was used to euthanize mice at the age of 30 weeks.

2.5. Analysis of protein levels by western blotting

Fibroblasts (6×10^5 cells) were passaged onto 10 cm diameter plates and left overnight. In some variants of the experiments, the cells were incubated in the presence of genistein (50 μM), to reduce the levels of GAGs, for 24 h. Fibroblasts were lysed with the lysis buffer (1 % Triton X-100, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.5, and a mixture of protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany; #05892791001 and #11873580001)) for 30 min and centrifuged (10 min, 12,000 rpm, 4 °C). When mice were used, they were euthanized with Morbital (2 ml/kg) at the age of 30 weeks. Following decapitation, the brains were extracted. Tissue samples were incubated with T-PER™ Tissue Protein Extraction Reagent (cat.no. 78510, Thermo Scientific™) and a mixture of protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany; #05892791001 and #11873580001)), and homogenized for 30 s with maximum speed using Bead Ruptor Elite (cat. No. SKU 19-040 E, Omni International) to obtain a homogenous mixture. The samples were then centrifuged for 10 min (12,000 rpm, 4 °C). Protein lysates were transferred to new Eppendorf tubes. Analysis of protein levels in lysates was conducted using Bradford reagent. Proteins were separated using the WES system (WES - Automated Western Blots with Simple Western; ProteinSimple, San Jose, California, USA), with a 12–230 kDa separation module (#SM-W003), and detected using an Anti-Mouse (#DM-002) or Anti-Rabbit ((#DM-001) detection module, according to the manufacturer's instructions. The Total protein module (#DM-TP01, ProteinSimple, San Jose, CA, USA) was used as a loading control. The following primary antibodies were used in the study: anti-α-synuclein, anti-β-amyloid, anti-tau, anti-p-tau and anti-TDP43 (1:50) (E4U2F, #51510 Cell Signaling; B-4; sc-28365, Santa Cruz Biotechnology; A-10; sc-390,476; Santa Cruz Biotechnology; PHF-13; sc-32275; Santa Cruz Biotechnology; G400, #3448 Cell Signaling).

2.6. Immunofluorescence

Fibroblasts (4×10^4 cells) were passaged onto coverslips in 12-well plates and left overnight. The next day, cells were fixed with 2 % paraformaldehyde in phosphate-buffered saline (PBS) and washed with 0.1 % Triton X-100 in PBS. The fibroblasts were then blocked with 5 % BSA and 1.5 % glycine in PBS for 1 h. Cells were incubated in primary antibodies anti-α-synuclein at dilution 1:100, anti-β-amyloid at 1:100, anti-tau at 1:400, anti-p-tau at 1:100 and anti-TDP43 at 1:100 (E4U2F, #51510 Cell Signaling; B-4; sc-28365, Santa Cruz Biotechnology; A-10;

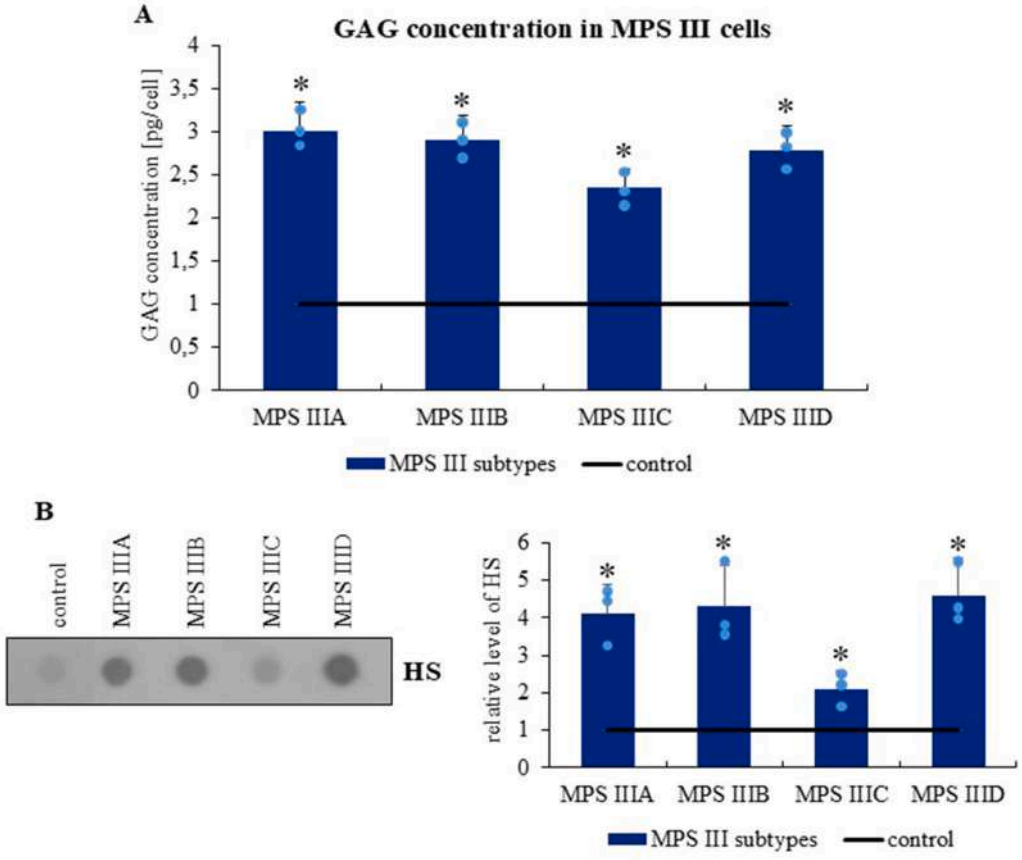

Fig. 1. Levels of total GAGs (A) and HS (B; representative blot and quantification) in fibroblasts derived from patients with MPS IIIA, B, C, and D, relative to control (healthy) cells. Bars represent mean values ± SD from three independent experiments. Results were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

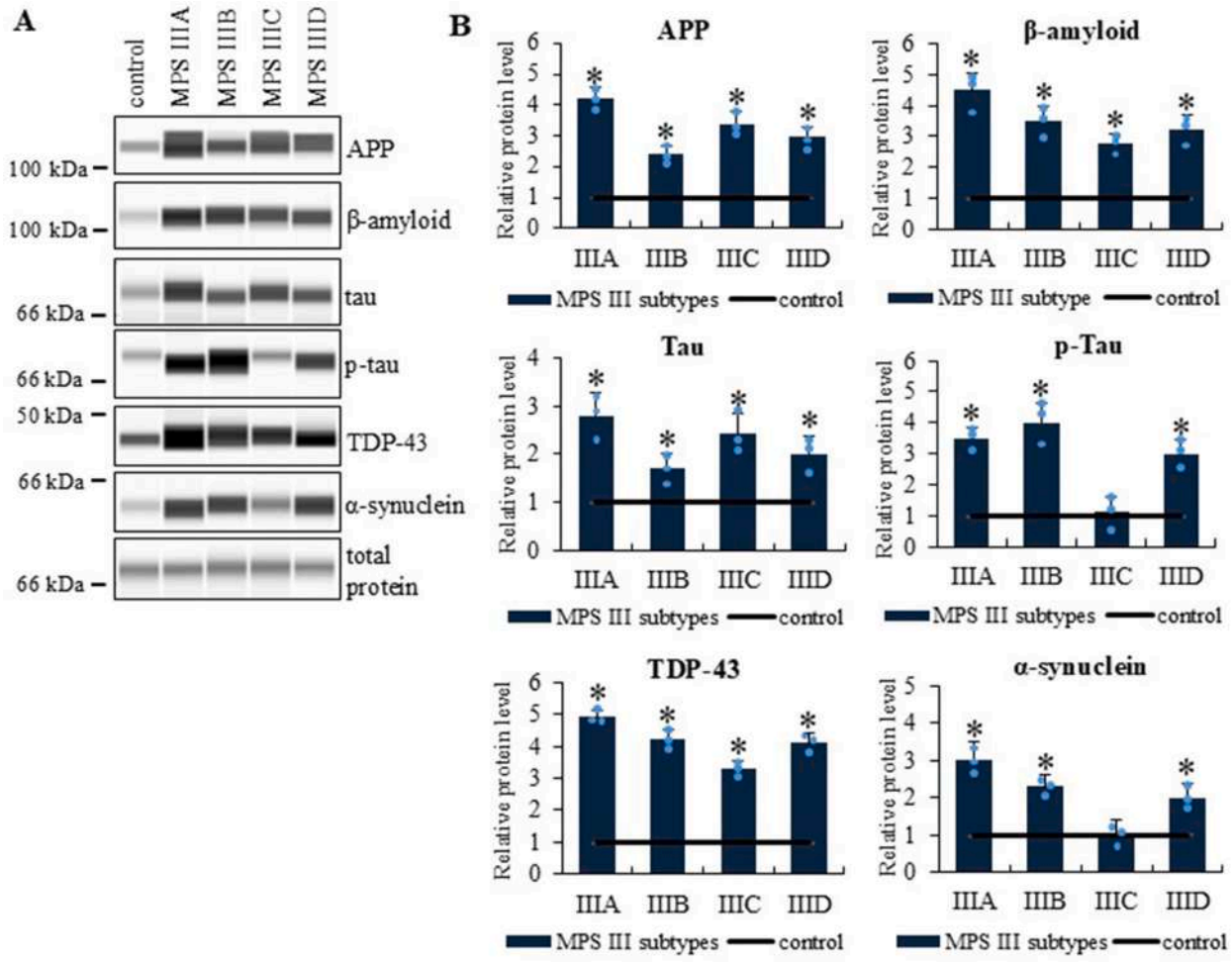

Fig. 2. Relative levels of APP, β-amyloid, tau, p-tau, TDP43 and α-synuclein in fibroblasts derived from patients with MPS IIIA, B, C, and D, relative to control (healthy) cells, as assessed by Western-blotting. In panel B, bars represent mean values ± SD from three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

sc-390,476; Santa Cruz Biotechnology; PHF-13; sc-32275; Santa Cruz Biotechnology; G400, #3448 Cell Signaling) in BSA overnight. The next day, the slides were washed 3 times with PBS and incubated in secondary anti-mouse or anti-rabbit antibodies (both 1:500) in PBS for 1 h. Cells were then washed 5 times with PBS. The coverslips were attached to glass slides using a mounting medium with DAPI dye. Images were taken using a Nikon Eclipse E800 microscope. Fifty images of each sample were analyzed. The exposition time was 2–3 s (depending on the protein), the gain level was 5, and intensity was 10. Analyses of fluorescence intensity and number of foci, considered as indicators of protein aggregates, were evaluated using the ImageJ software, according to instructions provided by the manufacturer. The fluorescence intensity and of number of foci (aggregates) were normalized per cell.

2.7. Measurement of glycosaminoglycan (GAG) levels using the Blyscan™ kit

Cellular GAG levels were measured in the obtained cell lysates using the Glycosaminoglycan Assay Blyscan™ kit (Biocolor Life Science Assays). The GAG levels were calculated according to the manufacturer's instruction.

2.8. Measurement of heparan sulfate (HS) levels using the dot-blot procedure

Cells were cultured and samples were prepared as described in the subsection 'Analysis of protein levels by Western blotting'. Following lysis of cells, protein concentration was determined, and macromolecules were fixed directly to the membrane, employing the dot/slot-blot apparatus (Bio-Rad). The blocking reaction was conducted with 5 % nonfat dry milk in the PBST buffer. Then, the membrane was incubated with monoclonal antibody against heparan sulfate (Novus Biologicals, USA; #NBP2-23523), diluted 1:500. Secondary antibody (goat anti-mouse antibody conjugated with horseradish peroxidase (Novus Biologicals, USA; #HAF008)) was added (dilution 1:500) and the incubation was conducted for 1 h at room temperature. For signal developing, the membrane was treated with a solution of substrates for horseradish peroxidase, and then exposed to the X-Ray film.

2.9. Statistical analysis

Statistical analysis of results obtained in biochemical and microscopic measurements was performed using one-way ANOVA with Tukey's *post-hoc* test. Statistica 12.5 software was used for statistical analysis. Differences between groups were considered significant when $p < 0.05$.

For statistical analyses of transcriptomic data, the R software version 3.4.3 was used. One-way ANOVA and post hoc Student's *t*-test with Bonferroni correction was employed to assess statistical significance between two normally continuous groups with $\log_2 (1 + x)$ values. To calculate the false discovery rate (FDR), the Benjamini-Hochberg method was used.

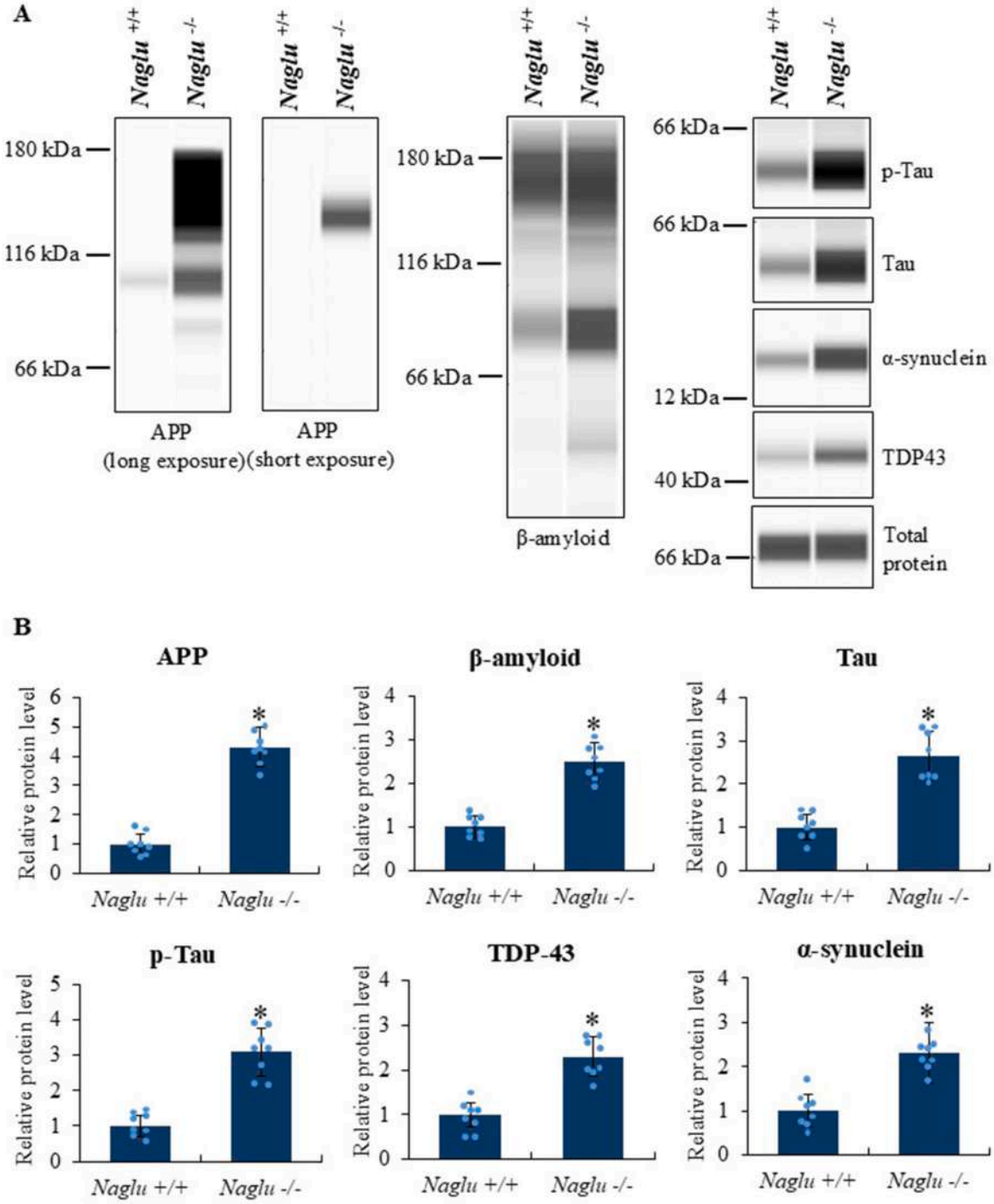

Fig. 3. Relative levels of APP, β-amyloid, tau, p-tau, TDP43 and α-synuclein in the brain of from 8 mice (n = 8; 4 males and 4 females). Results were normalized to the values measured for the control mice (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS IIIB and control mice.

3. Results

The study was conducted using fibroblast lines derived from patients with all subtypes of Sanfilippo disease (MPS IIIA, B, C and D). Although the use of fibroblasts might be considered a limitation of this study, the advantage was to use patient-derived cells, while the availability of human MPS neurons is negligible. This is especially important in the light of the fact that MPS III is a rare and severe disease. Moreover, there are many examples of works where it was demonstrated that cellular mechanisms investigated in fibroblasts reflect those occurring in other types of cells, including neurons, especially due to metabolic and biochemical relationships between fibroblasts and neurons, as analyzed recently [21].

Since MPS diseases are characterized by accumulation of GAG(s), we have confirmed that in all MPS III lines used their levels were significantly higher than in control cells (Fig. 1). Note that although HS is the primary GAG accumulating in Sanfilippo disease, there is also a secondary DS storage in MPS III cells, including fibroblasts [22]. Therefore, we considered that it is more appropriate to refer to GAGs, rather than solely to HS, to address the problem more comprehensively. Nevertheless, we also measured HS levels using specific antibodies and the dot-blot procedure. The profiles of GAG and HS levels were similar

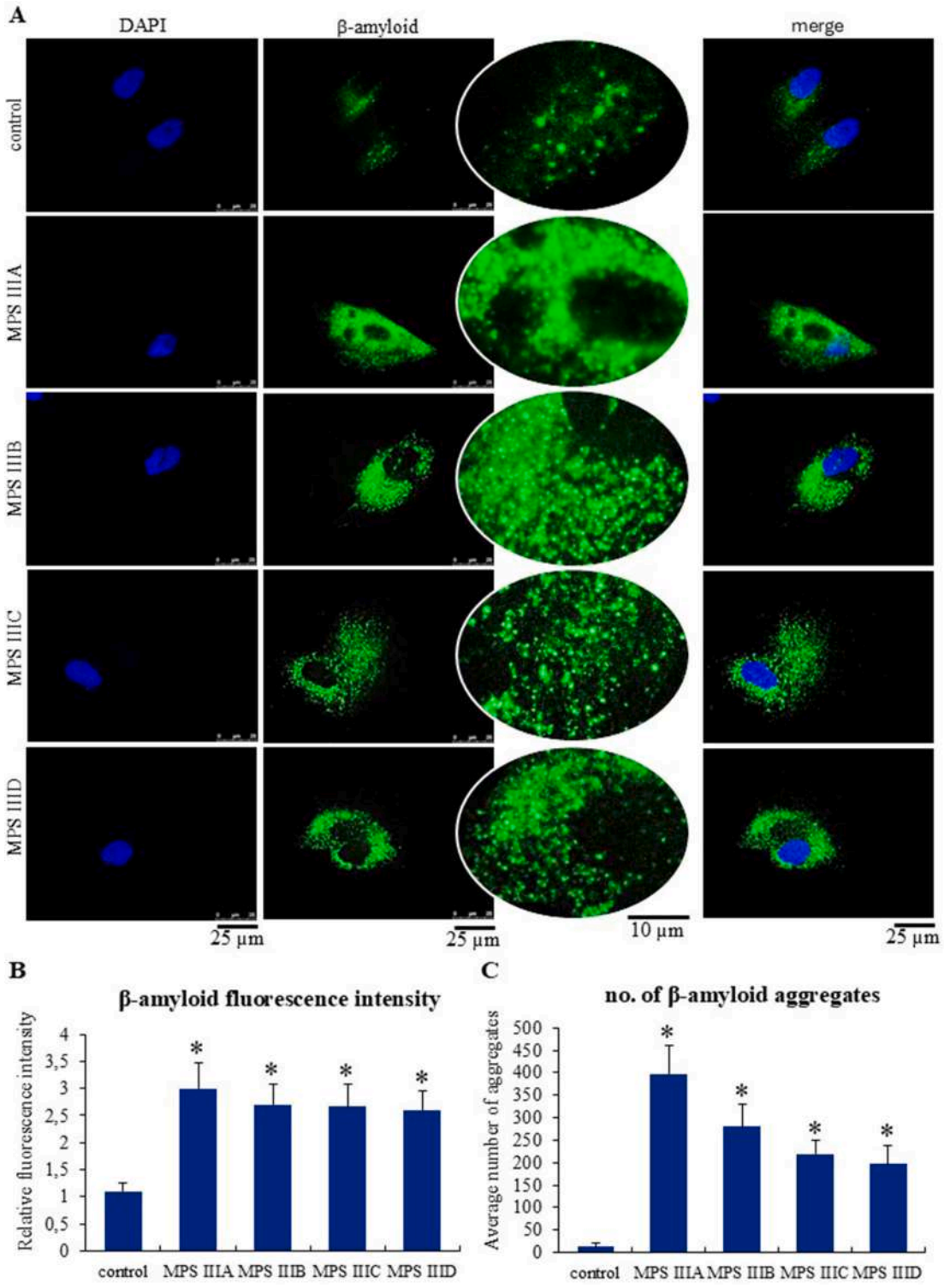

Fig. 4. Fluorescence intensity and number of aggregates formed by β-amyloid in fibroblasts derived from patients with MPS IIIA, B, C, and D, relative to control (healthy) cells. In panel A, scale bars indicating 25 μm or 10 μm (in regular and enlarged micrographs, respectively) are shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiment. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

among investigated cell lines, confirming the correctness of the methods used in these experiments (Fig. 1). The GAG/HS accumulation efficiency was the smallest in the MPS IIIC line, likely due to the highest residual activity of the deficient lysosomal enzyme (the residual activities of the deficient enzymes were lower than 1 % of the normal activities in MPS IIIA, B, and D lines, while >10 % in the MPS IIIC line, as reported recently using the same fibroblasts lines [7]).

