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Mukopolisacharydoza-plus (MPSPS): pierwsze badania molekularnego mechanizmu choroby w aspekcie opracowania terapii

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Mucopolysccharidosis-plus (MPSPS): the first study on the molecular mechanism of the disease in the light of therapy development

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Streszczenie w języku polskim

Mukopolisacharydozy (MPS) zaliczane są do grupy lizosomalnych chorób spichrzeniowych (LSD). Stanowią one schorzenie dziedziczne o charakterze postępującym, wynikające z całkowitego braku bądź znacznego obniżenia aktywności określonych enzymów lizosomalnych, odpowiedzialnych za degradację nierozgałęzionych łańcuchów polisacharydowych – glikozoaminoglikanów (GAG) [Nagpal et al. 2022]. Niezdegradowane GAG stopniowo gromadzą się w lizosomach, prowadząc do zaburzeń funkcjonowania komórek, tkanek, a z czasem całych narządów [Freeze et al. 2015]. Aktywność poszczególnych enzymów, w tym szlaku metabolicznym, jest ze sobą ściśle skorelowana – każdy enzym rozpoczyna działanie dopiero po zakończeniu reakcji przez poprzedni. W konsekwencji brak aktywności choćby jednego z nich skutkuje zaburzeniem całego procesu degradacji [Parenti et al. 2015; Trovão de Queiroz et al. 2016].

Dziedziczenie MPS ma najczęściej charakter autosomalny recesywny, z wyjątkiem MPS typu II (choroba Huntera), który jest sprzężony z chromosomem X. Częstość występowania poszczególnych typów jest bardzo zróżnicowana w zależności od regionu na świecie – szacuje się, że MPS I występuje z częstością około 1 na 88 000 żywych urodzeń, natomiast MPS VII z częstością około 1 na 2 111 000 urodzeń. MPS IX jest ekstremalnie rzadką postacią – dotychczas opisano jedynie cztery przypadki na świecie [Natowicz et al. 1996; Imundo et al. 2011; Tomatsu et al. 2018; Węgrzyn et al. 2022].

Jak dotąd opisano 14 typów i podtypów MPS, sklasyfikowanych na podstawie nieaktywnego bądź o szczątkowej aktywności enzymu lizosomalnego lub na podstawie gromadzącego się GAG [Zhou et al. 2020]. Obraz kliniczny obejmuje zarówno objawy wspólne dla wszystkich postaci choroby, jak i symptomy specyficzne dla konkretnych typów/podtypów MPS. Do symptomów opisywanych w prawie wszystkich typach/podtypach należą między innymi: organomegalia, charakterystyczne rysy twarzy, nieprawidłowości w obrębie układu szkieletowego, a w późniejszych etapach także zmiany w obrębie układu oddechowego oraz sercowo-naczyniowego. W części przypadków występują zaburzenia słuchu i wzroku. Upośledzenie funkcji poznawczych obserwuje się w MPS I, ciężkich postaciach MPS II, wszystkich typach MPS III oraz w MPS VII [Muenzer 2011a].

Pomimo, że poszczególne typy/podtypy MPS są spowodowane konkretną mutacją w różnych genach, prowadzą one do tego samego efektu biochemicznego – akumulacji GAG w lizosomach [Jakóbkiewicz-Banecka et al. 2009]. Jeszcze niedawno uważano, że gromadzenie GAG jest pierwotnym i jedynym czynnikiem patogenetycznym [Wraith 2013]. Najnowsze badania wskazują jednak, że MPS wiąże się także z dysfunkcją wielu procesów komórkowych kluczowych dla prawidłowego funkcjonowania komórek. Możliwe jest zatem, iż to zaburzenia molekularne – a nie sama akumulacja GAG – odgrywają zasadniczą rolę w patogenezie choroby. Zmiany te obejmują m.in.: zaburzenia apoptozy i autofagii [Tessitore et al. 2009; Brokowska et al. 2021], nieprawidłowości w transporcie pęcherzykowym [Gaffke et al. 2023], proteasomie [Pierzynowska et al. 2020], dysfunkcję

mitochondriów [Pshezhetsky 2015] oraz zaburzenia w obrębie cytoszkieletu [Parente et al. 2016] i przekazywaniu sygnału wewnątrz komórkowego [Pierzynowska et al. 2022]

Przez długi czas możliwości terapeutyczne u pacjentów z MPS ograniczały się wyłącznie do opieki paliatywnej. Alternatywną formą leczenia, wprowadzoną po raz pierwszy w 1980 roku, był przeszczep szpiku kostnego [Hobbs et al. 1981]. Skuteczność tej metody jest jednak ograniczona do przypadków, w których choroba została zdiagnozowana na bardzo wczesnym etapie, jeszcze przed wystąpieniem pierwszych objawów klinicznych. Terapia ta nie pozwala na odwrócenie istniejących deficytów neurologicznych, takich jak zaburzenia funkcji poznawczych, hiperaktywność czy problemy natury emocjonalnej, a także nie koryguje wszystkich zmian strukturalnych, m.in. powszechnie występujących deformacji szkieletowych [Weisstein et al. 2004]. Uważa się, że przeszczep szpiku kostnego jest nieskuteczny w leczeniu MPS typu II, III i IV [Sivakumur and Wraith 1999; Peters and Krivit 2000; Prasad and Kurtzberg 2010].

Wprowadzenie enzymatycznej terapii zastępczej (ERT), polegającej na dożylnym podaniu aktywnej formy nieaktywnego lub o szczątkowej aktywności enzymu, doprowadziło do pewnego ograniczenia w stosowaniu przeszczepów szpiku kostnego. Decyzję tę uzasadniał m.in. wysoki odsetek zgonów po pierwszym przeszczepie u pacjentów z MPS I, wynoszący około 15% [Boelens et al. 2007]. W ciągu ostatnich kilkunastu lat opracowano ERT dla MPS I, II, IVA, VI i VII, przy czym największy postęp odnotowano w przypadku MPS I [Wraith 2005; Wraith and Jones 2014; de Ru et al. 2011; Burton et al. 2025], co prawdopodobnie wynika z największej częstości występowania tego typu MPS. Podobnie jak w przypadku przeszczepu szpiku kostnego, kluczowe znaczenie ma wczesne rozpoznanie choroby i szybkie rozpoczęcie terapii [McGill et al. 2010; Schulze-Frenking et al. 2011; Pérez-López et al. 2017; Burton et al. 2025].

Korzyści wynikające z zastosowania ERT u pacjentów z MPS obejmują m.in.: poprawę ruchomości stawów, a w konsekwencji zdolności lokomocyjnych; poprawę funkcjonowania układu oddechowego; redukcję hepatomegalii oraz istotny spadek stężenia GAG w moczu [Wraith et al. 2007; Clarke et al. 2009; Harmatz et al. 2006; Muenzer et al. 2011b; Pérez-López et al. 2017]. Mimo to, ERT nie eliminuje wszystkich objawów choroby. Szczególnym ograniczeniem jest fakt, że podawany enzym nie przekracza bariery krew–mózg, co czyni terapię nieskuteczną w przypadku postaci z zajęciem ośrodkowego układu nerwowego, a także ograniczony wpływ enzymu na tkanki kostne [Chen et al. 2019; Rossi and Brunetti-Pierri 2024].

Z uwagi na konieczność opracowania terapii dla postaci neuronopatycznych, zaproponowano alternatywne podejście – terapię redukcji syntezy substratu (SRT) [Jakóbkiewicz-Banecka et al. 2009]. Metoda ta polega na zahamowaniu biosyntezy GAG w sytuacji, gdy ich degradacja jest upośledzona, co pozwala na przywrócenie równowagi pomiędzy ich powstawaniem a usuwaniem. Bezpośrednie zahamowanie aktywności enzymów syntetyzujących GAG jest niewykonalne, ponieważ ich substratami

są cukry proste uczestniczące w licznych procesach metabolicznych. Dlatego ukierunkowano działania na obniżenie ekspresji genów kodujących syntetazy GAG, co prowadzi do redukcji ich poziomu i w konsekwencji zmniejszenia tempa syntezy tych związków. Substancją wykorzystywaną w tym podejściu terapeutycznym jest trihydroksyizoflawon (genisteina) [Jakóbkiewicz-Banecka et al. 2009], naturalny związek z grupy izoflawonów który, z uwagi na szerokie spektrum działania, jest już stosowany w medycynie m.in. jako środek łagodzący objawy menopauzy oraz o potencjale przeciwnowotworowym.

Badania przeprowadzone z wykorzystaniem innego związku naturalnego – ambroksolu (4-((2-amino-3,5-dibromofenylo)metyloamino)cykloheksan-1-ol), wykazały, że pozytywnie wpływa on na funkcje komórkowe poprzez poprawę aktywności enzymów lizosomalnych, funkcji lizosomów oraz indukuje proces autofagii w modelu komórkowym mukopolisacharydozy typu III (MPS III) [Goker-Alpan et al. 2020]. Eksperymenty z wykorzystaniem ambroksolu, jako potencjalnego terapeutyku, przeprowadzono również modelu choroby Gaucher wskazują, że może on pomóc w zmniejszeniu nagromadzonych, toksycznych substancji przez aktywację ścieżki autofagia-lizosom [McNeill et al. 2014].

Na początku 2010 roku w Republice Sacha (Jakucja, Rosja) opisano kilku pacjentów z objawami przypominającymi zespół Hurler (ciężką postać MPS I), prowadzące do zgonu przed ukończeniem drugiego roku życia [Gurinova et al. 2014]. Początkowo schorzenie zostało sklasyfikowane jako „niezróżnicowana dziedziczna choroba metaboliczna" [Vasilev et al. 2020]. W 2017 roku, po szczegółowym scharakteryzowaniu obrazu klinicznego oraz ustaleniu etiologii, jednostka ta została opisana jako zespół mukopolisacharydozy plus (MPS-plus, MPSPS). U pacjentów zaobserwowano nadmierne wydalanie GAG z moczem, jednak nie było to wynikiem obniżonej aktywności enzymów lizosomalnych. Nazwa wskazuje, iż poza klasycznymi objawami typowymi dla konwencjonalnych postaci MPS, u chorych występują również inne cechy fenotypowe, m.in. wrodzone wady serca, zaburzenia czynności nerek oraz nieprawidłowości w obrębie układu krwiotwórczego [Faraguna et al. 2022; Sofronova et al. 2022]. Do chwili obecnej chorobę stwierdzono u kilkudziesięciu pacjentów, jednak dokładna ich liczba jest trudna do określenia, ponieważ niektórzy pacjenci mogli zostać ujęci kilkukrotnie w różnych publikacjach [Lipiński et al. 2022]. Większość zdiagnozowanych pacjentów pochodzi z populacji jakuckiej (Republika Sacha) [Kondo et al. 2017], natomiast opisano również przypadki w innych regionach świata – w Turcji [Dursun et al. 2017], oraz po jednym pacjencie z rejonu Morza Śródziemnego [Pavlova et al. 2022] i w Polsce [Lipiński et al. 2022]. Szacuje się, że częstość występowania MPSPS w populacji Jakutów wynosi około 1:12 100 żywych urodzeń [Kondo et al. 2017].