There have been reports linking GAG levels to the accumulation of protein aggregates [13,23,24]. Therefore, we aimed to determine the levels of APP, β-amyloid, tau, p-tau, α-synuclein, and TDP43 proteins in MPS III cell lines. To estimate levels of investigated proteins and to visualize the aggregates formed by these proteins, Western blotting and

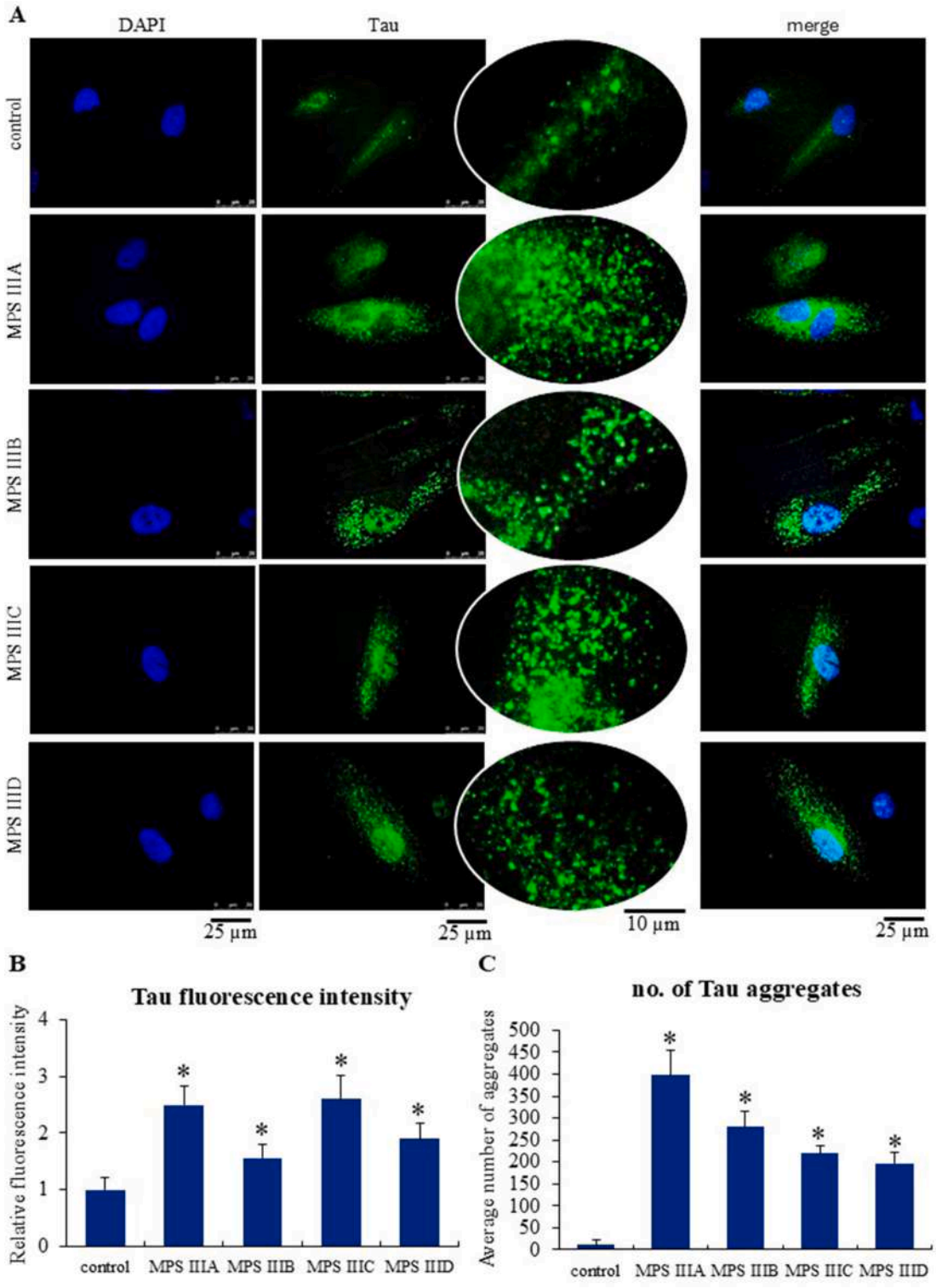

Fig. 5. Fluorescence intensity and number of aggregates formed by the tau protein in fibroblasts taken from patients with MPS IIIA, B, C and D compared to control (healthy) cells. In panel A, scale bars indicating 25 μm or 10 μm (in regular and enlarged micrographs, respectively) are shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

immunofluorescence analyses were carried out, respectively, using specific antibodies directed against the aforementioned proteins.

The results of these experiments indicated that levels of both β-amyloid precursor protein (APP) and mature β-amyloid were significantly elevated in all MPS III lines tested. Similar elevations were observed for the tau protein and TDP43. Large increases in levels of p-tau and α-synuclein proteins were also observed in MPS IIIA, B and D cells, but not the MPS IIIC line (Fig. 2; full-size blots to all relevant figures are shown in Supplementary Fig. S1).

To confirm that the changes in proteins' abundances, observed in MPS III fibroblasts, occur also at the organismal level, we have used a mouse model of MPS IIIB. This model allowed also to confirm the

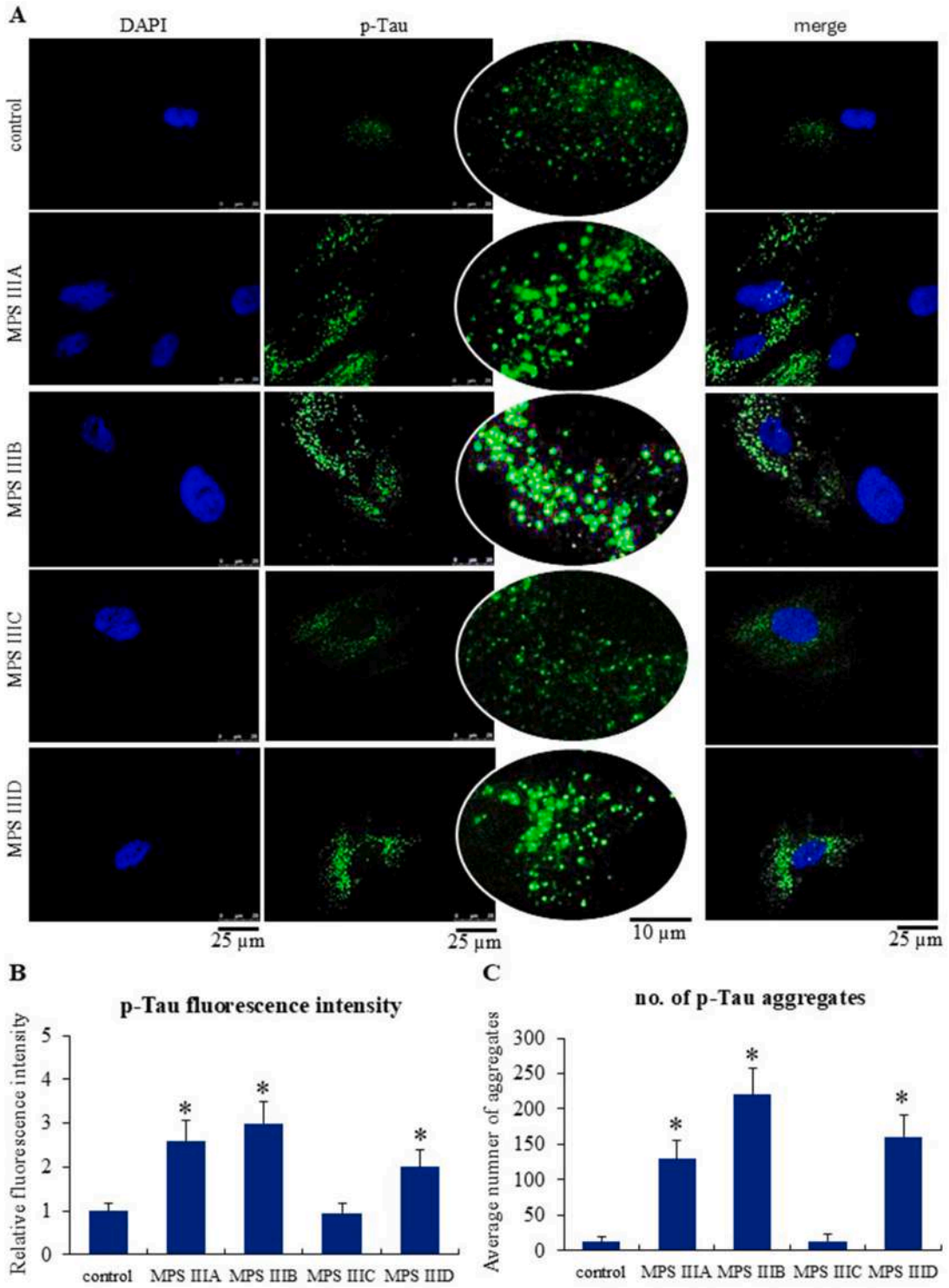

Fig. 6. Fluorescence intensity and number of aggregates formed by the p-tau protein in fibroblasts taken from patients with MPS IIIA, B, C and D compared to control (healthy) cells. In panel A, scale bars indicating 25 μm or 10 μm (in regular and enlarged micrographs, respectively) are shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

occurrence of the observed changes in neural cells. Analysis of the neural tissue isolated from MPS IIIB mice indicated a significant increase in the levels of all proteins tested relative to control (*Naglu*$^{+/+}$) mice (Fig. 3). No significant differences between sexes of animals were observed in these experiments, thus, all mice of the given genotype were analyzed together, within a single group.

During protein visualization by immunofluorescence, the fluorescence intensity and number of protein aggregates formed by β-amyloid, p-tau, TDP43 and α-synuclein were analyzed. The results of these experiments indicated an increase in fluorescence intensity for β-amyloid and an increased number of aggregates (visualized as specific foci) formed by this protein in all MPS III cell lines (Fig. 4). A similar situation was observed for the tau protein (Fig. 5). Increased fluorescence intensity and number of p-tau aggregates (foci) were noted in MPS

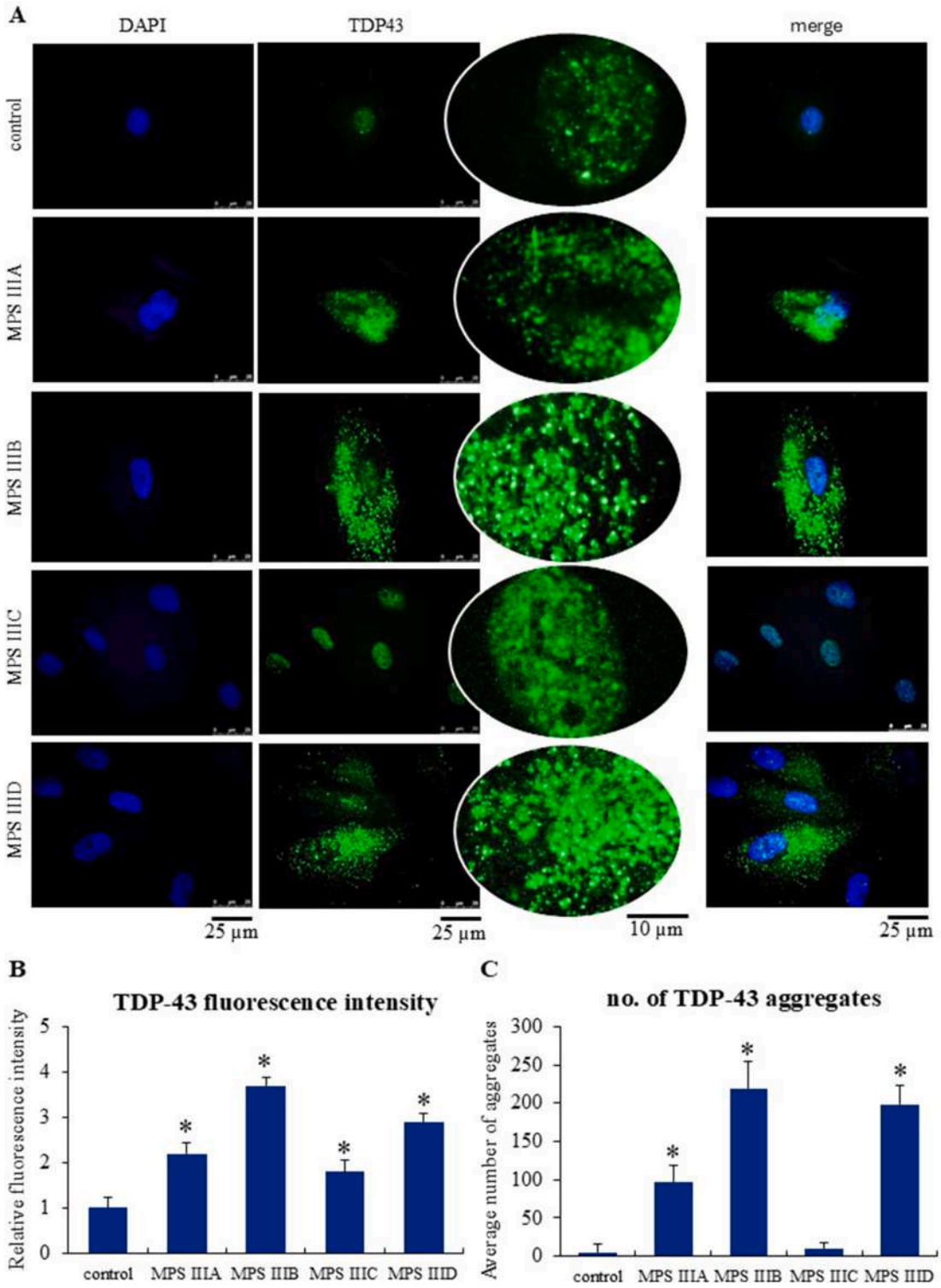

Fig. 7. Fluorescence intensity (total fluorescence was measured) and number of aggregates formed by TDP43 in fibroblasts derived from patients with MPS IIIA, B, C, and D, relative to control (healthy) cells. In panel A, scale bars indicating 25 μm or 10 μm (in regular and enlarged micrographs, respectively) are shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

IIIA, IIIB, and IIID cell lines, but not in the IIIC fibroblast line (Fig. 6). Similarly, protein aggregates (foci) formed by TDP43 and α-synuclein were not observed in the MPS IIIC cell line, despite being present in MPS IIIA, IIIB, and IIID fibroblasts (Figs. 7 and 8, respectively). The results of measuring the levels of individual proteins and the aggregates they form in MPS IIIA, B, C, and D cell lines are summarized in Table 3.

Since the cells of the MPS IIIC line have the lowest GAG levels among all the MPS III lines tested, and the lowest numbers of aggregates (foci) were recorded in the same line, the question about the relationship between the GAG levels and the levels of various proteins and number of aggregates in the cells appeared. To test the hypothesis about such a relationship, we decided to determine how could lowering the GAG

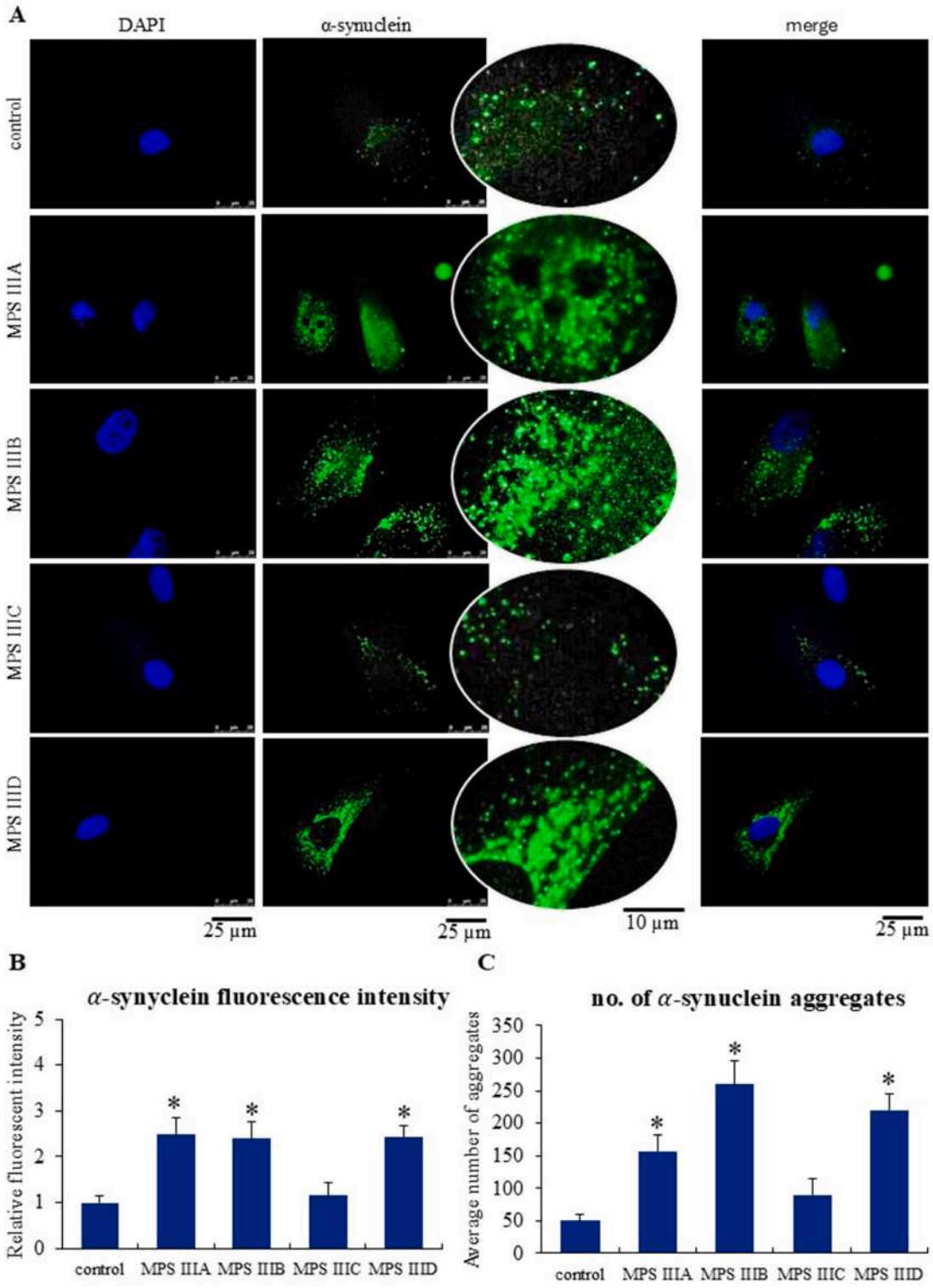

Fig. 8. Fluorescence intensity and number of aggregates formed by α-synuclein in fibroblasts derived from patients with MPS IIIA, B, C, and D, relative to control (healthy) cells. In panel A, scale bars indicating 25 μm or 10 μm (in regular and enlarged micrographs, respectively) are shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells.

levels affect the levels of the investigated proteins and the aggregates they form. In order to lower the levels of GAG in MPS III cells, we used genistein, one of the natural isoflavones, which, by inhibiting the activity of EGFR, impairs the specific signal transduction conducted by a kinase cascade, leading to a decrease in the efficiency of GAG synthesis, and resulting in lowering their levels in the cell [25]. Indeed, we demonstrated a significant decrease in GAG levels in all used MPS III lines treated with genistein (Fig. 9).

We found that genistein-mediated reduction of the GAG levels led to decreasing the levels of β-amyloid and its precursor (APP), as well as levels of the tau protein, to those observed in control cells, in all MPS III cell lines tested (Fig. 10). Normalization of the p-tau protein levels was

Table 3
Summary of the modulation of protein levels and the aggregates they form in all MPS III subtypes relative to control (healthy) cells.

Protein	Parameter	Effects in particular MPS III subtypes			
		A	B	C	D
APP	Protein level	↑	↑	↑	↑
	No. of aggregates	ND	ND	ND	ND
β-amyloid	Protein level	↑	↑	↑	↑
	No. of aggregates	↑	↑	↑	↑
Tau	Protein level	↑	↑	↑	↑
	No. of aggregates	↑	↑	↑	↑
p-tau	Protein level	↑	↑	–	↑
	No. of aggregates	↑	↑	–	↑
TDP43	Protein level	↑	↑	↑	↑
	No. of aggregates	↑	↑	–	↑
α-synuclein	Protein level	↑	↑	–	↑
	No. of aggregates	↑	↑	–	↑

* Abbreviations: ND – not detected; ↑, an increase in protein levels or the number of protein aggregates vs control cells; -, no change in protein levels or the number of protein aggregates vs control cells.

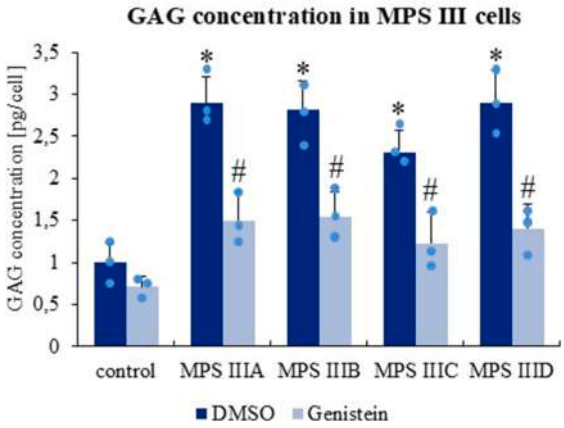

Fig. 9. Levels of GAG in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). Bars represent mean values ± SD from three independent experiments. Results were normalized to the value measured for the control cells without genistein treatment (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells, and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

also observed in MPS IIIA, B, and D lines, i.e. those where elevated levels of these proteins were described relative to healthy cells. The elevated levels of the TDP43 protein, previously observed in all MPS III fibroblast lines, also decreased under the influence of genistein, reaching values comparable to controls in the case of the MPS IIIA and IIID cells. However, there was no decrease in α-synuclein levels in any of the Sanfilippo disease fibroblast lines under the genistein treatment conditions (Fig. 10).

Fluorescence microscopy analysis also indicated a decrease in fluorescence intensity and lowering the number of aggregates (foci) formed by β-amyloid in MPS III cells incubated in the presence of genistein (Fig. 11). Similar results were observed for the tau protein (Fig. 12). The use of genistein also led to a significant reduction in the levels of fluorescence and the number of aggregates (foci) formed by p-tau in the lines where its excess was observed (MPS IIIA, B, and D) (Fig. 13). A similar phenomenon was detected for aggregates (foci) formed by TDP43. Under the influence of genistein, a decrease in the fluorescence intensity of this protein was noted in all MPS III lines, as was a decrease in the number of aggregates (foci) in the lines where it was originally elevated (i.e., MPS IIIA, B, and D) (Fig. 14). However, there was no decrease in the number of α-synuclein aggregates (foci) under the influence of genistein (Fig. 15). A summary of the effects of genistein on the levels of all the proteins listed above, and the numbers of protein aggregates (foci) they form in MPS IIIA, B, C and D fibroblast lines, is shown in Table 4.

In order to search for a common mechanism of protein aggregate deposition in MPS III and other neurodegenerative diseases in which the same proteins accumulate, a transcriptomic analysis was conducted using fibroblasts derived from patients with Sanfilippo disease. Based on the KEGG database, transcripts known for their roles in the development of diseases such as AD (KEGG: Alzheimer's disease [hsa05010 for Homo sapiens]), PD (KEGG: Parkinson's disease [hsa05012 for Homo sapiens]), and ALS (KEGG: Amyotrophic lateral sclerosis [hsa05014 for Homo sapiens]) were selected.

The results of these experiments indicated a high number of transcripts with changes in expression levels in MPS III cells, relative to healthy controls, which are also involved in the pathomechanisms of AD, ALS, and PD. The number of transcripts with altered expression in MPS III and known for their roles in the pathogenesis of AD is approximately 30 for the MPS IIIA cell line and an average of 20 for MPS IIIB, C, and D cell lines, with nearly 30 being transcripts that exhibit at least a 2-fold change in expression levels compared to control cells ($\log_2FC > 1$ or $\log_2FC < -1$). Examples of such transcripts include *APOE* and *MME*, encoding apolipoprotein E and membrane metal-endopeptidase, respectively. Changes in the expression levels of these and other genes linking MPS III to AD are indicated in a heatmap (Fig. 16). The list of genes with more than a 2-fold change ($\log_2FC > 1$ or $\log_2FC < -1$) along with the exact values of expression level changes in all lines of MPS III fibroblasts compared to controls is presented in Table S1.

Similarly, a large number of genes with altered expression in MPS III cells are known for their roles in the development of ALS symptoms. These numbers ranged from 13 (for the MPS IIIC cell line) to 30 (for the MPS IIIA cell line). An example of such a gene is *PFN1*, which encodes profilin, an actin-binding protein. The increase in the expression level of this gene is indicated in all tested MPS III cell lines. Changes in the expression levels of *PFN1* and other genes linking MPS III to ALS are shown on a heatmap (Fig. 17), while the list of genes with more than a 2-fold change ($\log_2FC > 1$ or $\log_2FC < -1$) along with the exact values of expression level changes in all MPS III fibroblast lines compared to controls is presented in Table S2.

The conducted analysis also indicated changes in gene expression patterns in MPS III cells that are associated with the pathogenesis of PD, which predominantly affected the MPS IIIA cells. The results showed significantly greater changes in the expression of certain PD-related genes in MPS IIIA fibroblasts, compared to other MPS cell lines, reaching up to 21 genes. These include *PARK7*, which is strongly down-regulated in MPS IIIA cells, and *UCHL1*, which is up-regulated in MPS IIIB and IIID fibroblast lines. These genes encode parkinsonism-associated deglycase (PARK7) and ubiquitin carboxy-terminal hydrolase L1, respectively. Changes in the expression levels of genes linking MPS III to PD are shown in a heatmap (Fig. 18). The list of genes with more than a 2-fold change ($\log_2FC > 1$ or $\log_2FC < -1$) along with the exact values of expression level changes in all MPS III cell lines compared to controls is presented in Table S3.

Many of the identified genes and their products are involved in the regulation of the formation or removal of toxic proteins or their aggregates from cells in AD, ALS, and PD. Their exact roles are discussed in the Discussion chapter.

4. Discussion

Until recently, it was thought that stored GAGs were the main, if not the only, cause of MPS. However, it turns out that enzyme replacement therapy (ERT) (used in clinical practice in MPS I, MPS II, MPS IVA, MPS VI and MPS VII), hematopoietic stem cell transplantation (HSCT) (used most often in the youngest patients with MPS I and II), gene therapy or

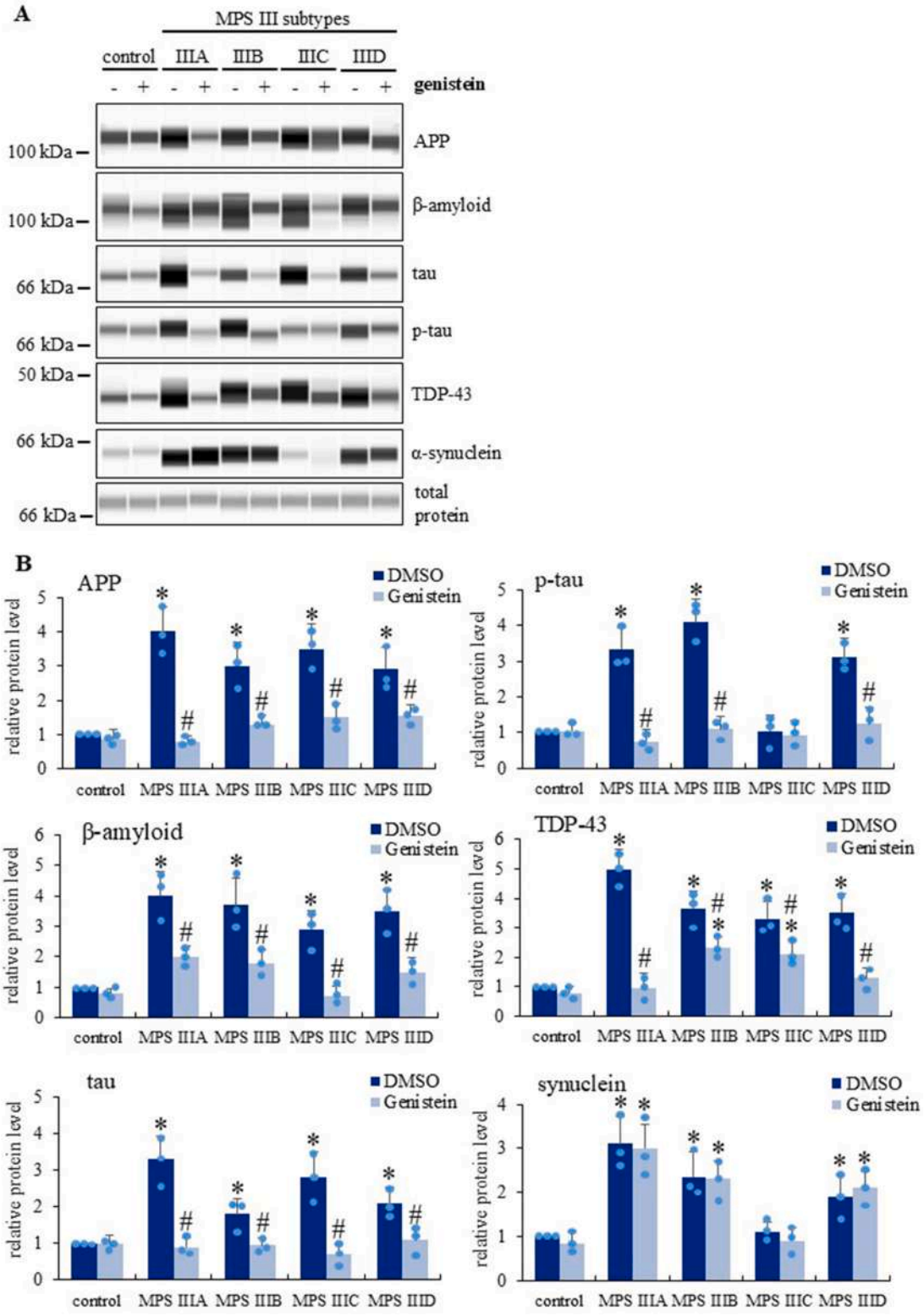

Fig. 10. Relative levels of APP, β-amyloid, tau, p-tau, TDP43 and α-synuclein in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control), as assessed by Western-blotting. Bars represent mean values ± SD from three independent experiments. Results were normalized to the values measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells, and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

substrate reduction therapy (SRT) (tested in preclinical studies and clinical trials) do not lead to complete correction of patients' symptoms, even if normalization of GAG levels is achieved. Moreover, a clear and strong argument against such a simple pathomechanism as the sole GAG storage is that their accumulation results in significant differences between symptoms of different MPS types and even subtypes [26–34].

MPS III (Sanfilippo disease) is characterized by four subtypes that have the primary HS (accompanied by a secondary DS) storage in

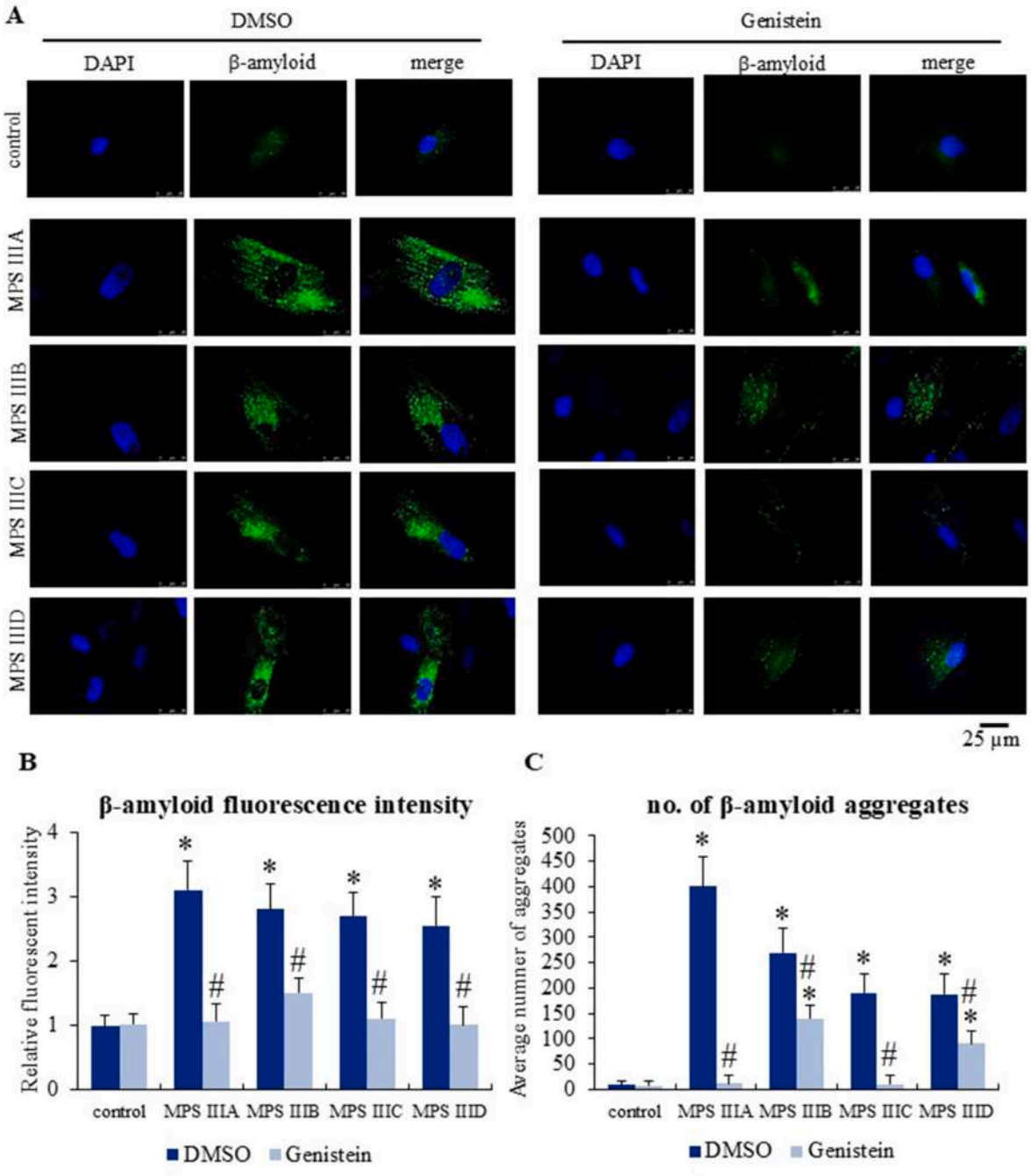

Fig. 11. Fluorescence intensity and number of aggregates formed by β-amyloid in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). In panel A, a scale bar indicating 25 μm is shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results in panel B were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells, and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

common. However, the subtypes differ in the frequency of individual symptoms resulting from central nervous system (CNS) damage such as epilepsy, dementia, autism spectrum disorders, behavioral changes, mental retardation, speech delay and others. They occur with variable severity and appear at different ages in patients with each subtype of MPS III [7,8].

It seems reasonable, therefore, to investigate pathogenic factors other than GAGs that may explain such a high variability of symptoms, especially those related to the nervous system. Similar symptoms, such as dementia or personality changes, appear in many patients suffering from other CNS diseases, like AD, PD, ALS or FTD. The accumulation of protein aggregates such as β-amyloid, p-tau, α-synuclein and TDP43 in cells is thought to be the cause of many of these diseases. These aggregates damage the normal functioning of nerve cells, leading to extensive neurodegeneration [9,10].

There are not many reports on the presence of protein aggregates in Sanfilippo disease. The results of studies performed with patients' brain scrapings or with mouse models of MPS IIIA and B can be considered contradictory, indicating the presence of aggregates of beta-amyloid, p-tau, alpha-synuclein or prion proteins [12–18] or their absence [18,19]. It also turns out that protein aggregates were found not only in nerve cells but also in the peripheral blood cells of MPS IIIB mice, showing a

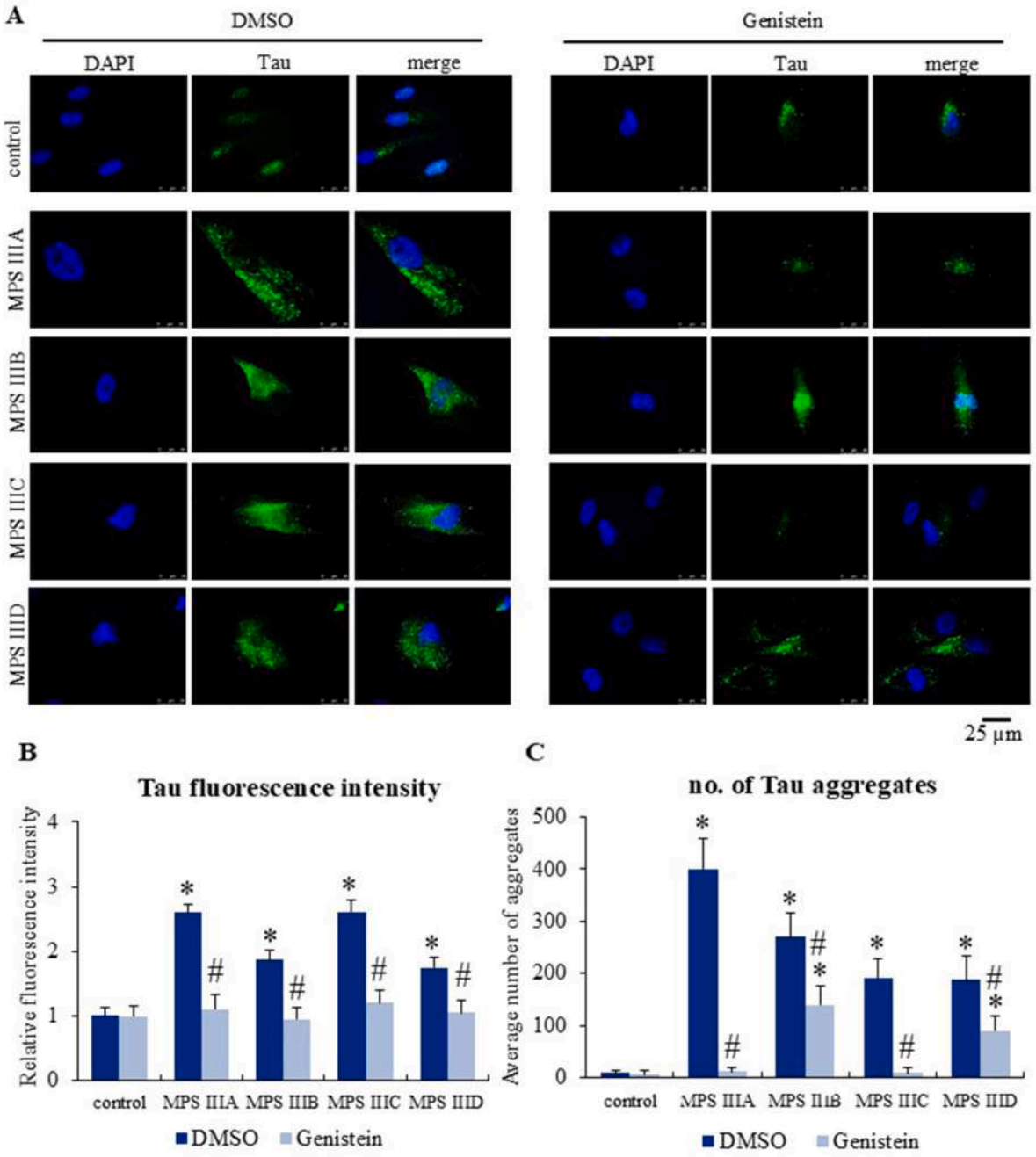

Fig. 12. Fluorescence intensity and number of aggregates formed by tau protein in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). In panel A, a scale bar indicating 25 μm is shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results in panel B were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

strong brain-blood correlation in amyloidosis and synucleinopathy. It was emphasized that molecular changes in blood may reflect the pathological state in CNS and provide a useful tool for identifying potential biomarkers of neurodegeneration for MPS III [14]. In addition, studies performed with a mouse model of MPS IIIA indicated that treatment of mice with CLR01 (an inhibitor of amyloid protein self-organization) reduced the pathological size of lysosomes which was accompanied by normalization of the efficiency of the autophagy process, reduction of CNS inflammation and improvement of memory deficits [12]. Protein aggregates were also found in MPS I which also has a neurodegenerative component [35]. On the other hand, experiments in which MPS IIIA mice were crossed with α-synuclein-deficient mice (Sncatm1Rosl/J) demonstrated that these double defective animals were behaviorally and histologically comparable to the MPS IIIA animals [36]. That study indicated that the rate, location and nature of protein aggregate depositions and inflammatory response in the brain of α-synuclein-deficient MPS IIIA mice mirrored those observed in MPS IIIA mice homozygous or heterozygous for the α-synuclein gene mutation.

Given the different results obtained by various research groups, the role of protein aggregates in the intensity of neurological symptoms in Sanfilippo disease is certainly worthy of thorough investigation. It is also worth mentioning that most studies have been performed using only MPS IIIA and IIIB models. Comparative studies indicating the presence or absence of aggregates of particular proteins in all subtypes of

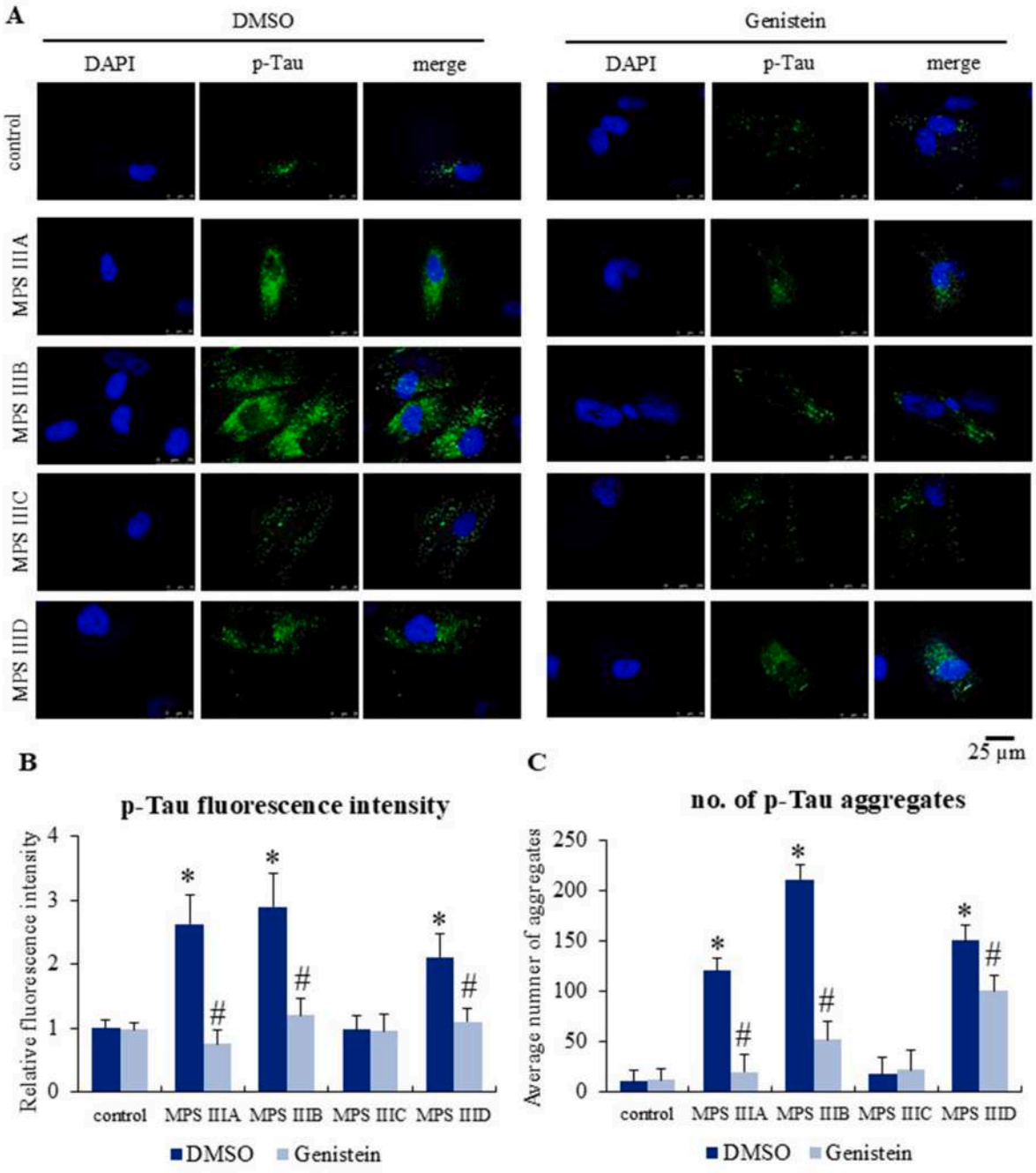

Fig. 13. Fluorescence intensity and number of aggregates formed by the p-tau protein in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). In panel A, a scale bar indicating 25 μm is shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results in panel B were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

Sanfilippo disease are lacking.

The purpose of our work was to comprehensively assess the levels of proteins known to fold abnormally in other neurological diseases such as AD (β-amyloid; p-tau), PD (α-synuclein), ALS and FTD (TDP43), to visualize the protein aggregates (observed as foci in fluorescent microscope) they form (if any), and to test if lowering the levels of GAGs in the cells might result in a reduction in the levels of the aforementioned proteins and the number of aggregates they form in fibroblasts derived from patients with all subtypes of MPS III (A, B, C, and D). Such an assessment has not yet been made simultaneously in all subtypes of Sanfilippo syndrome and has not been correlated with the severity of neurological symptoms present.