MPSPS dziedziczy się w sposób autosomalny recesywny i jak dotąd opisano dwie homozygotyczne mutacje, w genie *VPS33A*, prowadzące do jego powstania. Wariant c.1492C > T

(p.Arg498Trp) został opisany u wszystkich pacjentów pochodzących z Jakucji oraz Turcji [Dursun et al. 2017; Kondo et al. 2017; Faraguna et al. 2022], natomiast mutacje c.599G > C (p.Arg200Pro) opisano u pacjentów z rejonu Morza Śródziemnego oraz Polski [Pavlova et al. 2022; Cyske et al. 2025b - **Artykuł nr 3** wchodzący w skład tej rozprawy doktorskiej]. Porównując fenotyp wszystkich pacjentów z MPSPS można zauważyć, że mutacja c.1492C > T (p.Arg498Trp) prowadzi do powstania znacznie cięższych objawów oraz bardzo wczesnej śmierci pacjenta, natomiast w przypadku mutacji c.599G > C (p.Arg200Pro) objawy są znacznie łagodniejsze [Pavlova et al. 2019; Pavlova et al. 2022; Lipiński et al. 2022].

Pomimo identyfikacji mutacji prowadzącej do powstania MPSPS dokładny patomechanizm choroby pozostaje nieznany. Zaproponowano natomiast dwie hipotezy, które potencjalnie mogą wyjaśniać mechanizm choroby. Pierwsza z nich opiera się na badaniach potwierdzających nadmierne zakwaszenie lizosomów w wariancie p.Arg498Trp MPSPS. Enzymy lizosomalne w nadmiernie zakwaszonym środowisku nie funkcjonują prawidłowo i nie mogą rozkładać GAG, przez co gromadzą się one w lizosomach powodując szereg ciężkich objawów [Kondo et al. 2017]. Druga z hipotez dotyczy wariantu p.Arg200Pro MPSPS i skupia się na funkcji produktu białkowego genu *VPS33A* (białka VPS33A). Białko to jest składnikiem dwóch, dużych, wielofunkcyjnych kompleksów: homotypowego kompleksu fuzji i sortowania białek (HOPS) oraz kompleksu wiązanie wakuoli rdzeniowo-endosomalnych klasy C (CORVET). Oba te kompleksy uczestniczą w transporcie białek do lizosomów, a także w procesach autofagii i endozytozy [Wartosch et al. 2015; Sofronova et al. 2022]. Obniżony poziom białka VPS33A wykazano w przypadku obu opisanych wariantów MPSPS, jednak w obu przypadkach białko zachowało swoją aktywność biochemiczną, co wskazuje na to, że za powstające objawy odpowiedzialna jest obniżona ilość białka, spowodowana nadmierną jego degradacją w proteasomie, a nie brak jego aktywności. [Pavlova et al. 2022; Terawaki et al. 2025]. Kolejne badania wskazały, że za patomechanizm MPSPS może być odpowiedzialny zaburzony transport endosomalny, który spowodowany jest obniżonym poziomem białka VPS33A, a co za tym idzie GAG nie są efektywnie dostarczane do lizosomów w celu ich degradacji [Cyske et al. 2024a – **Artykuł nr 2**; Cyske et al. 2025b – **Artykuł nr 3**; Terawaki et al. 2025]. Takie badania zostały już wcześniej przeprowadzone na komórkowym (MPS I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, IX) oraz mysim modelu MPSI i wskazały na znaczne zaburzenia w transporcie pęcherzykowym, a także na poprawę tych parametrów po zastosowaniu genisteiny [Gaffke et al. 2023] Jeśli przedstawiona hipoteza byłaby prawdziwa, powinny nastąpić istotne zmiany w poziomach białek zaangażowanych w transport pęcherzykowy w MPSPS, a także w wybranych organellach komórkowych.

W związku z brakiem poznania dokładnego patomechanizmu, bardzo ograniczoną wiedzą na temat zaburzeń komórkowych w MPSPS oraz potrzebą opracowania skutecznego leku uzasadnionym jest zbadanie jakie procesy komórkowe mogą być upośledzone w MPSPS, co doprowadzić może do poznania dokładnego mechanizmu powstawania choroby oraz potencjalnych leków na MPSPS.

Dlatego celem mojej rozprawy doktorskiej stało się:

1) **Określenie jakie procesy komórkowe są zaburzone w MPSPS, a co za tym idzie poznanie patomechanizmu choroby**
2) **Przetestowanie dwóch związków naturalnych - ambroksolu i genisteiny, jako potencjalnych leków dla pacjentów z MPSPS**

W pracy wykorzystywałam komórki skórne – fibroblasty, pobrane od jedynej polskiej pacjentki z MPSPS z homozygotycznym wariantem c.599G>C (p.Arg200Pro) genu *VPS33A*. Nie miałam możliwości uzyskania większej liczby linii komórkowych MPSPS z uwagi na niezwykle małą liczbę pacjentów (jedynie kilkudziesięciu na świecie), bardzo dużą odległość i izolację geograficzną większości z nich (chorzy głównie zamieszkują regiony Jakucji – Syberii), a także niezwykle krótką długość życia chorych (większość umiera przed ukończeniem 2. roku życia). Te, a także wiele innych problemów związanych z chorobami rzadkimi zostały szerzej omówione w artykule Cyske et al. 2025 [Cyske et al. 2025a - **Artykuł nr 1**]. Jako kontrolę wybrałam komórki skórne pobrane od osoby zdrowej odpowiadającej wiekiem i płcią badanej pacjentce. W części doświadczeń zostały również wykorzystane linie komórkowe pobrane od pacjentów z chorobą Sanfilippo, jako porównanie do opisanych już i lepiej poznanych zaburzeń komórkowych w MPS.