Our results demonstrated the elevated levels of APP (β-amyloid precursor), β-amyloid, tau, and TDP43 proteins in all tested MPS III cell lines, and elevated levels of p-tau and α-synuclein in all these lines except the MPS IIIC fibroblast line (Fig. 2). These results were confirmed in the neural tissue of MPS IIIB mice (Fig. 3). Fluorescence microscopy studies also indicated a high number of protein aggregates (foci) formed by β-amyloid and tau in all lines tested and a high number of aggregates of p-tau, TDP43 and α-synuclein in all lines except the MPS IIIC cell line (Figs. 4–8). It is worth noting that the TDP43 protein has never before been studied in the context of MPS pathogenesis. These results are,

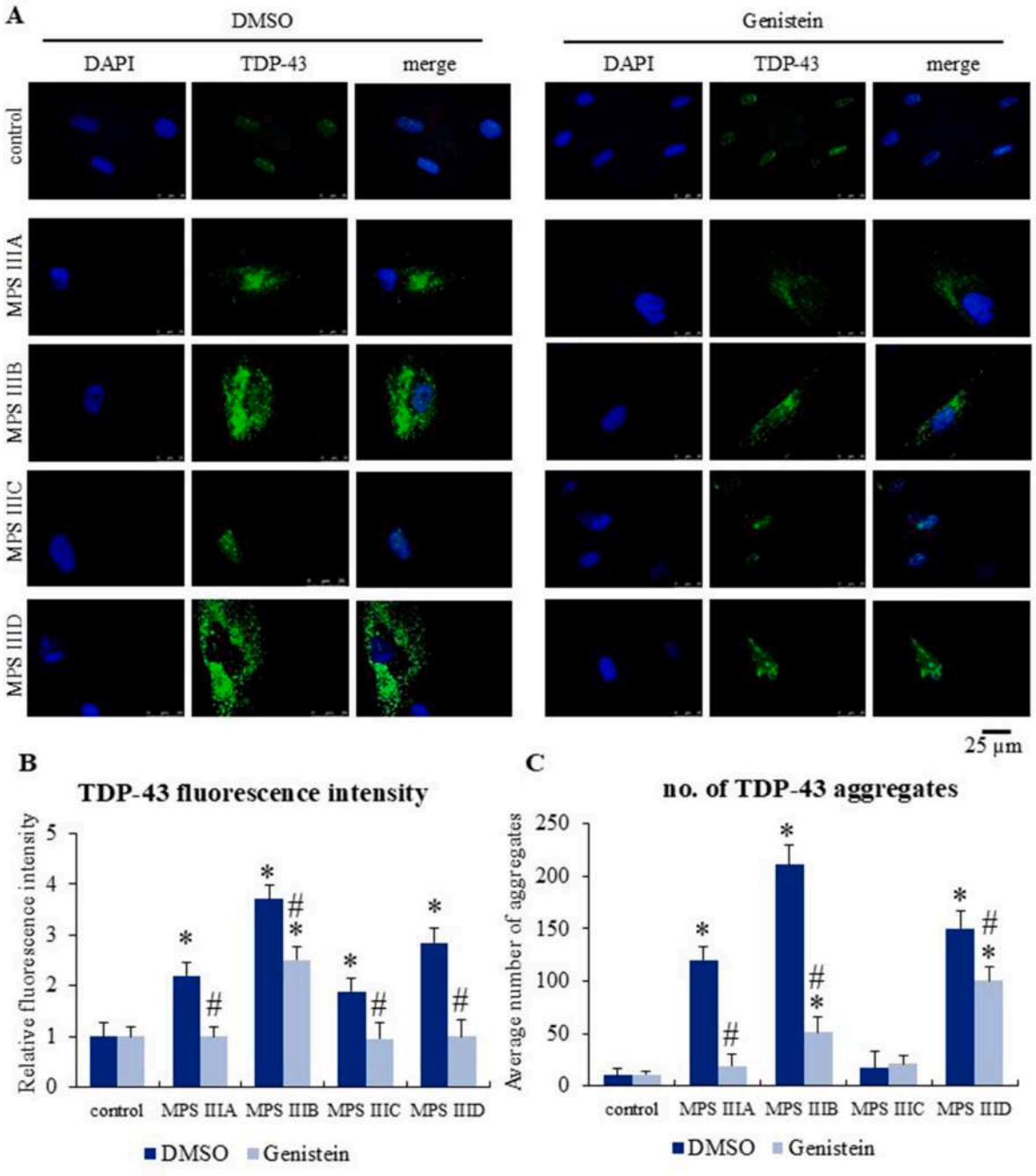

Fig. 14. Fluorescence intensity (total fluorescence was measured) and number of aggregates formed by the TDP43 protein in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). In panel A, a scale bar indicating 25 μm is shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD 50 images obtained in three independent experiments. Results in panel B were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS, and control cells and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-treated cells.

therefore, the first to indicate its elevated levels in Sanfilippo disease. In addition, it is worth noting that the number of aggregates (foci) formed by β-amyloid and the tau protein correlates with the severity of the disease, since MPS IIIA is considered the most severe in its course (the earliest onset, very rapid progression of symptoms, and short survival time) [37], and our study shows that it is in this subtype that the highest number of aggregates composed of these proteins was found. However, the use of only one MPS IIIA cell line indicates that this correlation needs to be confirmed in future studies.

Our attention was drawn to the results indicating the deposition of protein aggregates of p-tau, TDP43 and alpha-synuclein in MPS IIIA, B, and D cell lines, but not in the MPS IIIC line. The analyses of GAG levels clearly indicated that in the MPS IIIC cells the GAG storage is the lowest of all the cell lines tested (Fig. 1) (apparently due to the highest residual enzyme activity in this cell line among all tested fibroblasts [7]) which raises the question of the relationship of GAG levels to the level of individual protein aggregates. There are already reports in the literature about the possible role of GAGs in the formation of aggregates of various proteins. In a model of AD, the accumulation of HS and HS proteoglycans have been described as initiating events in the formation of β-amyloid aggregates [23]. Moreover, heparin was found to induce tau protein to form paired helical filaments composed of p-tau. In addition,

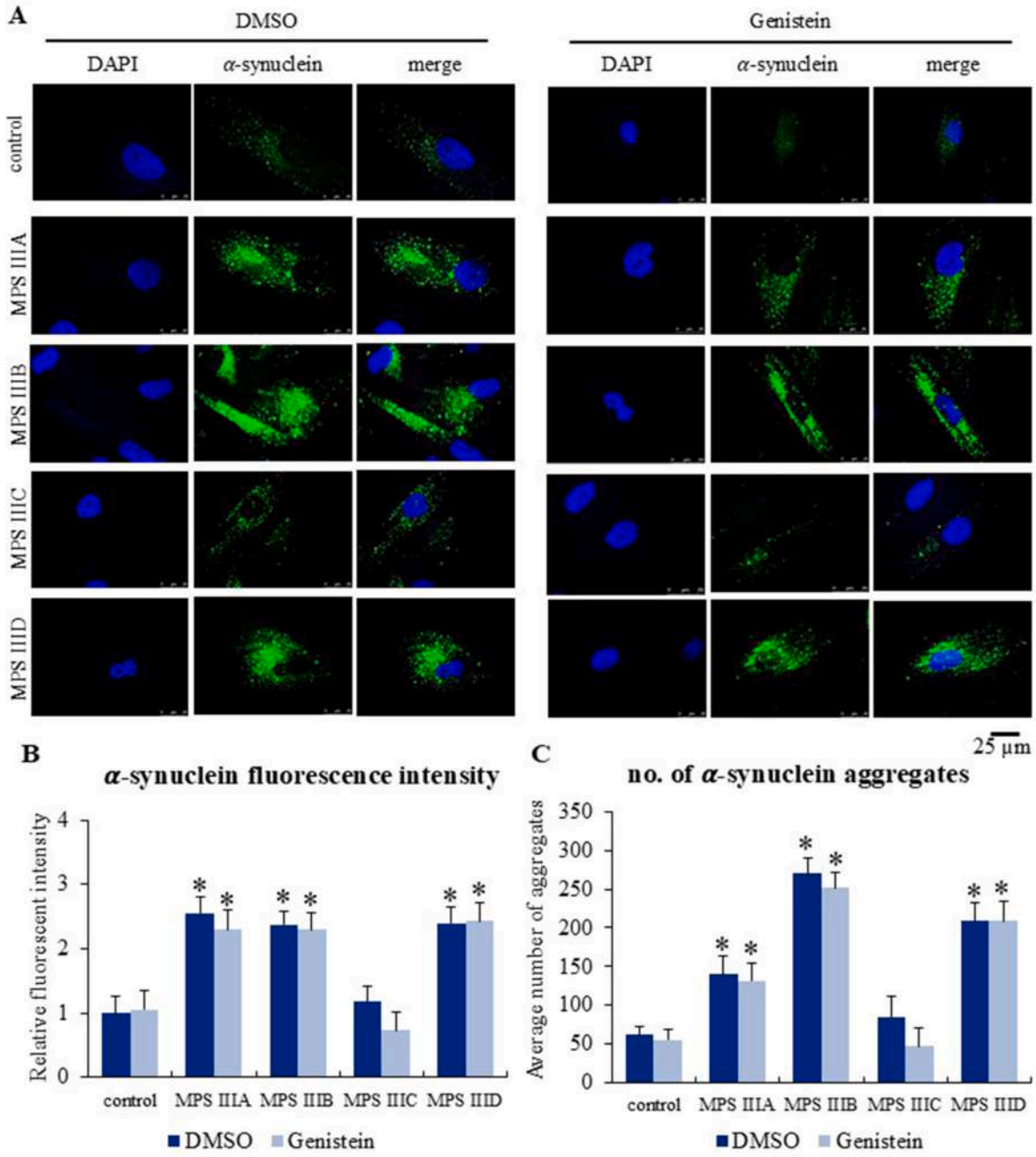

Fig. 15. Fluorescence intensity and number of aggregates formed by α-synuclein in fibroblasts derived from patients with MPS IIIA, B, C, and D, and control (healthy) cells treated with genistein (50 μM) or DMSO (control). In panel A, a scale bar indicating 25 μm is shown below the microscopic pictures. In panels B and C, bars represent mean values ± SD from 50 images obtained in three independent experiments. Results in panel B were normalized to the value measured for the control cells (assumed to be 1). (*) indicate statistically significant differences ($p < 0.05$) between MPS and control cells, and (#) indicate statistically significant differences ($p < 0.05$) between genistein-treated and non-genistein treated cells.

it was indicated that mutations resulting in impaired HS synthesis significantly reduced the accumulation of β-amyloid fibrils in the brain [23]. Similar studies have been carried out using a mouse model of MPS IIIA in which it was indicated that the deposition of multiple amyloid proteins in the brain follows primary GAG storage [13]. Those authors pointed to a pathological cascade of events initiated by the primary GAG accumulation and involving, in turn, protein aggregation and defects in autophagy [13]. The role of cathepsin B in this phenomenon has also been proposed. In a mouse model of MPS I, it was indicated that HS is responsible for the induction of cathepsin B activity in the brain which leads to β-amyloid deposition by APP metabolism [24]. Thus, the link between GAG levels and levels of toxic β-amyloid appears to be undeniable.

To further investigate this phenomenon, we conducted experiments using genistein, a natural EGFR inhibitor, resulting in reduced GAG levels in cells [25] (Fig. 9). The results of these experiments indicated that the reduction of GAG levels by genistein also led to reduction of abundance of the APP protein, β-amyloid, the tau protein, and TDP43. However, no reduction in the levels of α-synuclein was observed after genistein treatment (Fig. 10). Similarly, the number of protein aggregates (foci) formed by β-amyloid, the tau protein, p-tau, and TDP43 was reduced under the action of the applied flavonoid (Figs. 11–14) but not the number of aggregates of α-synuclein (Fig. 15). Our analysis clearly shows that GAG levels are related to the levels of APP, β-amyloid, tau,

Table 4
Effect of GAG reduction by genistein on protein levels and the aggregates they form in all MPS III subtypes relative to untreated cells.

Protein	Parameter	Effects of genistein in particular MPS III subtypes			
		A	B	C	D
APP	Protein level	↓	↓	↓	↓
	No. of aggregates	ND	ND	ND	ND
β-amyloid	Protein level	↓	↓	↓	↓
	No. of aggregates	↓	↓	↓	↓
Tau	Protein level	↓	↓	↓	↓
	No. of aggregates	↓	↓	↓	↓
p-tau	Protein level	↓	↓	–	↓
	No. of aggregates	↓	↓	–	↓
TDP43	Protein level	↓	↓	↓	↓
	No. of aggregates	↓	↓	–	↓
α-synuclein	Protein level	–	–	–	–
	No. of aggregates	–	–	–	–

* Abbreviations: ND, not detected; ↓, decrease in protein levels or the number of protein aggregates under the influence of genistein vs non-treated cells; -, no change in protein levels or the number of protein aggregates under the influence of genistein vs non-treated cells.

p-tau and TDP43 proteins, and number of the protein aggregates they form. However, no relationship was observed between GAG and the level and number of aggregates formed by α-synuclein.

The hypothesis about a role for GAG in the formation of protein aggregates would also be supported by studies on other neurodegenerative diseases that are caused by the aggregation of toxic proteins in nerve cells. The already mentioned studies with a model of AD, in which aggregates of β-amyloid and p-tau are characteristic, indicated high levels of GAG in patients [23]. A detailed study on this subject showed increased levels of chondroitin sulfate (CS) in prefrontal neocortical samples taken from AD patients [38]. 4-*O*-S CS was found in large amounts in senile plaques, while both 4-*O*-S and 6-*O*-S CS were found in neurofibrillary tangles of AD patients [39]. Moreover, in PD, the presence of CS (4-*O*-S and 6-*O*-S) with varying degrees of sulfation was detected in Lewy bodies [39].

HS proteoglycans have also been shown to be involved in the formation of amyloid precipitation [40]. In brains of AD patients, HS proteoglycan levels are increased significantly in areas with amyloid plaques and neurofibrillary tangles [41]. Examination of occipital neocortical and hippocampal tissue from AD patients revealed that β-amyloid plaques contained high levels of predominantly *N*-sulfated HS [42]. It was found that GAG proxy heparin has a remarkably high affinity for β-amyloid fibrils, which depend on the precise details of the fibril architecture [43]. HS also interacts with the tau protein by stabilizing its conformations and promoting phosphorylation [44]. Recent studies demonstrated that 3-*O*-S HS increased tau binding and promoted tau transport across membranes [45]. HS also has the ability to bind α-synuclein, affecting the conformation of the fibrils this protein forms. The proteoglycan of HS accelerates the formation of α-synuclein fibrils which leads to an increase in the level of the insoluble form of this protein [46]. It was also found that internalization of α-synuclein aggregates occurred in neuronal cells, a phenomenon that is strongly dependent on the degree of HS sulfation on the cell surface [47]. Therefore, the results of the studies presented in this report, as well as the literature data cited, indicate that sulfated GAGs uncover a major role in the formation of protein aggregates that are part of the pathogenesis of CNS diseases.

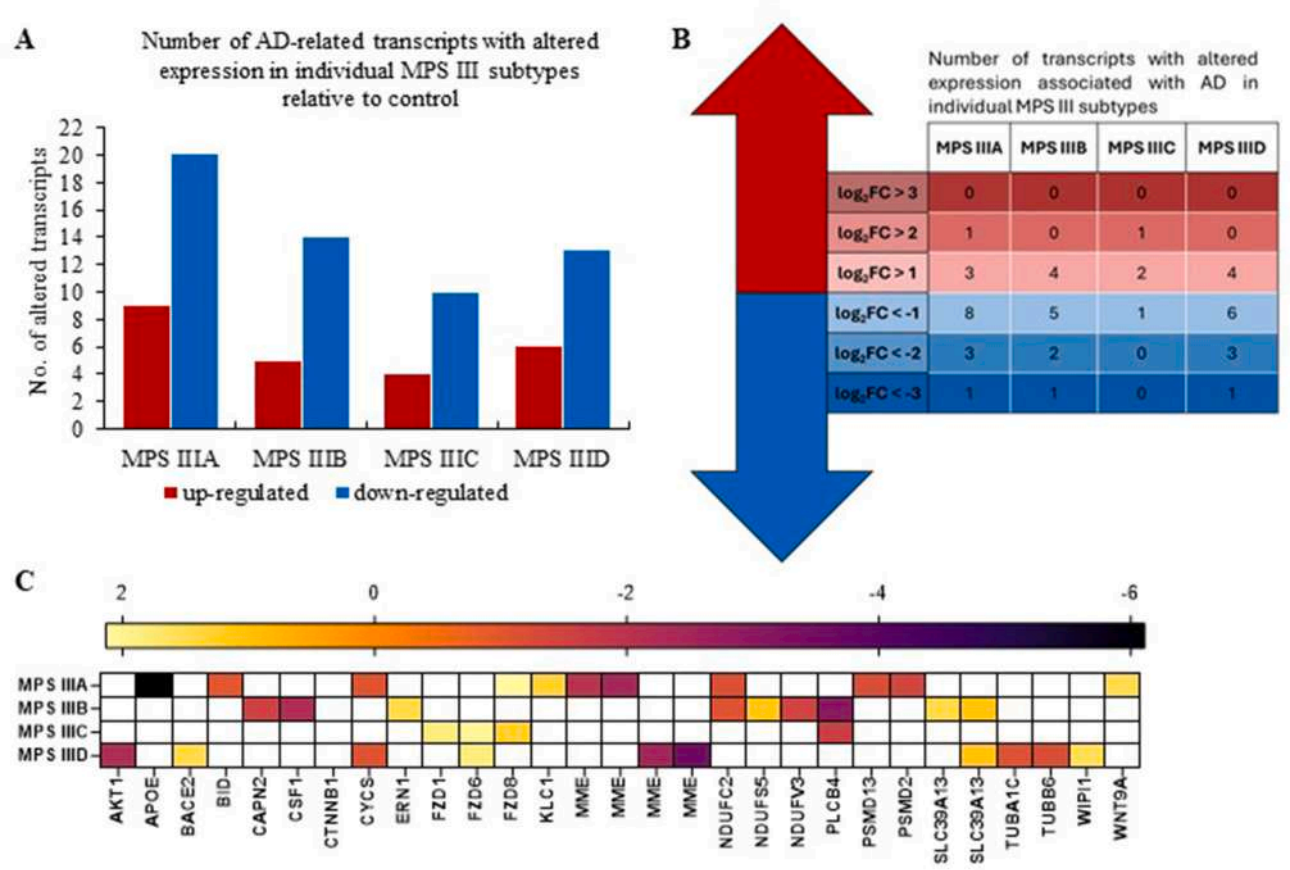

Fig. 16. Number of AD related-transcripts [hsa05010] undergoing changes in expression in different types of MPS III relative to control cells (A) and number of AD related-transcripts with altered expression depending on the level of fold-change (FC) value in different types of MPS III relative to control cells (B) as well as heatmap presenting differences in AD related-gene expression levels between different types of MPS III and control cells (C).

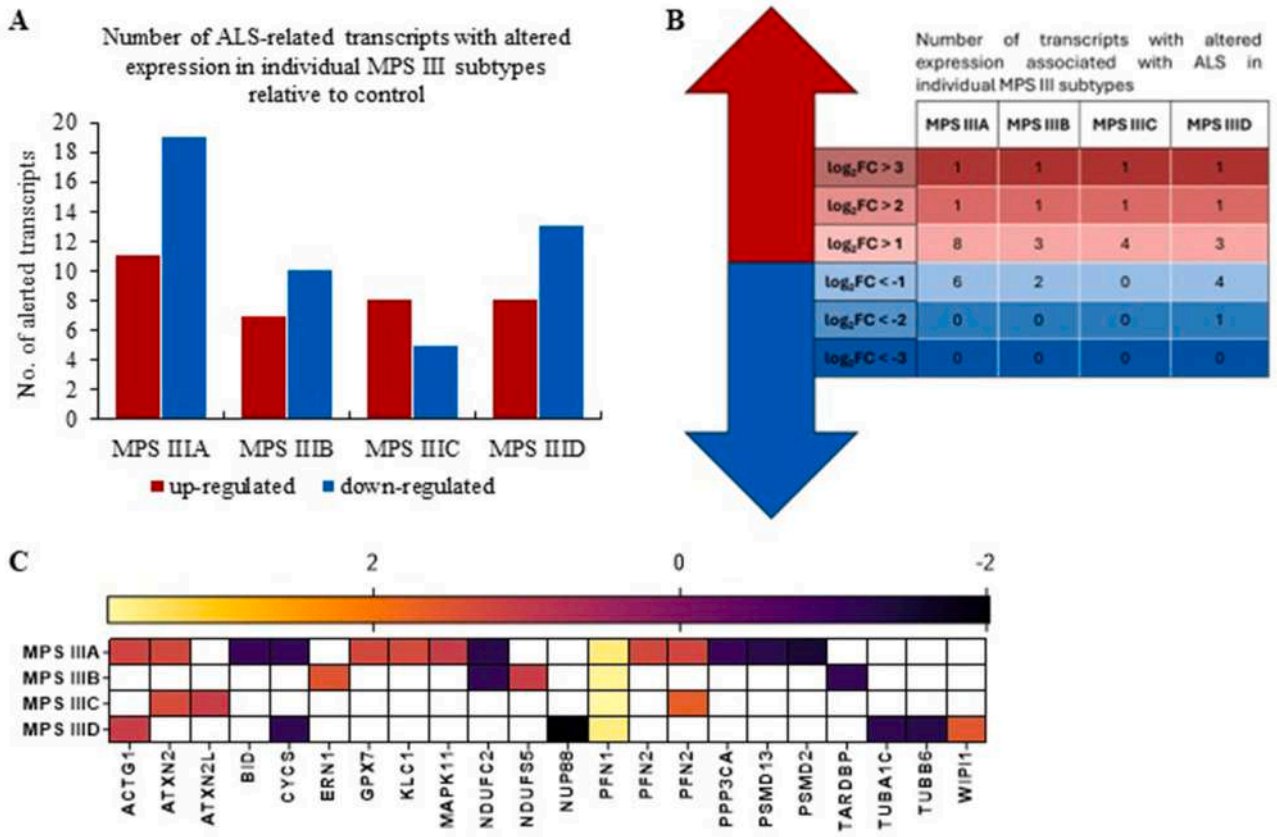

Fig. 17. Number of ALS related-transcripts [hsa05014] undergoing changes in in different types of MPS III relative to control cells (A) and number of ALS related-transcripts with altered expression depending on the level of fold-change (FC) value in different types of MPS III relative to control cells (B) as well as heat-map presenting differences in ALS related-gene expression levels between different types of MPS III and control cells (C).