1) Określenie jakie procesy komórkowe są zaburzone w MPSPS, a co za tym idzie poznanie patomechanizmu choroby

Aby przetestować hipotezę mówiącą o zaburzonym transporcie endosomalnym GAG, wynikającym z obniżonej ilości białka VPS33A i nieefektywnego dostarczania ich do lizosomów, w pierwszej kolejności określiłam poziom samych GAG w komórkach MPSPS z wariantem p.Arg200Pro genu *VPS33A* w komórkach kontrolnych oraz pacjenta cierpiącego na chorobą Sanfilippo (MPSIII). Eksperymenty, z wykorzystaniem metody dot-blotting, wykazały znacznie podwyższony poziom siarczanu heparanu (HS) w komórkach MPSPS w porównaniu do komórek kontrolnych. Zwizualizowałam również wewnątrzkomórkową lokalizację HS, co okazało się niezwykle interesujące, ponieważ obraz uzyskany po wybarwieniu komórek MPSPS znacznie różnił się o tego uzyskanego z komórek MPS III. Mianowicie, w MPS III HS był widoczny w postaci wielu skupisk według wcześniejszych danych literaturowych można sądzić, że są to agregaty HS w lizosomach natomiast w przypadku MPSPS HS był rozproszony równomiernie po całej komórce. Taki obraz może wskazywać na zaburzony transport i dostarczanie GAG do lizosomów.

Wcześniejsze badania, zarówno z wykorzystaniem wariantu p.Arg200Pro jak i p.Arg498Trp, wskazywały na obniżoną ilość białka VPS33A w MPSPS [Pavlova et al. 2022; Terawaki et al. 2025]. Zbadałam więc poziom tego białka w fibroblastach pobranych od polskiej pacjentki w porównaniu

z komórkami kontrolnymi. Stwierdziła drastycznie, bo o ponad 80%, obniżenie poziomu białka VPS33A w komórkach MPSPS w porównaniu do komórek kontrolnych. Wykorzystując metodę mikroskopii fluorescencyjnej również wykazałam zmniejszoną intensywność fluorescencji tego białka. Białko VPS16, tak samo jak VPS33A, jest jedną z podjednostek kompleksów HOPS i CORVET. Białka te ściśle ze sobą oddziaływują, dlatego określiłam również tego białka. Podobnie do wcześniejszego doświadczenia określającego poziom białka VPS33A, stwierdziłam także obniżony poziom białka VPS16 w MPSPS

Mikroskopia elektronowa pozwala na szerokie spojrzenie na ogólną morfologię komórki. Dla wcześniej opisanych typów/podtypów MPS, w obrazie tym, charakterystyczne są struktury „skórki cebuli" oraz „ciała zebrowate", są to specyficzne obrazy po których rozpoznać można lizosomy, w których zakumulowane są nierozłożone GAG [Piotrowska et al. 2006]. Dla pełnego oglądu komórek MPSPS wykonałam takie samo badanie, które wykazało znacznie podwyższoną Liczbę pęcherzyków o zmienionej strukturze w porównaniu do komórek kontrolnych. Jeśli jednak teoria mówiąca o zaburzonym transporcie pęcherzykowym jest prawdziwa, nie ma pewności czy na obrazie mikroskopowym widoczne pęcherzyki są lizosomami, jak w innych typach MPS, czy endosomami. Żeby odpowiedzieć na to pytanie wybarwiłam lizosomy z wykorzystaniem barwnika LysoTracker. Doświadczenia wykazały znacznie silniejszy sygnał w komórkach MPSPS niż w komórkach kontrolnych. Może to dowodzić zwiększonej liczbie lizosomów, jednak wcześniejsze eksperymenty dowiodły, że w MPSPS lizosomy są nadmiernie zakwaszone [Kondo et al. 2017; Pavlova et al. 2022], a wiadomym jest że barwnik LysoTracker wykazuje wyższe powinowactwo do organelli o niższym pH. Dlatego zadałam pytanie czy lizosomy są liczniejsze czy jedynie nadmiernie zakwaszone. Do uzyskania odpowiedzi wykorzystałam białko LAMP2, białko błonowe lizosomów i jeden z najczęstszych markerów w badań lizosomów. Zarówno poziom tego białka jak i intensywność fluorescencji była podwyższona na tej podstawie można stwierdzić, że lizosomy są liczniejsze w MPSPS, niezależnie od większego ich zakwaszenia.

Biorąc pod uwagę „rozsiany" obraz HS uzyskany z analiz mikroskopii fluorescencyjnej postawiłam pytanie czy GAG są efektywnie dostarczane do lizosomów w celu ich degradacji. Dlatego też określiłam kolokalizację lizosomów z HS w MPSPS oraz w MPS III i wykazałam, że w przypadku MPS III kolokalizacja była na znacznie wyższym poziomie niż w komórkach kontrolnych, jednak w MPSPS różnic tych nie było. Może to wskazywać, że GAG nie są dostarczane do lizosomów i mimo, że enzymy lizosomalne działają prawidłowo, GAG nie mogą być rozłożone. Niezwykle ciekawy okazał się kolejny eksperyment pokazujący kolokalizację HS z białkiem LAMP1, kolejnym białkiem błonowym lizosomów, w którym wynik był odwrotny. Mianowicie, kolokalizacja HS/LAMP1 była znacznie podwyższona w MPSPS w porównaniu z komórkami kontrolnymi, natomiast w MPS III obniżona. Mogłoby się to wydawać sprzeczne z poprzednim eksperymentem, jednak w literaturze opisane są badania mówiące o tym, że białko LAMP1 znajduje się również we wczesnych i późnych endosomach

[Cook et al. 2004]. Zatem prawdopodobne jest, że GAG zostają uwięzione w endosomach i nie są transportowane do lizosomów w celu degradacji.

Skoro białko VPS33A bierze udział w dojrzewaniu endosomów i ich transporcie do lizosomów [Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022], to zgodnie z testowaną hipotezą, poziom markerów endosomalnych powinien być podwyższony w komórkach MPSPS. Markerem wczesnych endosomów jest białko EEA1, które jest niezbędne do sortowania wczesnych i późnych endosomów, a także do sortowania na etapie wczesnych endosomów [Yap i Winckler 2022]. Podwyższony poziom tego białka oraz intensywność fluorescencji wykazałam w komórkach MPSPS w porównaniu do komórek kontrolnych, stosując odpowiednio metodę western-blotting oraz mikroskopię fluorescencyjną,. W tym przypadku również uzyskany wynik potwierdza postawioną hipotezę, a dodatkowo jest on zgody z eksperymentami przeprowadzonymi przez inny zespól na komórkach pacjenta z tą samą mutacją [Pavlova et al. 2022]. Idąc dalej tym tropem sprawdziłam poziom białka Rab7, które można uznać za marker późnych endosomów, ponieważ jest niezbędne do transportu późnych endosomów oraz do ich dostarczenia do lizosomów. Również ten eksperyment wykazał podwyższymy poziom tego biała, a także analizy mikroskopowe potwierdziły zwiększoną intensywność fluorescencji tego białka.

Wracając do wspomnianego powyżej założenia zakładającego, że HS gromadzi się w endosomach i nie może zostać przetransportowany do lizosomów, określiłam kolokalizację HS z białkiem EEA1. Na podstawie wyników, które uzyskałam, można stwierdzić zdecydowanie podwyższoną kolokalizację HS/EEA1 w komórkach MPSPS w porównaniu do komórek kontrolnych, co potwierdza postawioną hipotezę. Jednak nie można nie zwrócić uwagi na poziom kolokalizacji HS/EEA1 w MPS III, który był jeszcze wyższy niż w MPSPS, natomiast badania mówiące o wtórnym defekcie transportu pęcherzykowego w MPS zostały już wcześniej przeprowadzone [Gaffke et al. 2023]

2) Przetestowanie dwóch związków naturalnych - ambroksolu i genisteiny, jako potencjalnych leków dla pacjentów z MPSPS

MPSPS jak dotąd jest chorobą nieuleczalną, a z uwagi na niezwykle małą liczbę pacjentów oraz bardzo którą długość ich życia, opracowanie skutecznej terapii wydaje się być ogromnym wyzwaniem dla współczesnej nauki. Potencjalna terapia dla MPSPS musiałaby skupiać się na korygowaniu pierwotnych defektów molekularnych, ale oprócz tego również być bezpieczna w długoterminowym użyciu.

Wytypowałam dwa związki naturalne, które potencjalnie mogłyby stać się lekami dla MPSPS: ambroksol (4-((2-amino-3,5-dibromofenylo)metyloamino)cykloheksan-1-ol) oraz genisteina (5,7-dihydroksy-3-(4-hydroksyfenylo)-4H-1-benzopiran-4-on). W związki z tym, że oba były opisywane

jako inhibitory degradacji białek – odpowiednio degradacji związanej z siateczką śródplazmatyczną (ERAD) oraz degradacji proteasomalnej. Oba związki mają również właściwości przeciwzapalne oraz antyoksydacyjne, a także indukują proces autofagii [Dhanve et al. 2023; Cyske et al. 2024b; Ponugoti et al. 2024; Borah et al. 2025; Goswami et al. 2025; Pierzynowska et al. 2025], ale ambroksol łagodzi teżstres endoplazmatyczny i hamuje degradację ERAD [Bendikov-Bar et al. 2011; Dhanve et al. 2023], natomiast genisteina hamuje degradację proteasomalną poprzez modulację ubikwitynacji białek [Pierzynowska et al. 2020]. Zgodnie z wymaganiem stawionym lekom, które należy przyjmować przez całe życie, udowodniono, że oba związki są bezpieczne w długoletnim stosowaniu [Kim et al. 2013; Ghosh et al. 2021; Ponugoti et al. 2024]. Na podstawie powyższych danych wybrałam ambroksol i genisteinę jako potencjalne leki na MPSPS, a w przyszłości również na inne choroby związane z niestabilnością białek i zaburzeniami transportu endosomalnego, a także biorąc pod uwagę inne mechanizmy hamowania proteolizy przez ambroksol i genisteinę, określiłam czy zastosowanie terapii kombinowanej będzie skuteczniejsze niż wykorzystanie każdego związku osobno.

Pierwszym i jednym z najważniejszych parametrów przy poszukiwaniu nowych leków jest bezpieczeństwo. Dlatego takie doświadczenie było pierwszym, które wykonałam dla ambroksolu i genisteiny jako potencjalnych leków na MPSPS, mimo, że oba związki są już wykorzystywane od wielu lat w medycynie. Na podstawie wcześniejszych danych literaturowych wybrałam stężenia 100 μM dla ambroksolu i 50 μM dla genisteiny, które efektywnie hamują proteolizę [Bendikov-Bar et al. 2011; Pierzynowska et al. 2020]. Na podstawie testu żywotności komórek (MTT) wykazałam, że oba związki są bezpieczne dla komórek MPSPS we wskazanych powyżej stężeniach.