The role of GAGs in the formation of certain protein aggregates seems indisputable. Direct interactions of GAGs with aggregate-forming proteins have been demonstrated so far in the case of HS proteoglycans and beta-amyloid, tau protein, or alpha-synuclein [43–46]. It is very likely that such interactions occur only after GAGs exceed a certain concentration threshold in the cell. This situation has already been observed in the interactions between GAGs and the oxytocin receptor (OXTR) and estrogen receptor (GPER1) in fibroblasts taken from patients with MPS I [48].

Taking a closer look at the exact mechanism by which GAGs may regulate the levels of toxic proteins, transcriptomic studies were conducted. The results of these studies indicated disruptions in the expression of many genes in fibroblasts derived from patients with MPS III, which are also known for their roles in the pathogeneses of AD, ALS, or PD.

Examples of genes linking AD and MPS III include *APOE* and *MME*. The down-regulated (in the MPS III cell line) gene *APOE* encodes apolipoprotein E, whose various isoforms are involved in regulating the phosphorylation of tau protein or β-amyloid aggregation and clearance [49]. Similarly, the down-regulated (in MPS IIIA and IIID cell lines) gene *MME* encodes membrane metallo-endopeptidase (MME), which, like APOE, participates in β-amyloid clearance [50]. Interesting data also provide information about increased expression of the *PFN1* gene in all tested MPS III cell lines. Mutations in the *PFN1* gene are considered a cause of ALS. It has been suggested that the profilin 1 protein, encoded by *PFN1,* forms aggregates with other proteins (including myelin-binding protein) in the brain and spinal cord of ALS mice [51]. Neuropathological studies have shown that in the brains of patients with *PFN1* mutations, there is accumulation of TDP-43. It was indicated that expression of *PFN1* mutants induces TDP-43 accumulation and promotes the conversion of normal TDP-43 into its abnormal form [52]. Genes that exhibit altered expression in Sanfilippo disease are also implicated in the pathogenesis of PD. For example, significantly down-regulated (in the MPS IIIA cell line) is the *PARK7* (*DJ-1*) gene, which encodes the parkinsonism associated deglycase, also known as PARK, DJ-1 or Parkinson protein. Mutations in this gene lead to early-onset familiar PD. PARK7, with its chaperone activity, interacts with α-synuclein, modulating its aggregation state. It has been suggested that overproduction of this protein reduces α-synuclein dimerization, whereas mutations in the *PARK7* gene impair this process [53]. Another example could be the significantly up-regulated (in the MPS IIIB cell line) *UCHL1* gene, encoding one of the deubiquitinating enzymes. Literature reports indicate that its overexpression leads to the formation of inclusions containing components of the ubiquitin-proteasome system and α-synuclein [54]. As a result, proteasome activity is inhibited. It is suggested that the aggregation process may begin even before the inhibition of proteasome activity, but its inhibition accelerates this phenomenon, leading to less efficient degradation of misfolded proteins and thus creating a vicious cycle [54].

We hypothesize that in some cases, GAGs do not need to interact directly with proteins forming protein aggregates, but can regulate the expression of other genes encoding regulatory proteins or transcription factors. These, in turn, influence the expression of other genes such as *APOE, MME, PFN1, PARK7* and *UCHL1*, ultimately leading to enhanced protein aggregate deposition. Changes in the expression of the same genes in other neurodegenerative diseases, such as AD, ALS, and PD, highlight the fact that they may be heavily involved in the neurodegeneration processes.

A limitation of this study is that we have used one cell line per each subtype of MPS III. However, MPS III is a very rare disease with poor

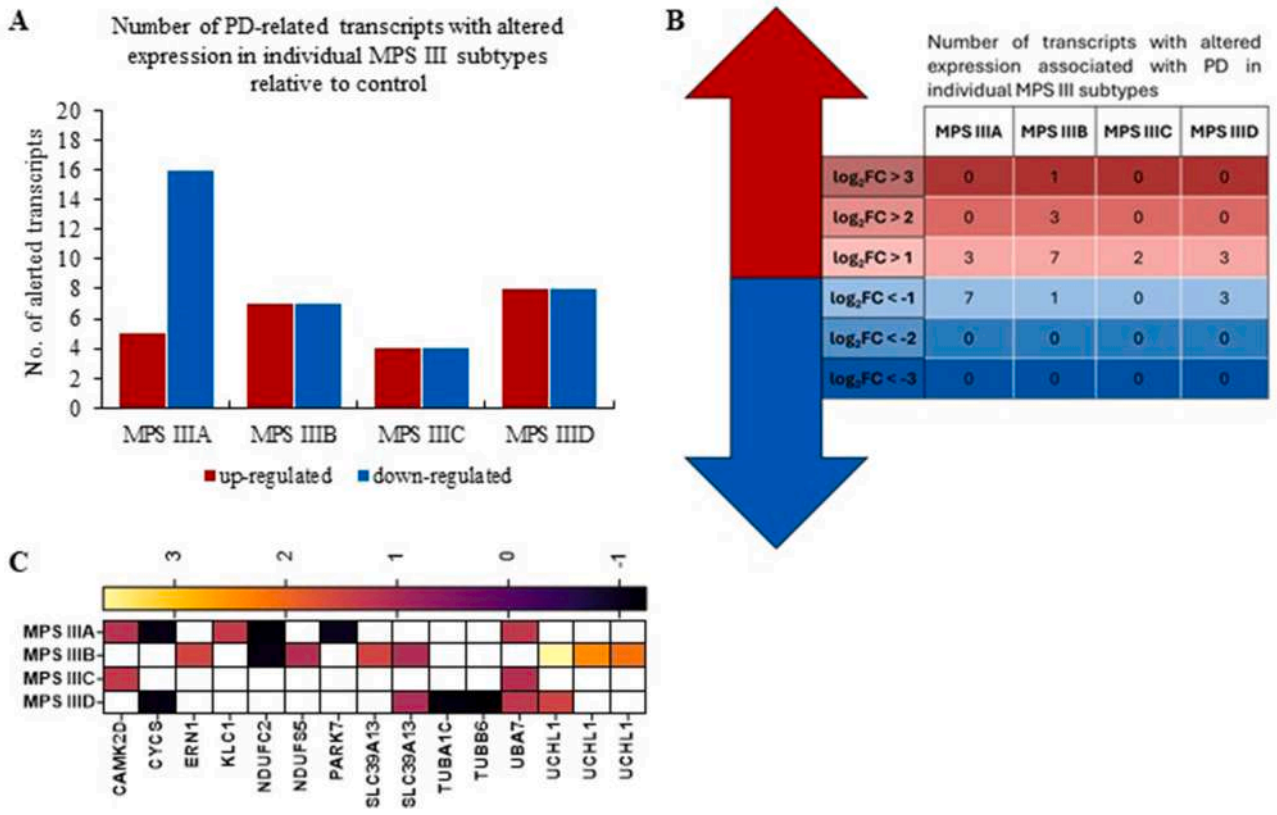

Fig. 18. Number of PD related-transcripts [hsa05012] undergoing changes in different types of MPS III relative to control cells (A) and number of PD related-transcripts with altered expression depending on the level of fold-change (FC) value in different types of MPS III relative to control cells (B) as well as heat-map presenting differences in D related-gene expression levels between different types of MPS III and control cells (C).

availability of biological materials. On the other hand, each experiment was repeated several times, and one should note that in most cases the obtained results were similar for all MPS III cell lines tested which indicates their reliability. The only considerable differences were observed in some experiments with the MPS IIIC line. These differences can likely be related to the fact that the residual activity of the deficient enzyme was significantly higher in the MPS IIIC cell line relative to other MPS III lines (A, B, and D) (>10 % activity of the normal value in the MPS IIIC cell line, and <1 % residual activity in other MPS III lines, as demonstrated previously for the same fibroblast lines [7]). In fact, this limitation was explained previously, indicating that the results of such experiments are reliable despite using one cell line per each MPS type [55].

5. Conclusions

In conclusion, this work describes comprehensively for the first time the problem of deposited protein aggregates in all subtypes of Sanfilippo disease which brings us closer to discovering the detailed mechanisms of the pathogenesis of this rare disease. Our results also indicate that GAGs are partly responsible for the formation of protein aggregates. These data are consistent with data on high levels of GAGs in other CNS diseases associated with protein aggregation which may indicate common mechanisms of pathogenesis for MPS type III and some other neurodegenerative diseases.

Ethics approval and consent to participate

All animal procedures were approved by the Local Ethics Committee for Animal Experiments in Bydgoszcz (application approval no. BUD13/2020) and carried out according to the guidelines of the European Communities Council Directive (2010/63/UE).

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A competing interest statement

The authors declare no conflict of interest.

CRediT authorship contribution statement

Karolina Wiśniewska: Writing – review & editing, Visualization, Validation, Investigation, Funding acquisition, Data curation. **Estera Rintz:** Investigation, Data curation. **Magdalena Żabińska:** Investigation, Data curation. **Lidia Gaffke:** Investigation, Data curation. **Magdalena Podlacha:** Investigation, Data curation. **Zuzanna Cyske:** Investigation, Data curation. **Grzegorz Węgrzyn:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Karolina Pierzynowska:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.150718.

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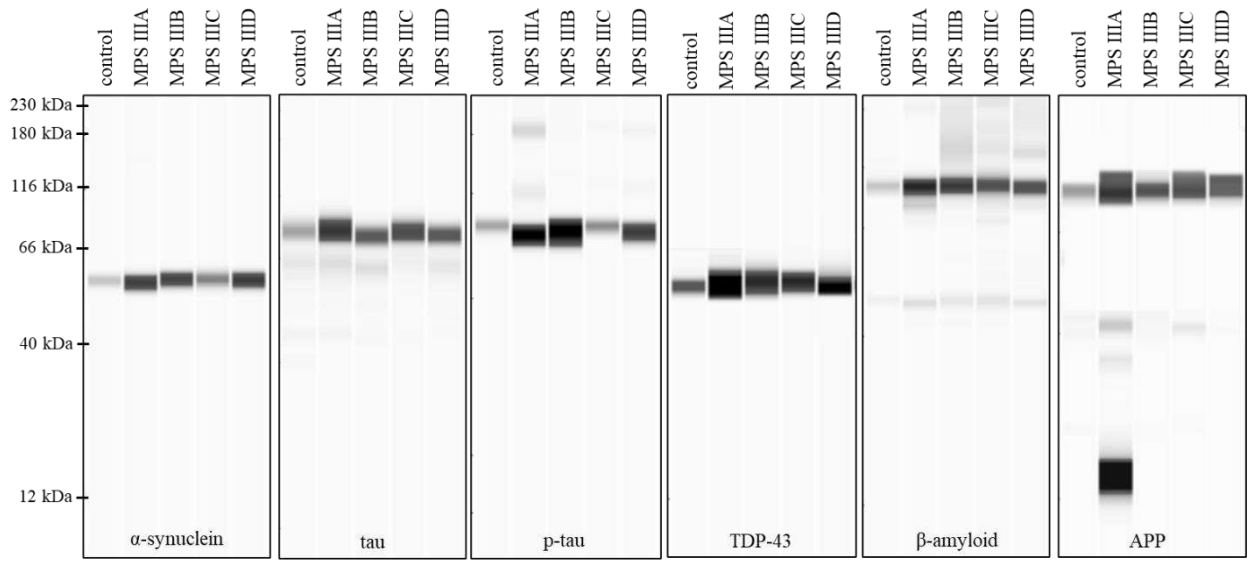
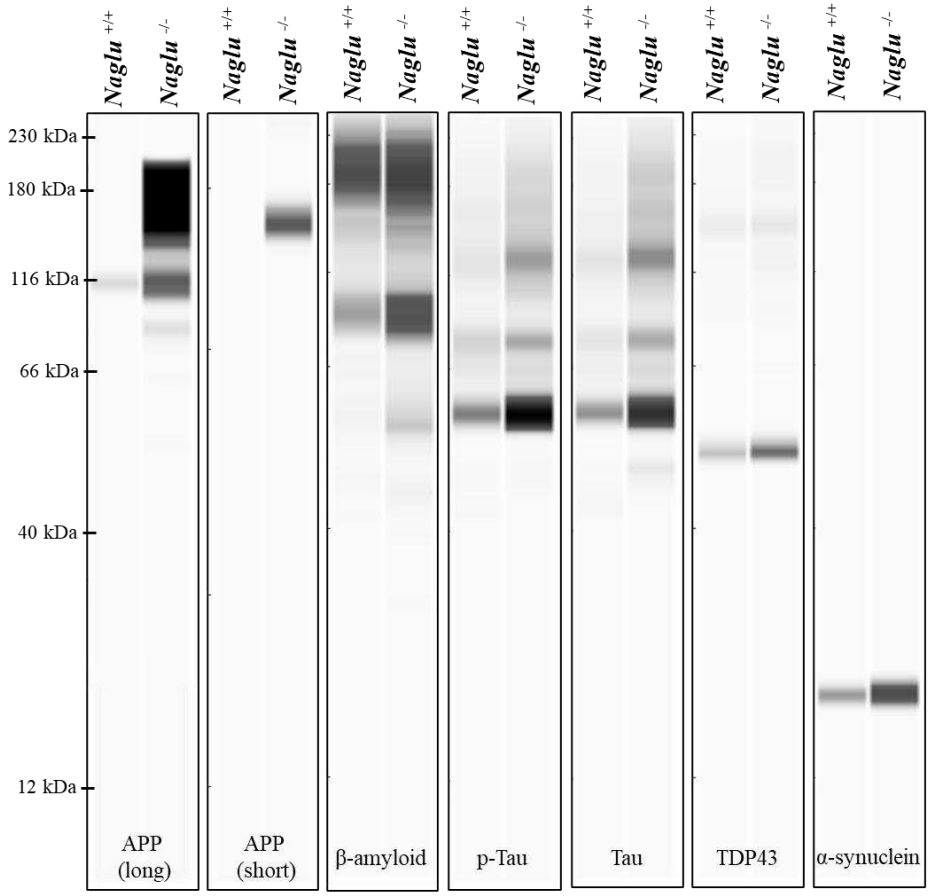
Supplementary

SUPPLEMENTARY DATA

A



B



C

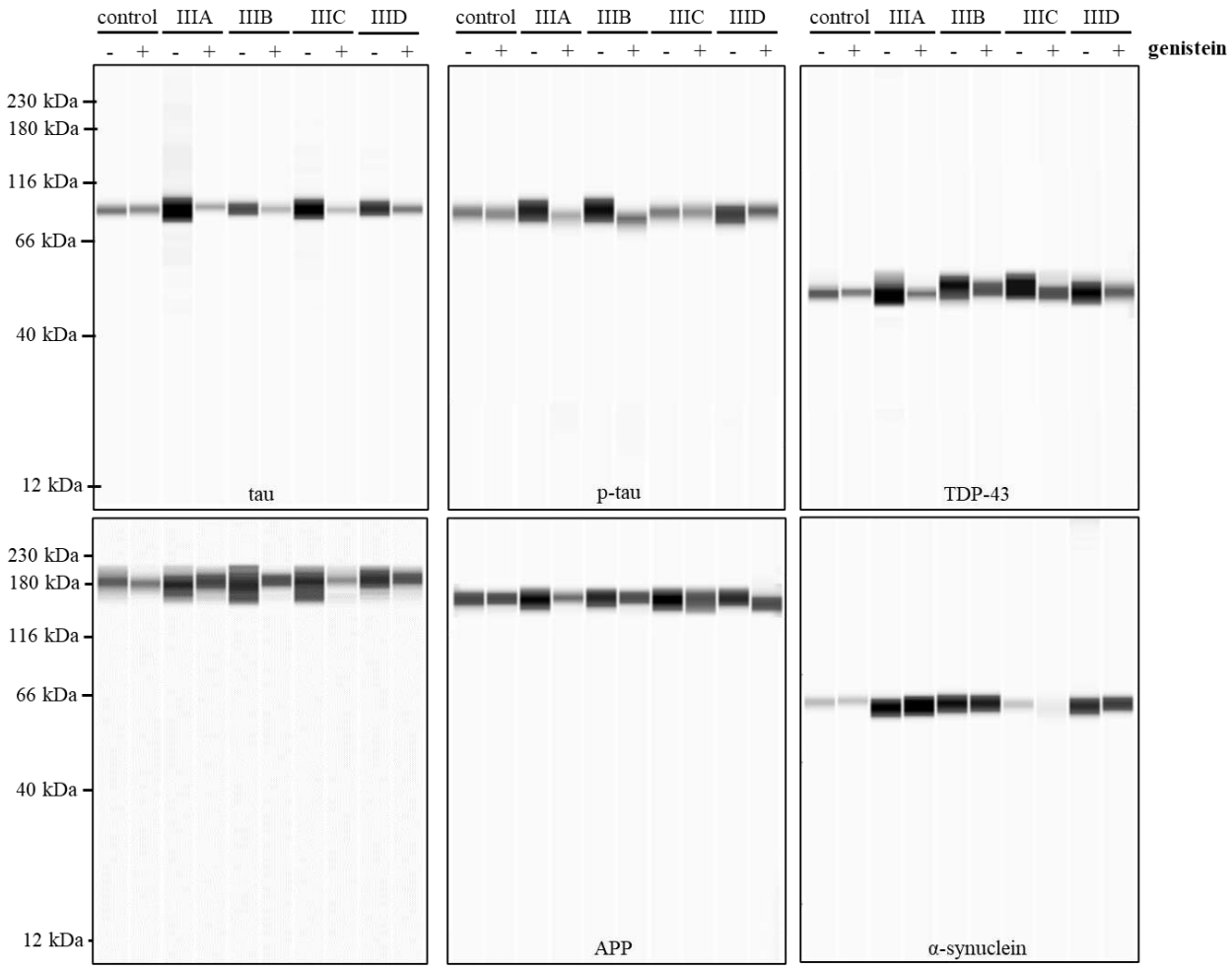


Figure S1. Uncropped blots to Figures 2 (A), Figure 3 (B), and Figure 10 (C).

Table S1. List of AD related-transcripts with more than a 2-fold change ($log_2FC>1$ or $log_2FC<-1$) in expression level (along with the exact values) in all subtypes of MPS III compared to controls.

Transcript[a]	logarithmic fold change values in gene expression levels in all subtypes of MPS III compared to controls			
	MPS III subtypes			
	MPS IIIA	**MPS IIIB**	**MPS IIIC**	**MPS IIID**
BACE2	-	-	-	1.48
FZD1	-	-	1.83	-
FZD6	-	-	2.05	1.83
FZD8	2.13	-	1.27	-
KLC1	1.29	-	-	-
NDUFS5	-	1.08	-	-
SLC39A13 (tr. 1)	-	1.48	-	-
SLC39A13 (tr. 2)	-	1.03	-	1.01
WIPI1	-	-	-	1.54
WNT9A	1.49	-	-	-
AKT1	-	-	-	-2.30
APOE	-6.11	-	-	-
BID	-1.02	-	-	-
CAPN2	-	-1.58	-	-
CSF1	-	-2.24	-	-
CYCS	-1.07	-	-	-1.11
ERN1	-	1.48	-	-
MME (tr. 1)	-2.02	-	-	-
MME (tr. 2)	-2.52	-	-	-
MME (tr. 3)	-	-	-	-2.54
MME (tr. 4)	-	-	-	-3.62
NDUFC2	-1.20	-1.11	-	-
NDUFV3	-	-1.52	-	-
PLCB4	-	-3.07	-1.65	-
PSMD13	-1.24	-	-	-
PSMD2	-1.47	-	-	-
TUBA1C	-	-	-	-1.15
TUBB6	-	-	-	-1.24

[a] When more than one transcripts were identified for a particular gene, they are marked as tr. 1, tr. 2, etc.

Table S2. List of ALS related-transcripts with more than a 2-fold change ($log_2FC>1$ or $log_2FC<-1$) in expression level (along with the exact values) in all subtypes of MPS III compared to controls.

Transcript[a]	logarithmic fold change values in gene expression levels in all subtypes of MPS III compared to controls			
	MPS III subtypes			
	MPS IIIA	**MPS IIIB**	**MPS IIIC**	**MPS IIID**
ACTG1	1.19	-	-	1.09
ATXN2	1.25	-	1.32	-
ATXN2L	-	-	1.05	-
ERN1	-	1.48	-	-
GPX7	1.27	-	-	-
KLC1	1.29	-	-	-
MAPK11	1.02	-	-	-
NDUFS5	-	1.08	-	-
PFN1	3.51	3.64	3.73	3.53
PFN2 (tr. 1)	1.26	-	-	-
PFN2 (tr. 2)	1.22	-	1.64	-
PPP3CA	-1.01	-	-	-
WIPI1	-	-	-	1.54
BID	-1.02	-	-	-
CYCS	-1.07	-	-	-1.11
NDUFC2	-1.20	-1.11	-	-
NUP88	-	-	-	-2.02
PSMD13	-1.24	-	-	-
PSMD2	-1.47	-	-	-
TARDBP	-	-1.06	-	-
TUBA1C	-	-	-	-1.15
TUBB6	-	-	-	-1.24

[a] When more than one transcripts were identified for a particular gene, they are marked as tr. 1, tr. 2, etc.

Table S3. List of PD related-transcripts with more than a 2-fold change ($log_2FC>1$ or $log_2FC<-1$) in expression level (along with the exact values) in all subtypes of MPS III compared to controls.

Transcript[a]	logarithmic fold change values in gene expression levels in all subtypes of MPS III compared to controls			
	MPS III subtypes			
	MPS IIIA	**MPS IIIB**	**MPS IIIC**	**MPS IIID**
CAMK2D	1.14	-	1.30	-
ERN1	-	1.48	-	-
KLC1	1.29	-	-	-
NDUFS5	-	1.08	-	-
SLC39A13 (tr. 1)	-	1.48	-	-
SLC39A13 (tr. 2)	-	1.03	-	1.01
UBA7	1.25	-	1.10	1.24
UCHL1 (tr. 1)	-	3.64	-	1.47
UCHL1 (tr. 2)	-	2.42	-	-
UCHL1 (tr. 3)	-	2.12	-	-
CYCS	-1.07	-	-	-1.11
NDUFC2	-1.20	-1.11	-	-
PARK7	-1.02	-	-	-
TUBA1C	-	-	-	-1.15
TUBB6	-	-	-	-1.24

[a] When more than one transcripts were identified for a particular gene, they are marked as tr. 1, tr. 2, etc.