Głównym problemem w przypadku MPSPS okazał się obniżony poziom białka VPS33A, co prowadzi do nieefektywnego transportu pęcherzykowego i uwięzienia GAG w endosomach. Potencjalny lek powinien więc podnosić poziom tego białka. Badania, które przeprowadziłam wykazały 4-krotnie obniżony poziom białka VPS33A w komórkach MPSPS w porównaniu do komórek kontrolnych. Natomiast podanie ambroksolu, genisteiny lub ich kombinacji spowodowało wzrost poziomu tego białka. Zastosowanie mieszaniny obu związków okazało się najbardziej efektywne i poziom białka VPS33A osiągnął około 80% wartości kontrolnej. Porównywalny wynik uzyskałam dla białka VPS16, które jest ściśle związane z białkiem VPS33A. Oba te wyniki wskazują, że ambroksol i genisteina podwyższają poziom białek VPS33A i VPS16 poprzez obniżenie aktywności ERAD (ambroksol) i proteasomu (genisteina) [Bendikov-Bar et al. 2011; Pierzynowska et al. 2020].

Eksperymenty, które przeprowadziłam potwierdziły znacznie podwyższony (około 3-krotnie) poziom HS w komórkach MPSPS w porównaniu do komórek kontrolnych [Cyske Z et al. 2025b – **Artykuł nr 3**]. Natomiast zastosowanie ambroksolu, genisteiny oraz ich mieszaniny obniżyło poziom HS, nawet do poziomu zanotowanego w komórkach kontrolnych. Aby określić czy degradacja HS zachodzi przez szlak lizosomalny zastosowałam chlorochinę (inhibitor, który blokuje fuzję

autofagosomu z lizosomem). Eksperymenty potwierdziły, że po zastosowaniu chlorochiny w stężeniu 10 μM zaobserwować można bardzo wysokie poziomy HS, niezależnie od obecności ambroksolu i genisteiny. Wynik ten wskazuje, że degradacja GAG w komórkach MPSPS po użyciu ambroksolu i genisteiny zachodzi przez szlak lizosomalny.

Wiadomo już że w MPSPS zaburzenia występują również w lizosomach, więc ponownie określiłam ich liczbę oraz intensywność fluorescencji z w wykorzystaniem barwnika LysoTracker, tym razem jednak określiłam również wpływ ambrosksolu i genistein na te parametry. Wykazałam, że liczba lizosomów oraz intensywność ich fluorescencji jest znacznie podwyższona w MPSPS w porównaniu do komórek kontrolnych, a leczenie ambroksolem, genisteiną oraz ich mieszaniną skutecznie obniżało liczbę lizosomów, natomiast zastosowanie mieszaniny obu związków najskuteczniej redukowało intensywność fluorescencji w MPSPS.

Aby potwierdzić normalizację transportu GAG do lizosomów określiłam kolokalizację HS z lizosomami (barwnik LysoTracker). Eksperymenty ponownie wykazały znacznie obniżony poziom ich kolokalizacji w komórkach MPSPS w porównaniu do komórek kontrolnych, ale także wzrost tego parametru po zastosowaniu ambroksolu, genisteiny oraz ich mieszaniny, przy czy zastosowanie mieszanki obu związków było najbardziej efektywne. Badając kolokalizację HS z LAMP2, białkiem błonowym lizosomów, zauważyć można obniżony poziom kolokalizacji w MPSPS w porównaniu do kontroli, ale również wzrost do poziomu porównywalnego z kontrola po zastosowaniu mieszaniny ambroksolu i genisteiny. Badania, które przeprowadziłam wcześniej sugerują, że w MPSPS GAG są uwięzione w endosomach z powodu wadliwego transportu endosomalnego [Cyske Z et al. 2025b]. Ponownie określiłam poziom białka EEA1 oraz jego kolokalizacje z HS, co pokazało, że poziom białka EEA1 jest znacznie podwyższony w MPSPS w porównaniu do komórek kontrolnych, jednak zastosowanie ambroksolu, genisteiny oraz ich mieszaniny spowodowało normalizację do poziomu nieodróżnialnego od kontroli. Wynik ten wskazuje na poprawę wydajności transportu pęcherzykowego w MPSPS po zastosowaniu terapii. Wniosek ten dodatkowo potwierdza określenie kolokalizacji HS z białkiem LAMP1 (jak wspomniałam wcześniej białko to znajduje się zarówno w lizosomach jak i endosomach) [Cook et al. 2024]. Eksperyment ten wykazał, jak poprzednio, zwiększoną kolokalizację HS/LAMP1 w komórkach MPSPS w porównaniu z kontrolą, ale również normalizację tego poziomu po zastosowaniu mieszaniny ambroksolu i genisteiny. Po raz kolejny wyniki wskazuje zatem na słuszność założen hipotezy o wadliwym transporcie endosomalnym GAG oraz na poprawę jego wydajności po zastosowaniu ambroksolu, genisteiny, a w szczególności ich mieszaniny.

Oprócz poziomu białka EEA1 (markera wczesnych endosomów) określiłam również poziom markerów późnych endosomów – białka Rab5 i Rab7. Białko Rab5 zaangażowane jest w dojrzewanie endosomów [Nagano et al. 2019], natomiast Rab7 jest niezbędne w transporcie endosomalnym i dostarczaniu makrocząsteczek do lizosomów [Marwaha et al. 2017; Mulligan i Winckler 2023].

Poziomy obu białek markerowych dla późnych endosomów były podwyższone w MPSPS w porównaniu do kontroli, a zastosowanie mieszaniny ambroksolu i genisteiny powodowało normalizacje tego parametru do poziomu kontroli (w przypadku Rab7) lub poprawę parametru (w przypadku Rab5).