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Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Wiśniewska, Karolina et al. "Comprehensive evaluation of pathogenic protein accumulation in fibroblasts from all subtypes of Sanfilippo disease patients." Biochemical and biophysical research communications vol. 733 (2024): 150718. doi:10.1016/j.bbrc.2024.150718

polegał na:

1. zaproponowaniu tematyki pracy
2. sfinansowaniu projektu w postaci otrzymanego grantu
3. przeprowadzeniu doświadczeń
4. analizie i interpretacji wyników
5. przygotowaniu figur i tabeli
6. asyście w przygotowaniu odpowiedzi na uwagi recenzentów

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polegał na:

1. pomocy w prowadzeniu doświadczeń
2. pomocy w opracowaniu wyników

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polegał na:

1. pomocy w prowadzeniu doświadczeń
2. pomocy w przygotowaniu figur i tabeli

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polegał na:

1. przygotowaniu biblioteki RNA-seq do analiz transkryptomicznych

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polegał na:

1. pomocy w prowadzeniu doświadczeń
2. pomocy w opracowaniu wyników

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1. recenzji wewnętrznej manuskryptu
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3. asyście w przygotowaniu odpowiedzi na uwagi recenzentów
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Behavioural disorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications

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REVIEW

Behavioural disorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications

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Abstract
Sanfilippo syndrome (MPS III) is one of the types of mucopolysaccharidoses (MPS), a group of inherited metabolic diseases in which the accumulation of glycosaminoglycans (GAGs) results from deficiency of different lysosomal enzymes. The hallmarks of MPS III are relatively minor somatic abnormalities with severe and progressive central nervous system (CNS) symptoms. An analysis of the literature showed that the biggest problems for carers of people with MPS III are behavioural disorders and sleep disorders. Despite extensive discussions on improving the quality of life of patients, little attention was paid to the families/carers of patients. The families/carers are providing appropriate medical and palliative care to the patient every day due to their loss of mobility, self-care skills, tube feeding, airway clearance and other supports continue to have an adverse effect on the quality of life of families/carers. However, a literature review of possible solutions showed that effective methods (both pharmacological and non-pharmacological) exist. The needs of carers of MPS III patients should receive as much attention as the search for new treatments. There are many options for dealing with such problems. The key issue is to identify the source of the problem and choose the most effective therapy. Alleviating behavioural disorders, pain complaints and sleep problems will have a positive impact not only on the quality of life of carers/families, but also on the patients themselves.

Keywords Sanfilippo syndrome · Sleep disturbance · Behaviour disturbances · Pain · Therapy

Introduction

Sanfilippo syndrome (MPS III) is one of the types of mucopolysaccharidoses (MPS), a group of genetically determined metabolic disorders. As a result of the deficiency in activity of specific hydrolases, degradation of glycosaminoglycans (GAGs) is inefficient, resulting in their accumulation in lysosomes [1].

Based on the type of stored GAGs and deficient enzyme, 12 types and subtypes of MPS are currently distinguished. In addition, based on the presence/absence of central nervous system symptoms, MPS can be divided into neuronopathic types (MPS I, II, III A-D, VII) and non-neuropathic ones (MPS IV A-B, VI, VII, X) [2]. The abnormalities observed in the course of the disease may vary depending on the MPS type/subtype, but facial dysmorphia, hepatosplenomegaly, vision problems, limited range of motion, cardiovascular or respiratory abnormalities as well as progressive character of the disease are typical for all MPS [3, 4]. Sometimes types classified as neuronopathic (i.e. MPS I or MPS II) present without nervous system abnormalities or these abnormalities appear very late or are mild in relation to the 'expected' abnormalities [2, 5]. The reason for this is not fully known, but the occurrence of attenuated types may be related to a higher level of residual dysfunctional enzyme activity, the ratio between accumulated GAGs or the occurrence of a specific mutation. [6–8].

Details of the classification and characteristics of MPS are presented in Table 1.

Currently, there is no effective therapy leading to a complete cure for any of the MPS types. Despite the lack of

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Table 1 Characteristics of classical/conventional types of MPS[a]

MPS type	Inheritance[a]	Defective gene	Deficient enzyme	Stored GAG[b]	Symptoms		Comments
					Typical for all MPS	Neurological[c]	
MPS I	AR	*IDUA*	α-L-iduronidase	HS, DS	Cardiorespiratory dysfunction, joint stiffness (expect MPS IV), skeletal problems (scoliosis, kyphosis, lumbar lordosis), hepatosplenomegaly, hip dysplasia, frequent infections of the respiratory system, hernia, vision and hearing problems, macroglossia, short stature, face dysmorphology changes in hair morphology, hirsutism	Impaired cognitive function, language, and speech abilities, behavioural abnormalities (excessive silencing), sleeping problems, and/or epileptic seizures	The division of MPS I into subtypes is based only on differences in the clinical picture.
MPS II	X-linked	*IDS*	Iduronidase-2-sulfatase	HS, DS		Developmental delay, mental retardation, and behaviour problems (aggression, overexcitability)	-
MPS IIIA	AR	*SGSH*	Heparan-N-sulfatase α-Nacetylglucosaminidase	HS		Developmental delay, cognitive impairment, behavioural disorders (impulsivity, aggression, anxiety disorders, autistic behaviour), sleeping problems	Relatively minor somatic disorders
MPS IIIB	AR	*NAGLU*	Heparan α-Glucosaminide	HS			
MPS IIIC	AR	*HGSNAT*	N-Acetylglucosamine-6-sulfatase	HS			
MPS IIID	AR	*GNS*	6-sulfatase	HS			

Table 1 (continued)

MPS type	Inheritance[a]	Defective gene	Deficient enzyme	Stored GAG[b]	Symptoms		Comments
					Typical for all MPS	Neurological[c]	
MPS IV A	AR	*GLANS*	N-Acetylglucosamine-6-sulfate sulfatase	C6S, KS	Cardiorespiratory dysfunction, joint stiffness (expect MPS IV), skeletal problems (scoliosis, kyphosis, lumbar lordosis), hepatosplenomegaly, hip dysplasia, frequent infections of the respiratory system, hernia, vision and hearing problems, macroglossia, short stature, face dysmorphology changes in hair morphology, hirsutism	Absence or mild neurological disorders	In patients with Morquio syndrome, other (non-skeletal) somatic symptoms are usually milder than with other types of MPS
MPS IVB	AR	*GLB1*	Iduronidase-2-sulfatase	KS			
MPS VI	AR	*ARSB*	N-acetylglucosamine-4- sulfatase (arylsulfatase B)	DS, C4S		In MPS VI neurological disorders are uncommon	Excessive joint mobility
MPS VII	AR	*GUSB*	β-Glucuronidase	HS, DS, C4S, C6S		Impaired cognitive, language, and speech abilities, behavioural abnormalities, sleep problems, and/or epileptic seizures	Symptoms that appear in the course of MPS VII (as long as they do not lead to the early death of the child) most often resemble diseases in the field of psychiatry/neurology
MPS IX	AR	*HYAL1*	Hyaluronidase	Hyaluronan		-	MPS VII is one of the two rarest types of MPS, as until now, only 4 cases were described
MPS X	AR	*ARSK*	Arylsulfatase K	DS		-	Difficulty sleeping, and macrocephaly without cognitive impairment, neurological abnormalities or learning difficulties

[a]*AR* autosomal recessive

[b]glycosaminoglycan (GAG) names: C4S chondroitin 4,6-sulfate; C6S chondroitin 6-sulfate; DS dermatan sulfate; HS heparan sulfate; KS keratan sulfate

[c]CNS disturbances can occur in non-neuronopathic types but they cause by secondary and tertiary pathological changes in cells and their incidence is not high

causal treatment, it is possible to conduct treatments focusing on the symptoms of the disease. Since MPS is a multisystem disease, potential therapies should be based on a multidisciplinary approach [9].

The quality of life of patients deteriorates due to the development of numerous disorders, but the disease also significantly affects the lives of families/guardians. Little is said about the fact that parents/carers of people with MPS are significantly more likely to suffer from depression, experience anxiety disorders and be at risk of post-traumatic stress [10]. They often isolate themselves from society. An important, and often overlooked, aspect is the relationship between healthy siblings and parents/sick siblings. The parents' attention will of course be focused primarily on the sick child. Over time, healthy siblings become involved in the care and at some point take on the role of caregivers [11]. From the point of view of both the sibling and the parent, negative emotions are certainly a difficult issue. Unintentional feelings of anger at certain situations/behaviours of the sick person can turn into feelings of guilt resulting from thinking 'how could I have thought/reacted that way, he/she is sick after all and it is not his/her fault'. Frustration resulting from helplessness, lack of information and limited possibilities not only to treat the illness itself, but to cope with everyday problems is also an important and yet often overlooked aspect of care [11, 12]. The biggest difficulties, significantly affecting daily functioning, are sleep problems and behavioural disorders that worsen as the disease progresses [13–17]. The purpose of this review is to highlight possible causes of behavioural and sleep disorders in MPS III patients and to identify possible measures to alleviate them.

Mucopolysaccharidosis type III—Sanfilippo syndrome

Unlike other MPS types, in Sanfilippo syndrome there are severe CNS abnormalities with relatively minor somatic symptoms [18]. The former ones include cognitive and motor dysfunction, behavioural problems, speech disorders, sleep problems, aggression, hyperactivity, and extreme risk-taking behaviour [9, 13, 19]. Emerging symptoms often resemble autism spectrum disorders (ASD) or attention-deficit/hyperactivity disorder (ADHD) [2, 15].

The course of the disease can be divided into 3 stages (Fig. 1) [9]. The first symptoms appear between 1–6 years of age [2, 14]. At this stage, the child's development is slowed down or stopped [2]. Facial dysmorphia may occur, accompanied by frequent upper respiratory infections, ear infections and diarrhoea [20]. Between 2–4 years, cognitive deterioration progresses, sleep problems begin, and the child's ability to focus deteriorates. Behavioural problems such as aggression, irritability, hyperactivity, tantrums or anxiety attacks with panic attacks become increasingly frequent [8, 15]. Such symptoms may resemble ASD. Around the age of 10, general quieting/indifference to the environment, progressive impairment of motor functions, dementia, and dysphagia are noticed [8] Later, epilepsy may appear, including generalized tonic–clonic seizures, tonic, focal and myoclonic seizures, and status epilepticus without seizures [8]. Patients' life expectancy ranges between 2 and even 6/7 decades of life.

Sanfilippo syndrome is one of the MPS types which is divided into subtypes (Table 1). The subdivision is based on the enzyme whose defect underlies the disease, but in all 4 subtypes, HS accumulates. One might therefore assume that, since we are moving within one type and the same GAG accumulates, the different MPS III subtypes would not differ significantly from each other. However, this is not true. First of all, the course of the disease for subtypes A and B is usually severe, and patients usually live into their teenage years. Subtypes C and D are milder in the course, and as a result, patients' life expectancy can be longer [2]. Moreover, variation in frequency, age and type of symptoms is observed between subtypes. The characteristic thickening of facial features or hepatomaegaly is much more frequently observed in types IIIA (92% and 56%) and IIIB (94% and 56%) than in type IIIC patients (85% and 39%) Language delay, abnormal behaviour (like hyperactivity, impulsivity/proneness to take dangerous actions, aggression, anxiety disorders) are more common in MPS type IIIA (93% and 75%) and IIIC (92% and 77%) than in MPS type IIIB (88% and 69%). Abnormal ASD or epilepsy predominates in the clinical presentation of patients with types IIIA (29% and 17%) and IIIB (19% and 13%) whereas it is not as common in patients with MPS IIIB (8% of patients) [21].

As mentioned earlier, the variation between subtypes also relates to the timing of symptom onset. In MPS IIIA, the earliest visible/manifested symptoms are developmental delay and/or behavioural (around 2–3 years of age). Hyperactivity begins to draw attention around 4 years of age, while speech disorders between 5–6 years of age. A similar age of onset applies to patients with MPS IIIB [21]. Epilepsy appears in MPS IIIA patients at an average age of 7 years, compared to 12.5 years for MPS IIIB patients. First signs of dementia appear before age of 6 years in 83% of type A patients but only 24% and 33% of type B and C patients, respectively [22].

Behavioural disorders in MPS III—possible causes

There are currently no registered therapies for MPS III, and available treatments are limited to symptomatic treatment [19]. In Sanfilippo syndrome, the range of abnormalities

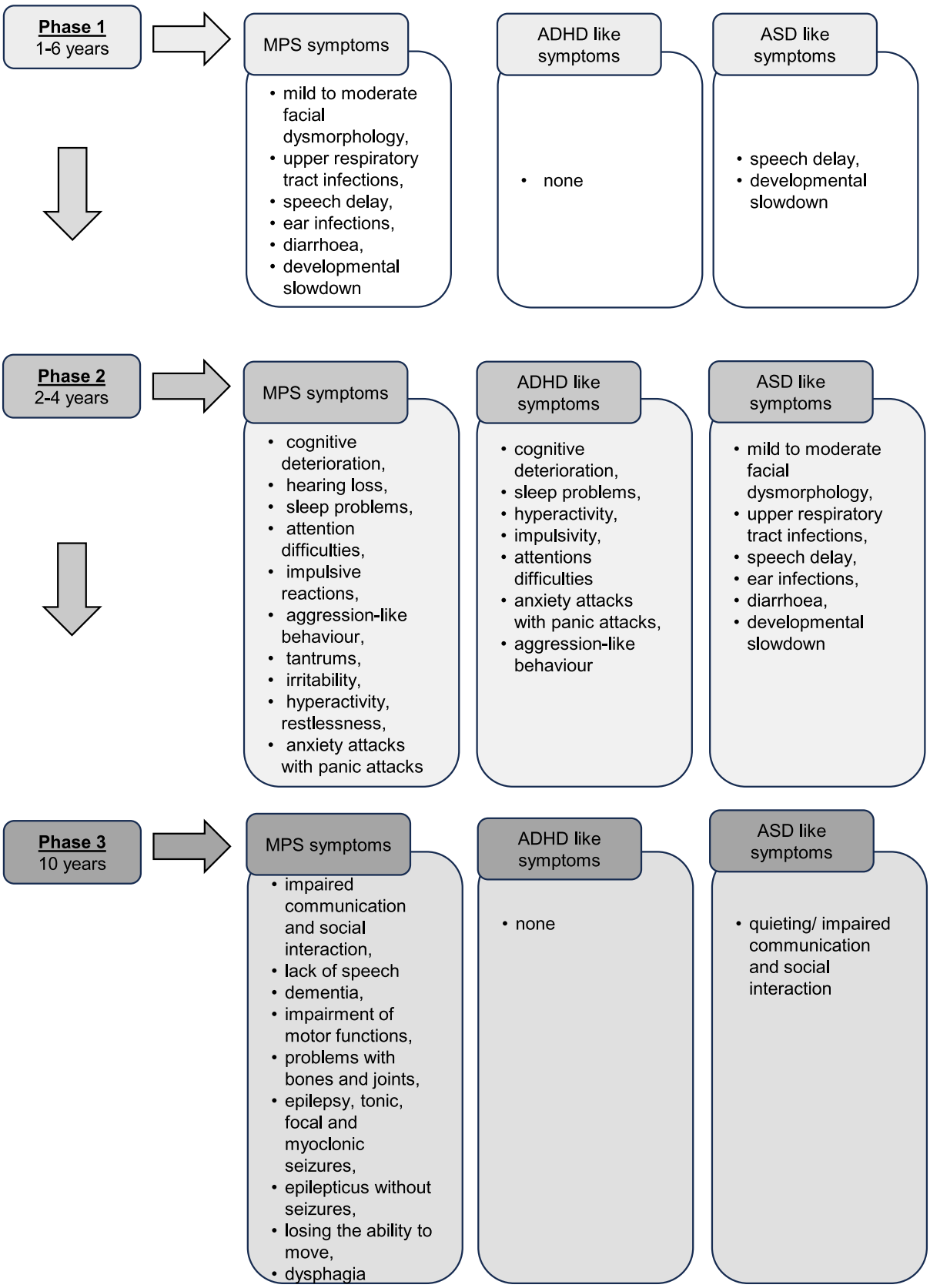

Fig. 1 Stages of MPS III syndrome (patients without treatment) with typical symptoms and signs of ADHD-like/ASD-like symptoms that may lead to misdiagnosis

is extensive: behavioural abnormalities, neurocognitive abnormalities, skeletal deformities, cardiovascular abnormalities, respiratory abnormalities, ear-nose-throat (ENT) and gastrointestinal problems [2]. Undoubtedly, all of the aforementioned disorders are serious problems, but as noted previously, from the point of view of caregivers of patients with MPS III, behavioural disorders have the biggest impact on the quality of care [13].

Molecular mechanisms of behavioural disorders

Transcriptomic analysis showed changes in the expression profiles of behaviour-related genes in patients suffering from all types and subtypes of MPS [23]. Abnormalities also included genes related to locomotory behaviour, feeding behaviour or social interaction. Among the genes with altered expression in MPS III were those coding for OXTR, EIF4A3, and HOMER2 proteins, whose dysregulations are associated with ASD, mental retardation, or anxiety [23].

Among genes with altered expression in MPS III, there were more that may influence abnormal behaviour or CNS disorders. Such genes include *RPL10* (abnormal expression of this gene is observed in patients with autism, syndromic intellectual disability and epilepsy), *RARRES2* (detected in blood of children with idiopathic epilepsy), *NME2* (associated with ADHD) or *IRF2BP* (neurodevelopmental disorder with regression, abnormal movements, loss of speech) [22, 24].

Pain

Behavioural abnormalities can result directly from CNS damage due to GAG accumulation, but this is only one possible cause. Other factors, like pain, may influence (induce or exacerbate) the abnormal behaviour problem. Pain accompanies patients with all types of MPS, but the highest prevalence/severity was observed in MPS III (52.9%) [25].

Nociceptive pain results from the accumulation of GAGs in the skeletal system, leading to bone deformity, and joint swelling and stiffness. Such changes may result in craniofacial dimorphisms, clawed hands, thoracolumbar scoliosis, 'cocked' chest, dwarfism, and muscle contractures. All of the abnormalities mentioned above are accompanied by chronic pain [26]. With age and disease progression, abnormalities worsen and pain increases. GAG accumulation is also the cause of hepatosplenomegaly, which can cause visceral pain [27]. Patients may also suffer from toothache resulting from decay, unerupted teeth, malocclusion or gingival overgrowth [13, 26, 28, 29].

GAG storage leads to activation of inflammatory mediators, the cause of neuralgic pain sensation [25, 30]. This type of pain may be associated with peripheral neuronopathy, exemplified by carpal tunnel syndrome. Spinal cord injuries are accompanied by chronic neuronopathic pain. Hypersensitivity, pain hypersensitivity, and allodynia are also observed [26, 28]. Pain complaints were more frequently reported in paediatric patients with cognitive impairment. Compared to the group without intellectual disability, where 30% patients reported pain complaints, 50% patients with intellectual disability reported pain complaints. [28]. This makes it difficult to determine whether the patient is actually experiencing pain and to determine the type and source of the pain [13].

It is also worth mentioning that pain may arise in MPS III patients from disorders recognized as secondary or tertiary effects of GAG storage. For example, various infections (particularly ear infections), gastrointestinal problems (like diarrhea or constipation), and headaches (which may reflect hydrocephalus or other problems) cause a pain-related discomfort in patients [2]. In such cases, eliminating the direct cause of the pain by using available drugs (for example, like antibiotics in bacterial infections; diarrhea-inhibiting or laxative agents in diarrhea or constipation, respectively or dietary modifications if any food intolerance is the problem) may provide a considerable relief, provided that the cause of the pain has been properly determined.

Hyperactivity, aggression, elopement, and a lack of feeling danger

Hyperactivity is one of the most characteristic features of behavioral abnormalities among MPS III patients [2]. This feature does appear at the age of several years, while it is very disturbing for both patients and caregivers [20]. No moment to catch a breath causes exhaustion and significantly disturbs a daily life. Aggression is another disturbance which makes the behavior of MPS III patients hardly acceptable. It seems plausible, however, that hits, blows and screams, expressed by Sanfilippo syndrome patients, do not arise from the desire to hurt someone, but rather they are effects of hyperactivity and a need to do anything, even hitting anybody. Thus, such a feature is sometimes called a “pseudo-aggressive behavior”, rather than a true aggression [9].

Perhaps elopement is another reflection of hyperactivity, combined with a lack of understanding the situation in meaning of physical and social conditions [2, 9]. Nevertheless, elopement may cause serious problems influencing safety, as a lack of control and care on a Sanfilippo syndrome patient might lead to serious incidents, even life threatening ones. Similar situations might be caused by a lack of feeling a danger by MPS III patients, causing their disregarding any difficulties, such as obstacles on the way, deep holes or others [9].

The molecular mechanisms of the behavioral disturbances described in this subsection remain unclear. One proposal was related to possible interactions of partially degraded

HS, through their reactive chemical moieties, with other molecules, including neural transmitters [9]. Another cause might be dysregulation of expression of genes involved in baharioral regulations [23]. However, these hypotheses require further testing, and other mechanisms cannot not be excluded.

ADHD and ASD

There can be cases where symptoms of MPS III, may be misdiagnosed for (ADHD or ASD) (Fig. 1).

Typical symptoms of ADHD are inattention, motor hyperactivity and impulsivity. In addition, ADHD increases the risk of other psychiatric disorders. Impulsivity and emotional disturbances negatively influence social behaviours, making it difficult to function properly [31, 32]. ADHD-like symptoms can occur in the course of various metabolic disorders, including Sanfilippo syndrome. The similarity of symptoms MPS III to those of ADHD results in frequent misdiagnosis of Sanfilippo syndrome as ADHD [2]. Sleep difficulties are also common in MPS III and ADHD and may be a cause of mild cognitive impairment in people with ADHD, while in patients with MPS III in whom these disorders are one of the symptoms, they may contribute to the patient's overall deterioration [31].

MPS III and ASD are distinct conditions, but they can share some symptoms, leading to potential confusion or misdiagnosis [2]. ASD is characterized by challenges in social interaction, communication, and repetitive behaviours, and has a different ethology than MPS III. The misdiagnosis occurs especially at initial phases of MPS III where the symptoms are often not recognized as typical for MPS III and their occurrence is assigned as ASD. ASD has unclear aetiology and both environmental and genetic factors are known to lead to abnormalities in the developing brain [33]. Symptoms include impaired communication and social interaction (including emotional disturbances, and difficulties with non-verbal communication), reduced interest and repetitive behaviours [34]. Similar abnormalities occur in patients with MPS III, so patients are often diagnosed as having ASD [34]. Clinical underdiagnosis or misdiagnosis of children with MPS III can be influenced by the diversity among MPS III patients [22]. Symptoms can differ across different MPS III subtypes, leading to varying phenotypes within this group, ranging from severe to unusually mild [35]. The more severe forms of MPS III are more likely to manifest at earlier ages, and clinical signs and symptoms alone may not suffice for a diagnosis, which should be confirmed through biochemical analyses. Behavioural difficulties typically emerge between 3 and 5 years of age, and they are characterized by restlessness, destructiveness, anxiousness, and aggressive behaviour [36].

Furthermore, a factor hindering a prompt diagnosis is the relatively late administration of genetic tests, meanwhile genetic testing leaves no doubt about the diagnosis and allows to avoid errors. However at the early stages of the disease, it is used only in families with the known history of the disease [37]. A good solution in such a situation is for the psychologist to provide a symptomatic diagnosis rather than a nosological one, especially at the early stages of a child's life. The psychologist can then describe the child's functioning, noting the presence of autism-like symptoms. This approach allows for the detection of developmental abnormalities over time and, at the same time, opens the possibility for further exploration.

Neurotransmitters and hormones

Stress responses, ability to focus attention, learning and memory, sleep, mood, behaviour are all regulated by the action of the endocrine system, and its disruption (can cause various sorts of abnormalities [38–40]. Neurotransmitters that can influence the above-mentioned processes include gamma-aminobutyric acid (GABA), dopamine, and serotonin. [41–43]. The actions of hormones are also important for the body to function [44, 45].

Low levels of GABA are correlated with aggressive behaviour, increased vulnerability to stress and anxiety [46, 47]. The relevance of its level to sleep remains debatable [46]. The action of GABA in ADHD is also being investigated significantly, and reduced GABA levels may be associated with attention deficits [48–50]. Indeed, developing therapy based on the regulation of glutamic acid levels can have a positive impact on patients (treatment efficacy depends on patient age) [51, 52].

Serotonin regulates brain functions related to cognitive function, learning, and mood [41, 43]. Deficiency of serotonin or its receptors may therefore be associated with cognitive impairment, mood deterioration and aggression [41, 42, 53]. In addition, reduced levels of serotonin are found in the brains (grey matter and brainstem) of people with ASD [53, 54].

Dopamine, like serotonin, is associated with mood regulation, cognitive function and concentration [55, 56]. Impairment of the dopaminergic system can result in cognitive impairment, behavioural disorders, developmental delay learning and memory difficulties [40, 57–59]. Dopamine level abnormalities or mutations in the coding gene are observed in patients with ADHD. Anxiety and difficulties with concentrating result from increased dopamine reuptake. Cognitive dysfunction, on the other hand, may be due to a dysfunctional motivation centre. Mutations in the dopamine receptor genes have also been linked to cognitive impairment in ADHD patients. [60–62]. In ASD, a possible role for the dopaminergic system (both disorders related

to dopamine itself and its receptors) in the development of behavioural disorders or impaired social interaction is assumed. However, the abnormalities observed in patients can only be partly related to and explained by abnormalities of the dopaminergic system, as this system can exhibit both excessive and under-signalling properties [56, 63]. It was showed that autistic-like behaviours in a mouse model of MPS IIIA may be related to dopamine D1 receptor activity [64]. According to the authors, HS storage leads to modification of its function and causes proliferation of mesencephalic dopamine neurons originating during embryogenesis, which translates into the occurrence of autistic-like behaviours in mice [64].

The importance of oxytocin (OT) and arginine vasopressin (AVP) and their receptors in anxiety, psychiatric disorders, social dysfunction has also been reported [44, 45]. Oxytocin is associated with the formation of emotional behaviour, social bonds and deriving satisfaction from them [65]. Reduced oxytocin levels are observed in patients with both ASD and ADHD. This can result in increased aggression, reduced ability to feel empathy, and emotional recognition deficit [66, 67]. The action of vasopressin is linked to social behaviours including social preference, memory and social bonding [68]. In newborns later diagnosed with ASD, vasopressin levels were lower than in healthy children and may serve as markers for screening in the future [69].

Given current knowledge of the importance of endocrine changes in other diseases, the behavioural disorders and developmental delay observed in patients with MPS III are perhaps somehow related to endocrine abnormalities. Unfortunately, there is little knowledge about endocrine abnormalities in Sanfilippo syndrome, in the context of neurological problems. It is known that genes related to signal transduction are dysregulated in MPS over and above this, abnormal levels of oxytocin are observed in the cells of patients with MPS [70]. Most works, however, focused on growth hormone-related disorders [71].

On the other hand, based on the available literature, it can be speculated that cognitive impairment, social and emotional behaviour disturbances in Sanfilippo syndrome may at least in part be related to abnormalities in neuronal signalling and the endocrine system.

Other causes

Characteristics of MPS III patients include aggressive behaviour, reactions that are inappropriate to the situation and actions/behaviours that lead to health risks [2, 13, 14]. These types of behaviour/reactions are essentially "normal" if one considers the age of the patient (most often children) and the nature of other abnormalities that occur during the course of the illness. Aggressive behaviour, frustration and excessive irritability may result from an inability to express one's needs or to satisfy them independently. In the course of the disease there is a developmental delay, difficulty and loss of the ability to speak, and over time there is a disruption and eventually a loss of the ability to swallow food [18, 27]. Motor function also deteriorates, including loss of mobility which is a source of frustration and aggression for children [13]. Over time, the motor impairment become so severe that the patient is no longer able to function independently. Because communication is impaired, it is difficult to determine whether the child's behaviour is due to pain or whether the cause lies elsewhere [2, 28].

Sensory disorders such as hypersensitivity, pain sensitisation, allodynia as well as well as sleep problems can affect concentration problems and aggression [28, 72].

Hyperactivity and impulsivity observed in patients with MPS III may provoke unsafe behaviours [13]. Carers of children with Sanfilippo syndrome report that children show an increased tolerance to pain. There are also doubts about whether the child feels pain at all, which is also not insignificant for potentially dangerous behaviour [19]. Studies conducted to date show that disturbances in the circadian rhythm of sufferers may also explain the problem of hyperactivity [73].

Sleep disorders

Sleep disturbance is a typical symptom of MPS III [74–76]. Research on sleep quality in patients with MPS III shows that sleep disturbances can affect up to 87–92% of patients [8, 36, 74] and include difficulty falling asleep, waking during the night and early mornings, nocturnal insomnia and long daytime sleep duration [15, 76]. In children with Sanfilippo syndrome, problems may include staying awake all night, wandering, laughing, singing or reciting nursery rhymes [77, 78]. Sleep difficulties may result from breathing problems, night-time seizures or be caused by alterations in the circadian rhythm of melatonin or pain [3, 73].

Breathing difficulties occur in all types of MPS, due to anatomical abnormalities (both upper and lower airways and abnormal thoracic structure), spinal cord injury (which can affect respiratory muscle weakness), recurrent respiratory infections or heart failure (fluid build-up in the lungs). Patients with MPS often develop obstructive sleep apnoea (throat muscles relax and block the airway) [76, 79, 80].

Seizures and epilepsy can occur in neuronopathic types of MPS, with seizures occurring in 26–52% of patients with Sanfilippo syndrome [20]. Seizures may occur during the day as well as at night and may therefore cause sleep disturbances [3, 81].

Both children and adults with MPS III exhibit abnormal circadian rhythms, which can translate into sleep problems. A 2015 study by Mumford et al. found that children with

MPS III show increased activity during the night/early morning hours (between midnight and 6 am) compared to healthy children. At the same time, these results are consistent with the observation of hyperactivity in the second phase of the disease. The rhythm of children with MPS III shows considerable fragmentation and, for older patients, the period of increased activity falls during the daytime [73]. Abnormalities of the 'biological clock' may be related to the diurnal rhythm of melatonin secretion. This neurohormone regulates the sleep–wake rhythm; in healthy individuals, its increased secretion occurs at night and decreases during the day. In MPS III, this cycle is reversed, with melatonin secreted in greater amounts during the day and decreased levels at night. [73, 82]. This explains why patients do not sleep through the night (they have difficulty falling asleep, often wake up) and sleep long during the day [73]

The patient's sleep problems also become a cause of insomnia for their carers. Stress related to the occurrence of convulsions or nocturnal apnoea causes a decrease in the quality of sleep, the period of sleepless nights (to soothe and put the child to sleep) is significantly prolonged. A sleepless night can translate into worsening of the patient's mood/behaviour the following day [13]. Thus, it can be said that sleep disorders are both one of the symptoms of the disease and at the same time they could be one of the causes of behavioural disorders.

Therapies

As described earlier, the behavioural disturbances observed in MPS III (aggression, lack of concentration, tendency towards dangerous behaviour, etc.) and sleep problems may have many causes [78]. To achieve the best possible therapeutic effects, it is important to identify the cause of the abnormal behaviour/sleep disturbance and select appropriate treatment measures [2, 78, 83].

The proposed therapies only involve the possibility of influencing some of the abnormalities observed in MPS, which may (to the authors' knowledge) be one of the causes of the behavioural disorders. It should be remembered that currently causative treatments for MPS are not possible, and no therapies have been registered for MPS type III. For a summary of the causes of conduct disorders and potential therapies, see Table 2.

Pain

Pain is one of the factors that can influence the patient's (especially paediatric) behaviour [84, 85]. It is clear that the treatment of pain should, as far as possible, consist of identifying and removing the source of the pain. Children or people with limited communication skills may not be able to specify the location and describe the type of pain they are experiencing [26, 86]. According to the literature, the pain usually concerns the joints (most commonly the hips) and the back, but there are many more possibilities [25, 30].

For patients with cognitive impairment/intellectual impairment, it is recommended to use specific questionnaires to assess pain. The choice of the appropriate method depends on the patient's age and ability to cooperate [26, 86]. If examination in the outpatient setting does not identify the cause, more detailed investigations should be ordered [25, 28]. For example, MPS III patients have more frequent ear infections, bone and joint disorders, and dental diseases, causing additional pain. Thus, a full ophthalmic examination, an assessment by an orthopaedic surgeon with X-rays or an assessment by a dentist may be helpful [3, 14].

The type of treatment used depends on both the cause and the age of the patient and the response to the therapy. Both pharmacological and non-pharmacological agents can be used [26, 28, 30].

Pharmacological treatment

According to the World Health Organization (WHO), non-steroidal anti-inflammatory drugs (NSAIDs), opioids or paracetamol can be used for pain relief in patients with MPS. The choice of drug depends on the type of pain experienced (neuropathic or nociceptive), the source, intensity and duration of pain (Table 3). The individual patient's preference (perception of side effects/self-perception after the drug) is also taken into account [3, 26, 86]. Neuropathic pain (resulting from damage to the nervous system) is more difficult to control; patients with this type of pain often do not respond to conventional treatments. In this case, tricyclic antidepressants may be helpful (note that these drugs have many side effects) or anticonvulsants [87]. By including such drugs in therapy, it is possible to alleviate central neuropathic pain, peripheral pain and visceral hypersensitivity.

Cannabinoids are used for the treatment of certain types of pain (chemotherapy-related, neuropathic pain, AIDS-related neuropathy, or multiple sclerosis), but due to possible side effects and little data on efficacy and effects on children and adolescents, their use in paediatric patients is currently debatable. The Canadian Paediatric Society recommends that the medical use of cannabis in children should be assessed on a case-by-case basis and always after a comprehensive discussion of potential benefits, risks and treatment goals [88]. Some studies suggest that intravenous administration of zoledronic acid or pamidronate acid may reduce bone pain and improve patient mobility [26, 86, 89].

Paracetamol (acetaminophen), a commonly used analgesic and antipyretic agent, is used for the control of mild pain. Due to its low rate of side effects, paracetamol is the drug of the first choice, especially in paediatric patients.

Table 2 Summary of possible causes of behavioural disturbances and potential therapies [a]

Etiopathogenesis	Cause of behavioural changes [b]	Therapies
Skeletal abnormalities (bone deformity, thoracolumbar scoliosis, dwarfism, muscle contractures, kyphosis, genu valgum, joint contractures, stiffness, arthralgia, arthritis, cervical cord compression, cervical cord compression, craniovertebral canal stenosis with spinal cord compression cervical myelopathy) Toothache (decay, unerupted teeth, malocclusion, gingival overgrowth, malocclusion) Headaches (alterations in intracranial pressure or symptoms of normal-pressure hydrocephalus) Abdominal pain (acid reflux, ulcers, gastrointestinal discomfort, constipation, hepatosplenomegaly) Peripheral neuronopathy (carpal/ tarsal tunnel syndrome, spinal cord injuries) Avascular necrosis of the femoral head Hypersensitivity Allodynia Inflammation	Pain	Removing the cause of the pain (if possible) is fundamental Pharmacological: The choice of drug depends on the type of pain experienced (neuropathic or nociceptive), the source, intensity and duration of pain.[c] Cannabinoids Some studies suggest that intravenous administration of Zoledronic acid or pamidronate acid may reduce bone pain and improve patient mobility Antidepressants Non- Pharmacological: Interventions Acupuncture Acupressure Transcutaneous Electrical nerve stimulation (TENS) Physical therapies Surgical procedures
Abnormalities in levels of: GABA, glutamic acid, serotonin, dopamine, melatonin secretion Changes in oxytocin, arginine vasopressin and their receptors levels	Neurotransmitters and hormones [d]	Pharmacological: Oral intake of GABA Riluzole, MPEP, Baclofen SSRI Intranasal administration of oxytocin Administration of arginine vasopressin Mood stabilisers Antipsychotic Non- pharmacological: None
Breathing problems (deformity of the skeletal system, narrowing of the upper airway, respiratory muscle weakness, restrictive pulmonary disease, obstructive sleep apnoea, night-time seizures) Alterations in the circadian rhythm of melatonin Pain Hyperactivity	Sleep disorders	Pharmacological: Administration of melatonin Benzodiazepines Chloral hydrate Antihistamines Surgical intervention Clobazam Carbamazepine CPAP (continuous positive airway pressure) Non- Pharmacological: Behavioural therapy Fixed bedtime Constant day/ activities routine Reduce different types of stimuli in the bedroom Peaceful environment at home

Table 2 (continued)

Etiopathogenesis	Cause of behavioural changes [b]	Therapies
Genetic and environmental factors influence the developing abnormalities	Behavioural disturbances (similar to ADHD and ASD)	Pharmacological: Risperidone Valproic acid Atomoxetine Methylphenidate Non- Pharmacological: Therapies involving behaviour management training: (educate parents to manage their child's behaviour, techniques to improve behavioural problems, an individual course of study or to provide appropriate facilities at the place of study, cognitive training, improve working memory, regular physical activity, attention training using special computer programmes,, exercises to improve: hand–eye coordination, eye trackers or gesture recognition sensors, JASPER (joint attention, symbolic play, engagement and regulation intervention), a type of therapy based on play-based sessions)

[a]The proposed therapies only involve the possibility of influencing some of the abnormalities observed in MPS, which may (to the authors' knowledge) be one of the causes of the behavioural disorders. It should be remembered that currently causative treatments for MPS are not possible and no therapies have been registered for MPS type III

[b]primary cause is GAG accumulation

[c]Details on typical MPS pain and its treatment in Table 3

[d]Endocrine/neurotransmitter abnormalities in MPS III have not yet been thoroughly investigated. Their relevance to the observed disorders is based by the authors on the similarities of the symptoms of diseases in which such disorders are described to those of patients with MPS III

NSAIDs include compounds used to treat mild to severe pain, especially if the cause is inflammation (they are more effective than paracetamol) [26, 86]. Most commonly, drugs from this group are used for patients suffering from bone and joint pain, headaches, or migraines [28]. Long-term use of NSAIDs is not recommended due to their adverse effects. For moderate to severe pain, opioid drugs are recommended (can be combined with paracetamol and NSAIDs). For severe chronic/episodic pain, strong drugs based on pure opioid agonists, fentanyl/methadone or morphine are recommended for chronic pain and episodic pain, respectively. Moderate pain can be relieved with weak oral opioids, like tramadol, or stronger longer-acting opioids, like oxycodone at low doses [28].

Non-pharmacological treatment

Pain management can also include a non-pharmacological approach. This type of treatment should be used alongside pharmacological treatment as part of a multidisciplinary therapy. Literature data include various methods, like psychological interventions, acupuncture and acupressure, transcutaneous electrical nerve stimulation (TENS), and physical therapies [28, 82, 90]. Psychological therapies usually include behavioural or cognitive-behavioural therapies. Sessions can be conducted in groups (for children or children with parents) or individually. Research has shown that this type of non-pharmacological treatment helps to reduce the intensity of chronic pain and, to some extent, to reduce disability in children with mixed chronic pain conditions [91]. Distraction-based strategies, the use of relaxation techniques and breathing exercises can also be helpful. An interesting method is animal-assisted therapy (AAT), based on the human-animal relationship. This type of therapy helps to reduce pain to some extent, improve cognitive function, behaviour and social interaction [26, 92, 93]. It should be noted that AAT is targeted, conducted by a qualified person with appropriately trained animals [94]. Physiotherapy may also be helpful [101–103]. There are also references in the literature to the use of heat/cold applications, osteopathy, massage and chiropractic in controlling pain in paediatric patients. Acupuncture as a pain management procedure has been used successfully in chronic headache, osteoarthritis, chronic back pain and neck pain [95, 96]. The efficacy of this type of therapy in paediatric patients has so far been confirmed in two studies [97]. Some studies pointed to the positive effects of TENS for pain management and allodynia [90, 98].

Abnormalities that develop in MPS can lead to a situation where surgical intervention is necessary. Surgeries help to treat carpal tunnel syndrome, hip dysplasia, trigger fingers, dental dysplasia, scoliosis or spinal cord compression [3,

Table 3 Types of pain occurring in MPS and ways of alleviating them

Group of drugs (low power- high power)	Drug	Kind of pain	Observed effect	Dosage and route of administration	Restrictions
Non-opioid analgesics, Low power	Acetaminophen	mild to moderate pain (with opioids—moderate and severe pain)	antipyretic analgesic, reduction of pain, fever, inflammation and stiffness, remarkably inhibiting GAG synthesis, (tested with genistein)	po: 20 mg/kg initially, then 15 mg/kg every 4–6 h rectal: 30–40 mg/kg initially, then 15–20 mg/kg every 4–6 h iv: weight < 10 kg: 7.5 mg/kg every 6 h weight > 10 kg: 15 mg/kg every 6 h	impaired liver function, impaired renal function Maximum dose: 90 mg/kg/day (60 mg/kg/day if present risk factors)
NSAID (Non-steroidal anti-inflammatory drugs), Low power	Ibuprofen	mild and occasional pain	more effective than Acetaminophen in those situations in which inflammation is the major cause of pain	po: < 6 months: 5 mg/kg every 6–8 h 6 months: 10 mg/kg every 6–8 h rectal: weight > 6 kg, 60 mg suppository every 8 h weight > 12 kg, 125 mg suppository every 8 h	maximum dose: 40 mg/kg/day
	Ketoprofen			po, rectal or iv: 3 mg/kg every 8–12 h	maximum dose: 9 mg/kg/day
NSAID, Moderate power	Naproxen/ Piroxicam	moderate pain	helpful in ear infections (ENT) issues, osteoarticular pain, headache, migraine attacks	po: 5–10 mg/kg every 8–12 h	maximum dose: 20 mg/kg/day
NSAID, High power	Ketorolac	severe neuropathic pain	-	po: 0.2 mg/kg (max 10 mg) every 4–6 h iv, im: 0.5 mg/kg start, then 0.2–0.3 mg/kg every 4–6 h	Maximum dose: 3 mg/kg/day if administered for long periods—side effects on gastric and medullar sites, reversible inhibitory effect on platelet aggregation
	Indomethacin	moderate pain	antipyretic, analgesic, reduction of pain, fever, inflammation, stiffness, remarkably inhibiting GAG (tested with genistein)	po, iv: 1 mg/kg every 8 h maximum dose: 3 mg/kg/day	maximum dose: 3 mg/kg/day
Opioids, Weak	Codeine	mild to moderate pain		po, rectal: 0.5–1 mg/kg every 4–6–8 h	NO if < 12y-old NO for 12–18 y-old if: recent tonsillectomy and or adenoidectomy, bad respiratory function
	Tramadol	chronic moderate/severe pain		po: 0.5–1 mg/kg every 4–6–8 h iv: 1 mg/kg every 3–4 h iv: continuous infusion 0.3 mg/kg/h	caution in patients with epilepsy

Table 3 (continued)

Group of drugs (low power- high power)	Drug	Kind of pain	Observed effect	Dosage and route of administration	Restrictions
Opioids, strong	Oxycodone	chronic pain		po: 0.1–0.2 mg/kg every 8–12 h	drowsiness, constipation, light-headedness, dizziness, headache, strong addictive potential
	Methadone	episodic severe pain		po: 0.05–0.1 mg/kg every 8–12 h (schedule modifying based on the duration therapy)	immune system hyperactivation, reduced circulating lymphocytes, increased blood viscosity, strong addictive potential
	Fentanyl	chronic pain		bolus 1–2 mcg/kg (max 5 mcg/kg with spontaneous breathing), continuous infusion 0.1 mcg/kg/h intranasal: 1–2 mcg/kg	cough, strong addictive potential
	Morphine	episodic severe pain		CLORIDRATE iv: Bolus 0.05–0.1 mg/kg every 2–4 h, Continuous infusion 0.02–0.03 mg/kg/h SOLFATE (po): Early release: 0.15–0.3 mg/kg every 4 h Slow release: 0.3–0.6 mg/kg every 8–12 h	strong addictive potential
Bisphosphonates	Pamidronate	chronic pain, pain from bone fractures	reduction in bone pain and improvements in mobility	intravenous, monthly for a year	
TNF-α inhibitor	Adalimumab	-	improve ROM (range-of-motion), physical function, and possibly pain	dose of 20 mg (weight 15- < 30 kg) or 40 mg (weight ≥ 30 kg) administered subcutaneously every other week, 16 weeks	well tolerated, without serious adverse events
Antidepressants anticonvulsants	Tricyclic, Gabapentin and Carbamazepine	neuropathic and mixed pain (visceral hyperalgesia, central and peripheral neuropathic pain, headache, insomnia)	anaesthetics, adjuvant therapies		
Cannabinoids	trans-Δ-9-tetrahydrokannabinol (THC), cannabidiols (CBD)	neuropathic pain	refractory chemotherapy-related nausea and neuropathy, spasticity in multiple sclerosis, AIDS-related neuropathy	inhalation (vaporized, smoked), orally, or oromucosal Rarely determine the window between the doses at which drugs produce analgesia and side-effects	adverse physiological and physical effects (psychomotor and cognitive impairment, anxiety and panic attacks, acute psychosis, paranoia, dry mouth, blurred vision, palpitations, tachycardia, and postural hypotension)

28, 37]. For patients with MPS, surgical procedures performed under general anaesthesia carry additional risks due to respiratory abnormalities (megaloglosia, airway narrowing, dorsal and cervical curvature changes) can significantly impede effective ventilation/intubation. Although studies indicate that such complications are unlikely in MPS III patients, the risk still exists. A multidisciplinary assessment of the efficacy of the planned procedure and preoperative simulation is necessary before performing procedures requiring sedation [99].