Eksperymenty przeprowadzone przez inne zespoły [Terawaki et al. 2025] wskazały, że autofagia nie jest upośledzona w wariancie p.Arg498Trp MPSPS. Natomiast wyniki badań, które uzyskałam niedawno jasno pokazują, że proces ten jest zaburzony w wariancie p.Arg200Pro MPSPS [Cyske Z et al. 2025b]. Zarówno ambroksol jak i genisteina są znanymi induktorami procesu autofagii, określiłam ich wpływy na stymulację tego procesu w MPSPS w wariancie p.Arg200Pro. Określiłam poziom białka LC3-II (markera procesu autofagii) i stwierdziłam, że zarówno ambroksol jak i genisteina znacząco zwiększają poziom tego białka, co wskazuje na skuteczną stymulacje autofagii, a proces ten jest najbardziej efektywny po zastosowaniu mieszaniny obu związków. Degradacja zakumulowanego materiału białkowego jest niezwykle ważna w kontekście potencjalnej terapii MPSPS, dlatego indukcja procesu autofagii przez ambroksol i genisteinę jest niezwykle pomocna w kontekście opracowania potencjalnej terapii z wykorzystaniem tych związków.

Rezultaty moich badań opisanych w tym rozdziale zostały przedstawione w manuskrypcie artykułu, który został przesłany do redakcji czasopisma *Mammalian Genome* i w momencie składania tej rozprawy doktorskiej znajdował się w recenzjach [Cyske et al. – **Artykuł nr 4**].

Wnioski

Niniejsza praca doktorska dostarcza dowodów eksperymentalnych popierających hipotezę o zaburzonym transporcie endosomlanym, spowodowanym przez obniżony poziom białka VPS33A, co jest podstawą molekularnego mechanizmu MPSPS. Terapia z wykorzystaniem ambroksolu i genisteiny, którą proponuję jako potencjalnie skuteczną w leczeniu MPSPS, skutkuje podwyższeniem poziomu białka VPS33A, co poprawia prawie wszystkie zaburzone parametry lub nawet normalizuje je do poziomu nieodróżnialnego od kontroli. Zaproponowana terapia w przyszłości ma potencjał stać się przydatna również w leczeniu innych chorób związanych z niestabilnością białek i zaburzonym transportem endosomalnym.

Summary in English

Mucopolysaccharidoses (MPS) belong to the group of lysosomal storage disorders (LSDs). They are a progressive hereditary disorder resulting from the complete absence or significant reduction in the activity of specific lysosomal enzymes responsible for the degradation of unbranched polysaccharide chains – glycosaminoglycans (GAGs) [Nagpal et al. 2022]. Undegraded GAGs gradually accumulate in lysosomes, leading to dysfunction of cells, tissues, and eventually entire organs [Freeze et al. 2015]. The activity of individual enzymes, including those in the metabolic pathway, is closely correlated – each enzyme begins to act only after the previous one has completed its reaction. As a result, the lack of activity of even one of them disrupts the entire degradation process [Parenti et al. 2015; Trovão de Queiroz et al. 2016].

MPS inheritance is most often autosomal recessive, with the exception of MPS type II (Hunter syndrome), which is X-linked. The prevalence of individual types varies greatly depending on the region of the world – it is estimated that MPS I occurs at a rate of approximately 1 in 88,000 live births, while MPS VII occurs at a rate of approximately 1 in 2,111,000 births. MPS IX is an extremely rare form – only four cases have been reported worldwide to date [Natowicz et al. 1996; Imundo et al. 2011; Tomatsu et al. 2018; Węgrzyn et al. 2022].

To date, 14 types and subtypes of MPS have been described, classified on the basis of inactive or severely impaired lysosomal enzyme activity or on the basis of GAG accumulation [Zhou et al. 2020]. The clinical picture includes both symptoms common to all forms of the disease and symptoms specific to particular types/subtypes of MPS. Symptoms described in almost all types/subtypes include organomegaly, characteristic facial features, skeletal abnormalities, and, in later stages, changes in the respiratory and cardiovascular systems. In some cases, hearing and vision impairments occur. Cognitive impairment is observed in MPS I, severe forms of MPS II, all types of MPS III, and MPS VII [Muenzer 2011a].

Although individual types/subtypes of MPS are caused by specific mutations in different genes, they lead to the same biochemical effect - the accumulation of GAG in lysosomes [Jakóbkiewicz-Banecka et al. 2009]. Until recently, it was believed that GAG accumulation was the primary and only pathogenic factor [Wraith 2013]. However, recent studies indicate that MPS is also associated with the dysfunction of many cellular processes that are essential for normal cell function. It is therefore possible that molecular disorders, rather than GAG accumulation alone, play a key role in the pathogenesis of the disease. These changes include, among others: apoptosis and autophagy disorders [Tessitore et al. 2009; Brokowska et al. 2021], abnormalities in vesicular transport [Gaffke et al. 2023], proteasome [Pierzynowska et al. 2020], mitochondrial dysfunction [Pshezhetsky 2015], and disorders within the cytoskeleton [Parente et al. 2016] and intracellular signalling [Pierzynowska et al. 2022].

For a long time, therapeutic options for patients with MPS were limited to palliative care. An alternative form of treatment, first introduced in 1980, was bone marrow transplantation [Hobbs et al. 1981]. However, the effectiveness of this method is limited to cases where the disease was diagnosed at a very early stage, before the first clinical symptoms appeared. This therapy does not reverse existing neurological deficits, such as cognitive impairment, hyperactivity, or emotional problems, nor does it correct all structural changes, including common skeletal deformities [Weisstein et al. 2004]. Bone marrow transplantation is considered ineffective in the treatment of MPS types II, III, and IV [Sivakumur and Wraith 1999; Peters and Krivit 2000; Prasad and Kurtzberg 2010].