ADHD and ASD

Patients with MPS III are often diagnosed with ADHD and/or ASD. It is possible to effectively manage the symptoms associated with these disorders, but it is important to know if they co-occur with other conditions that may affect the effectiveness of the chosen therapy.

Pharmacological treatment

Few studies have investigated the use of medications for the treatment of disorders like depression, mood disorders, affective-bipolar disorder (BD) (symptoms similar to behavioural disorders in MPS III) in Sanfilippo syndrome, and results are sometimes conflicting. Neuroleptics, although they can lead to improvement, are not recommended. Their use is associated with many side effects, including increased hyperactivity and aggression [78].

One of the medications used to correct the disorders of people with ASD is risperidone. This drug belongs to the Second Generation Antipsychotics and works by alleviating disorders such as aggressive behaviour, frequent mood changes, reduced irritability and hyperactivity. It also improves speech impairment and an emerging state of lethargy in people with ASD [100]. In MPS III with ASD, the use of risperidone appears to have a positive effect, but this may be preceded by an increase in aggression [101–104]. In a specific case, physicians successfully used valproic acid in the treatment of ASD in a patient with MPS IIIB to stabilise mood disorders [103].

When ADHD is diagnosed, treatment with atomoxetine can be tried. Studies indicated that this compound is well tolerated by patients with MPS III. Significant improvements in aggression have been observed in patients with MPS IIIB [102]. However, there are no studies confirming the effects of atomoxetine on hyperactivity and impulsivity (however, the compound is known to improve the ability to focus attention and to have a positive effect on hyperactivity in people diagnosed with ADHD) [102]. The effect of methylphenidate (the most commonly used drug to treat ADHD) on patients with MPS III is also unknown. However, studies with MPS I and II patients indicated that this compound can be used successfully in patients with a mild phenotype, without causing drowsiness, and improving cognitive function [78]. Therefore, it might be worth trying this drug also in MPS III. There are also no studies on the efficacy of both compounds in MPS IIIB, IIIC, and IIID patients [102]. Early treatment and appropriate management of patients with MPS III can significantly enhance the quality of life for both the patients and their families [37, 105]. Regrettably, they are often misdiagnosed in the early stages of their condition. It is common for these children to receive a diagnosis of ADHD or ASD. This misdiagnosis happens due to the symptoms that manifest initially, such as hyperactivity and speech delay or even the complete absence of speech development [20]. Additionally, these children often experience developmental delays that can obscure or amplify emerging MPS III symptoms. Similarities in symptoms resulting in misdiagnosis may lead to the implementation of inappropriate therapy [101, 106]. Although improvement can be achieved initially, the development of the disease and other symptoms means that the effect is not long-lasting. Only the use of methods appropriate for the comprehensive alleviation of MPS III symptoms may allow relative control of the disease [102, 106].

Non-pharmacological treatment

There are many non-pharmacological treatment options for people with ADHD and ASD; however, there is a lack of research on their use in patients with MPS III diagnosed with ASD and/or ADHD. There is one report in the literature about a patient diagnosed with MPS IIIA using applied behaviour analysis (ABA) [107]. ABA has been used successfully with ASD and ADHD patients, and in the case of the patient in question, its implementation has also been successful. [107–110]. The data collected indicated that the use of ABA aided communication skills and cognitive function [93]. Improvements were noted in learning, behavioural motor skills and a reduction in behavioural problems Clearly, much more research is needed into the use of ABA and other non-pharmacological therapies used in ADHD and ASD in MPS III patients. However, it is possible that this simple approach may be able to help both patients and their families to some extent. Therefore, possible non-pharmacological treatment strategies for patients with ADHD and ASD are described below.

In the case of ADHD, 3 types of psychosocial therapies can be distinguished. The first group are therapies involving behaviour management training. First-line treatment (for patients aged about 4 years) is to educate parents to manage their child's behaviour so that undesirable behaviour is discouraged and desirable behaviour is reinforced [111]. These types of therapies also aim to help caregivers better understand the child, improve techniques to

improve behavioural problems and support/strengthen the caregiver-child relationship [111]. Behavioural management intervention also describes educational measures. It is possible to qualify a child to plan an individual course of study or to provide appropriate facilities at the place of study [112, 113]. Children with ADHD have difficulty establishing relationships, so behavioural management intervention also includes support in this area [114]. The second group of therapies are training interventions, where the focus is on the education of the child/teenager. Cognitive training, which aims to improve working memory, can be helpful. However, this effect can be achieved without affecting the improvement of ADHD symptoms [115]. Another option is organisational skills training to learn time management and build collaboration skills [116]. The third group includes activities to ensure regular physical activity. It is thought that this can have a positive impact on the patient's wellbeing, but also on cognitive function, memory and other ADHD symptoms [117].

Attention training using special computer programmes, activities, video games, exercises to improve hand–eye coordination, eye trackers or gesture recognition sensors are also possible [118]. This type of therapy is called biofeedback, a method that involves measuring the patient's brain activity, processing selected parameters (so that they are understandable to the patient) and transmitting the information to the user. In this way, the brain activity associated with a particular condition becomes perceptible to the patient and enables him or her to learn self-regulation. Games or video boards are used to visualize the parameter. The reward received when a certain level of the selected parameter is reached is important for the effectiveness of the training. [119]. Neurofeedback training can be implemented based on the theta/beta (TBR), sensory-motor rhythm (SMR), and slow cortical potential (SCP) protocol. This type of training can effectively reduce deficits in inattention, hyperactivity and sleep quality [120].

People with ASD, like people with ADHD, also have difficulty establishing social relationships and adapting to their environment. Occupational therapy can help people with ASD to improve their daily functioning in terms of self-care, work/education and social participation. This type of therapy can also have a positive effect on behavioural problems, motor coordination or sensory processing difficulties. [121]. According to a study by Sterman et al. adults with ASD advocate supportive, goal-oriented therapies to help develop self-presentation and autonomy. They are critical of the concept of therapies aimed (in their view) at 'fixing' the child and adapting the child to the environment rather than adapting the environment to the child [122].

Another option is JASPER (joint attention, symbolic play, engagement and regulation intervention), a type of therapy for preschool/school-aged children based on play-based sessions [123]. In this way, it is possible to improve social communication (including improved language skills), increase flexibility and range of play (which may translate into his adaptability), broaden his abilities and develop his interests [124].

There are also opportunities unrelated to the therapy of patients stricto sensu, which can significantly influence their comfort (and thus their behaviour) while facilitating their care. Wood et al. [125] and Cermak et al. [126] described various types of modifications to the environment (the description related to the emergency room and the dentist's office) that significantly improved the functioning of people with ASD. Facilities introduced included sensory adaptations i.e. lower intensity light, 'soothing' sounds, small manipulative toys or other seating options (rocking chairs). The changes made helped to reduce sensory discomfort and behavioural distress in people with ASD. Similar solutions and observations were described by Litwin and Sellen [127] in relation to the paediatric emergency room. The use of sensory adaptations resulted in improved comfort for patients and, consequently, for their caregivers. It is possible that similar solutions may be equally effective for MPS III patients presenting with ASD. Collaboration between occupational therapists and interior design can be considered when designing an environment that addresses specific sensory needs. [128]. It is worth noting that such facilities do not have to involve high costs, but should nevertheless be tailored to individual needs as far as possible. [128].

Neurotransmitters and hormones

At present, endocrine and neurotransmitter abnormalities are a poorly understood aspect of MPS. However, they are known to be important in the pathology of disorders such as ADHD and ASD and may be correlated with the occurrence of cognitive impairment in neurological disorders [45, 49, 129, 130]. As described earlier, aggression, mood disorders and other behavioural changes occurring in both MPS III and other neurological/psychiatric disorders may also be related to endocrine function and neurotransmission. For some of these changes, it may be possible to achieve improvement using appropriate medication.

Studies on gamma-aminobutyric acid (GABA) supplementation for stress reduction and improved sleep quality have yielded inconclusive results. Oral intake of GABA reduces stress markers of both the autonomic and peripheral nervous system, but the range of doses taken by patients to achieve an improvement effect is very large [46]. However, most research confirms the positive effect of oral supplementation on stress [46]. Some of the drugs used to treat ASD or ADHD, such as riluzole, MPEP or baclofen, are GABA receptor agonists [49]. However, studies on the effect of GABA supplementation in MPS III are lacking.

Drugs in the serotonin reuptake inhibitor (SRRI) group are primarily used to treat depression [131]. However, it is possible to use them in ASD or ADHD. Administration of citalopram correlated with improvements in hyperactivity, aggression, and attention span. The ability to perform specific/assigned tasks also improved [132, 133]. Recently published preclinical studies suggested that for MPS III (specifically MPS IIIA), the use of fluoxetine (a compound from the SSRI group) may have a positive therapeutic effect [134].

Impaired oxytocin levels have been observed in individuals with ASD, and although in younger children intranasal administration appears to improve social function the overall efficacy in improving the underlying symptoms of the disease is debatable and requires further research [135, 136]. In contrast to oxytocin, research into the effects of arginine vasopressin (AVP) regulation appears promising. So far, administration of AVP helps to improve social relationships, the ability to read emotions and express them, reduces the propensity to take risky actions [137]. However, there are no studies on the use of oxytocin or vasopressin in the treatment of MPS III.

Studies using a mouse model of MPS IIIA (indicating a role for the dopamine D1-like recptor in autistic-like behaviour in MPS IIIA) have shown that the use of the dopamine D1-like receptor antagonist SCH-23390 corrects autistic-like behaviours in young adult mice. [64].

The efficacy of mood stabilisers and antipsychotics in patients with MPS has been tested. However, it is difficult to assess the efficacy of treatment due to inconclusive results. In addition, the studies did not specify which MPS III subtype the study participants had [78]. In one such experiment, behavioural improvement was achieved for 55% of patients [75].

Sleep disorders

As mentioned earlier, sleep problems are both one of the symptoms of MPS III and a possible cause of the disorders [8, 13]. Like pain, sleep disorders can have a variety of causes and, as with pain, it is essential to identify the source of the problem in order to apply appropriate treatment [3, 73]. In general, the treatment of sleep disorders should be based on a multidisciplinary approach [3].

Pharmacological treatment

Available literature indicates that melatonin is most effective against sleep problems in patients with MPS III [74]. It is well tolerated by patients, has no significant number of side effects and there is no development of tolerance with long-term use. Treatment with melatonin may not be effective if sleep disturbances are associated with pain sensations [73]. However, if no improvement is achieved despite the absence/treatment of the source of pain, an increase in dose or a change of drug may be necessary. Benzodiazepines may be a second choice, but their use requires additional caution [74]. Sleep apnoea is rarely a cause of sleep problems in patients with MPS III but patients with episodes of snoring should undergo a diagnostic workup for respiratory disorders. Benzodiazepines inhibit the respiratory centre which, in combination with sleep apnoea, could lead to the death of the patient. Another possibility is the administration of chloral hydrate or antihistamines [78].

If the cause of sleep problems is a breathing disorder, it is also important to determine what the source is. Deformity of the skeletal system (including the thorax and spine) may require surgical intervention, as may obstructive sleep apnoea resulting from narrowing of the upper airway. Respiratory muscle weakness or restrictive pulmonary disease can be corrected with continuous positive airway pressure (CPAP) by mask or ventilatory support systems [80].

Epileptic seizures are very common in patients with MPS III, but no association was seen between nocturnal seizures and sleep disturbances, although in some cases a seizure occurring at night disturbed sleep. In patients with MPS II, epileptic seizures were controlled with clobazam and carbamazepine. In patients with MPS III, clobazam significantly reduced their incidence, but the emergence of sedation and patient instability led to abandonment of this therapy. [80].

Patients with MPS often suffer from recurrent respiratory infections, which obviously worsen the quality of breathing which affects the quality of sleep [79].

Non-pharmacological treatment

Behavioural therapy can provide support for drug therapy. Strategies as simple as introducing a fixed bedtime and some routine in the activities before going to bed can be helpful. After some time, a certain time of day and activities will begin to be associated with sleep, which may make it easier to fall asleep [74]. Reducing the impact of different types of stimuli in the bedroom (light, sound, even removing toys) can have a positive impact on the process of falling asleep and sleep itself. Even a progressive intellectual disability should not exclude simple techniques [74, 80].

Taking care of the environment also helps, the child will not accidentally get hurt and therefore the parents' sleep comfort will improve. Modifications included sharing a bedroom with the child, sleeping harnesses or barriers to prevent the child from leaving the bed (patients.

can sleepwalk), installing a camera in the child's room [80].

Individual treatment trials (ITTs)

Off-label medicines are therapeutics that are prescribed for a different dose, disease/indication or age group than the registered one. Such therapies are used in up to approximately 70% of in-patients (paediatric and adult) in European countries. This use of drugs is the basis of the ITTs method; when dedicated therapies prove ineffective, a non-targeted drug can be used experimentally [138]. In oncologic patients or osteoarthritis, ITTs are an increasingly recommended approach [139, 140]. Also among patients suffering from rare diseases, for which the need for therapies is great, such an approach could improve the treatment process [141].

In addition, clinical trials in rare diseases are difficult due to their heterogeneity and small number of patients, therefore ITTs could be a good solution to these problems. ITTs would allow the treatment to be tailored to the patient's needs and response [142, 143].

A survey by Wiesinger et al. of 27 experts from 11 different countries and centres and the data collected show that although this treatment approach needs to be refined, it has a growing number of supporters (both among clinicians and patients and their families). Fourteen of the 27 professionals asked for their opinion on the use of ITT had used it and 6 of them had used it with MPS patients [138]. Although the European Medicines Agency recommends the repurposing of medicines for the treatment of rare diseases as an option for highly personalised medical care, clinicians are rarely chosen to use this option citing insufficient training and knowledge in this area and the need for an effective tool to assess the risks and benefits of selected treatments as the reason [144]. Additional difficulties may be the denial of medical reimbursements due to the off-registration use of the drug and obtaining approval from the ethics committee [138].

A decision analysis framework (DAF) scheme for MPS in the context of the use of immunomodulators has been proposed [138]. The general procedure involves 3 steps: a comprehensive literature analysis of promising treatment targets and therapeutics, a quantitative risk–benefit assessment (RAB) of selected compounds and allocation of phenotypic profiles with quantitative assessment [138, 145].

Concluding remarks

Despite extensive discussions on improving the quality of life of patients, some attention was paid to the families/carers of patients. Few studies, however, focus on the day-to-day problems of patients and their families. According to papers focusing on this topic, behavioural and sleep disorders are among the more problematic disorders accompanying the disease, especially in neuronopathic types of MPS such as Sanfilippo syndrome. There are many options for dealing with such abnormalities. The key issue is to identify the source of the problem and choose the most effective therapy. Pharmacological treatment can and should be supported by non-pharmacological therapies (if this does not adversely affect the patient's health and life). However, it is important to remember that alternative therapies should not be used instead of pharmacological treatment. Undoubtedly, the overriding aim of ongoing research should be to learn as much as possible about the disease in order to develop the best possible treatments. However, as long as the only possible treatment is symptomatic, looking for measures to help patients and their families to improve their quality of life should be an equally important aspect of MPS III research.

Our work aimed to identify treatment options for the most problematic disorders in MPS III patients from a caregiver's perspective. However, it is important to be aware that there are many more problems affecting the quality of life of families. MPS are rare diseases and therefore knowledge of them, not only medically, but also socially, is limited. This translates into a lack of/limited opportunities for support, knowledge and help with care and quality of life for families.

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polegał na:

1. zaproponowaniu tematyki pracy
2. selekcji i doborze literatury
3. przygotowaniu figur i tabeli
4. przygotowaniu pierwotnej wersji manuskryptu
5. rewizji manuskryptu po uwagach recenzentów
6. przygotowaniu odpowiedzi na uwagi recenzentów

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polegał na:

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1. pomocy w przygotowaniu figur i tabeli
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Publikacje i działalność naukowa

Dorobek naukowy

1. Bębnowska, D., Hrynkiewicz, R., Wiśniewska, K., Żabińska, M., Rintz, E., Pierzynowska, K., & Niedźwiedzka-Rystwej, P. (2024). Apoptosis activation during *Lagovirus europaeus*/GI.2 infection in rabbits. *Frontiers in microbiology*, *14*, 1308018. https://doi.org/10.3389/fmicb.2023.1308018

2. Wiśniewska, K., Wolski, J., Anikiej-Wiczenbach, P., Żabińska, M., Węgrzyn, G., & Pierzynowska, K. (2025). Behavioural disorders and sleep problems in Sanfilippo syndrome: overlaps with some other conditions and importance indications. *European child & adolescent psychiatry*, *34*(6), 1795–1816. https://doi.org/10.1007/s00787-025-02661-5

3. Bębnowska, D., Rzeszotek, S., Kolasa, A., **Wiśniewska, K.,** Żabińska, M., Szulc, A., Cyske, Z., Pierzynowska, K., Wilk, A., & Niedźwiedzka-Rystwej, P. (2025). Evaluation of autophagic and apoptotic markers during infection with animal virus causing hemorrhagic fever in rabbits. Frontiers in Microbiology, 15. https://doi.org/10.3389/fmicb.2024.1517725

4. **Wiśniewska, K**., Żabińska, M., Szulc, A., Gaffke, L., Węgrzyn, G., & Pierzynowska, K. (2024). The Role of Gene Expression Dysregulation in the Pathogenesis of Mucopolysaccharidosis: A Comparative Analysis of Shared and Specific Molecular Markers in Neuronopathic and Non-Neuronopathic Types of the Disease. *International Journal of Molecular Sciences*, *25*(24), 13447. https://doi.org/10.3390/ijms252413447

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18. **Wiśniewska, K.,** Gaffke, L., Krzelowska, K., Węgrzyn, G., & Pierzynowska, K. (2022). Differences in gene expression patterns, revealed by RNA-seq analysis, between various Sanfilippo and Morquio disease subtypes. *Gene*, *812*, 146090.

19. Pierzynowska, K., Cyske, Z., Gaffke, L., Rintz, E., Mantej, J., Podlacha, M**., Wiśniewska, K.,** Zabińska, M., Sochocka, M., Lorenc, P., Bielańska, P., Giecewicz, I., & Węgrzyn, G. (2021). Potencjał autofagii indukowanej przez genisteinę w leczeniu chorób neurodegeneracyjnych [Potential of genistein-induced autophagy in the treatment of neurodegenerative diseases]. *Postepy biochemii*, *67*(2), 117–129

Projekty badawcze

Kierownik projektów:

- PRELUDIUM 23 ”Rola profiliny w patogenezie mukopolisacharydoz” (2024/53/N/NZ3/02910) (2025 r.- 2028 r.)

- „Znaczenie zaburzeń peroksysomów w patogenezie zespołu chorobowego NBIA” Uniwersytet Gdański (533-BG10-GS0D-24) (2024 r.- 2025 r.)

- „Akumulacja β-amyloidu, TDP-43, α-synukleiny i białka tau w komórkach pacjentów ze wszystkimi podtypami mukopolisacharydozy typu III” Uniwersytet Gdański (539-D020-B080-23) (2023 r.- 2024 r.)

- ”Wpływ obniżenia poziomu siarczanu heparanu na nadekspresję genów PFN1 i MFAP5 w aparacie komórkowym choroby Sanfilippo” Uniwersytet Gdański (533-0C20-GS32-21) (2021 r.- 2022 r.)

Główny wykonawca projektów:

- „Interakcje białek z produktami częściowej degradacji siarczanu heparanu: znaczenie w patogenezie neuronopatycznych typów mukopolisacharydoz” Fundacja Sanfilippo (559-D020-0174-23) (2022 r.- 2025 r.)

- „Wpływ eksperymentalnej terapii z użyciem modulatorów autofagii na procesy zapalne w układzie obwodowym i ośrodkowym w podstawowych odmianach NBIA (PKAN, MPAN, BPAN)” Stowarzyszenie NBIA Polska (zadania 559-D020-1016-21-P9) (w toku)

Wykonawca:

- „Stymulacja autofagii przez genisteinę w leczeniu choroby Alzheimera: mechanizmy i efekty badane przy użyciu genetycznych (komórkowych i zwierzęcych) modeli choroby” (2024/53/B/NZ2/00225) (2024r. – 2028r.)

- „Zmiany w procesach komórkowych jako kluczowe defekty w patogenezie dziedzicznych chorób metabolicznych z grupy mukopolisacharydoz” (2017/25/B/NZ2/00414) (2018 r.-2022 r.)

- „Molekularny mechanizm zaburzeń ferroprotezy w mukopolisacharydozie typu I i ich wpływ na przebieg choroby” NCN (2022/47/D/NZ2/03095) (2022 r.- 2026 r.)

Konferencje

- Wiśniewska K.
„Differences in gene expression patterns between Sanfilippo disease subtypes”
V International Sopot Youth Conference 2021 entitled Where the World is Heading 11.06.2021r. Sopot, Poland (on-line) (poster)

- Wiśniewska K.
„Similarities of organellar disorders between neuronopathic types of mucopoisaccharidoses and neurodegenerative diseases and neurological disorders”
Congress of Young Science International and Multidisciplinary Congress of Young Science 6-8.07.2023 Gdańsk, Poland (poster)

- Wiśniewska K.
„Comprehensive evaluation of the accumulation of pathogenic proteinsin fibroblasts of patients with Sanfilippo disease”
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- Wiśniewska K.
„Pathogenic protein accumulation in fibroblasts of patients with Sanfilippo disease (all subtypes)”
9th National Conference on Molecular Biology 14-15.03 2024 Łódź, Poland (wystąpienie ustne)

- Wiśniewska K.
„The importance of peroxisomal dysfunction in the pathogenesis of NBIA disorders”
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- Wiśniewska K.
„The significance of peroxisomal dysfunction in the pathogenesis of neuredegeneration whith brain iron accumulation (NBIA)"
VI Zjazd Polskiego Towarzystwa Biologii Medyczne 19-21.09, 2024 Warszawa, Poland (poster)

- Wiśniewska K.
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- Wiśniewska K.
„Gene expression dysregulation in Sanfilippo and Morquio Syndromes: the impact of PFN1 on glycosaminoglycan accumulation”
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Nagrody

III miejsce w sesji posterowej „The significance of peroxisomal dysfunction in the pathogenesis of neuredegeneration whith brain iron accumulation (NBIA)"
VI Zjazd Polskiego Towarzystwa Biologii Medyczne 19-21.09, 2024 Warszawa, Poland (poster presentation)