The introduction of enzyme replacement therapy (ERT), which involves the intravenous administration of the active form of an inactive or partially active enzyme, has led to some restrictions on the use of bone marrow transplants. This decision was justified, among other things, by the high mortality rate after the first transplant in patients with MPS I, amounting to approximately 15% [Boelens et al. 2007]. Over the past dozen or so years, ERT has been developed for MPS I, II, IVA, VI, and VII, with the greatest progress being made in the case of MPS I [Wraith 2005; Wraith and Jones 2014; de Ru et al. 2011; Burton et al. 2025], which is probably due to the highest prevalence of this type of MPS. As with bone marrow transplantation, early diagnosis of the disease and rapid initiation of therapy are crucial [McGill et al. 2010; Schulze-Frenking et al. 2011; Pérez-López et al. 2017; Burton et al. 2025].

The benefits of ERT in patients with MPS include improved joint mobility and, consequently, locomotor ability; improved respiratory function; reduction of hepatomegaly; and a significant decrease in urinary GAG concentration [Wraith et al. 2007; Clarke et al. 2009; Harmatz et al. 2006; Muenzer et al. 2011b; Pérez-López et al. 2017]. Nevertheless, ERT does not eliminate all symptoms of the disease. A particular limitation is the fact that the administered enzyme does not cross the blood-brain barrier, which makes the therapy ineffective in cases involving the central nervous system, as well as the limited effect of the enzyme on bone tissue [Chen et al. 2019; Rossi and Brunetti-Pierri 2024].

Due to the need to develop a therapy for the neuronopathic form, an alternative approach has been proposed – substrate reduction therapy (SRT) [Jakóbkiewicz-Banecka et al. 2009]. This method involves inhibiting GAG biosynthesis when their degradation is impaired, which allows the balance between their formation and removal to be restored. Direct inhibition of GAG-synthesizing enzymes is not feasible because their substrates are simple sugars involved in numerous metabolic processes. Therefore, efforts have been focused on reducing the expression of genes encoding GAG synthetases, which leads to a reduction in their levels and, consequently, a decrease in the rate of synthesis of these compounds. The substance used in this therapeutic approach is trihydroxyisoflavone (genistein) [Jakóbkiewicz-Banecka et al. 2009], a natural compound from the isoflavone group which, due to its broad spectrum of activity, is already used in medicine, among others, as a remedy for menopausal symptoms and for its anti-cancer potential.

Studies conducted using another natural compound, ambroxol (4-((2-amino-3,5-dibromophenyl)methylamino)cyclohexan-1-ol), has shown that it has a positive effect on cellular functions by improving the activity of lysosomal enzymes and lysosomal function, and induces autophagy in a cellular model of mucopolysaccharidosis type III (MPS III) [Goker-Alpan et al. 2020]. Experiments using ambroxol as a potential therapeutic agent were also conducted in a model of Gaucher disease, indicating that it may help reduce the accumulation of toxic substances by activating the autophagy-lysosome pathway [McNeill et al. 2014].

In early 2010, several patients with symptoms resembling Hurler syndrome (a severe form of MPS I) were described in the Republic of Sakha (Yakutia, Russia), leading to death before the age of two [Gurinova et al. 2014]. Initially, the condition was classified as an "undifferentiated hereditary metabolic disease" [Vasilev et al. 2020]. In 2017, after detailed characterization of the clinical picture and determination of the etiology, this entity was described as mucopolysaccharidosis plus (MPS-plus, MPSPS). Patients were found to have excessive GAG excretion in their urine, but this was not due to reduced lysosomal enzyme activity. The name indicates that, in addition to the classic symptoms typical of conventional forms of MPS, patients also have other phenotypic features, including congenital heart defects, renal dysfunction, and hematopoietic abnormalities [Faraguna et al. 2022; Sofronova et al. 2022]. To date, the disease has been diagnosed in several dozen patients, but the exact number is difficult to determine because some patients may have been included several times in different publications [Lipiński et al. 2022]. Most of the diagnosed patients come from the Yakut population (Sakha Republic) [Kondo et al. 2017], but cases have also been reported in other regions of the world – in Turkey [Dursun et al. 2017], and one patient each from the Mediterranean region [Pavlova et al. 2022] and Poland [Lipiński et al. 2022]. It is estimated that the prevalence of MPSPS in the Yakut population is approximately 1:12,100 live births [Kondo et al. 2017].

MPSPS is inherited in an autosomal recessive manner, and to date, two homozygous mutations in the *VPS33A* gene have been described as causing the disorder. The c.1492C > T variant (p.Arg498Trp) has been described in all patients from Yakutia and Turkey [Dursun et al. 2017; Kondo et al. 2017; Faraguna et al. 2022], while the c.599G > C (p.Arg200Pro) mutation has been described in patients from the Mediterranean region and Poland [Pavlova et al. 2022; Cyske et al. 2025b - **Article No. 3** included in this PhD thesis]. Comparing the phenotype of all patients with MPSPS, it can be seen that the c.1492C > T (p.Arg498Trp) leads to much more severe symptoms and very early death, while in the case of the c.599G > C (p.Arg200Pro) mutation, the symptoms are much milder [Pavlova et al. 2019; Pavlova et al. 2022; Lipiński et al. 2022].

Despite the identification of the mutation leading to MPSPS, the exact pathomechanism of the disease remains unknown. However, two hypotheses have been proposed that could potentially explain the mechanism of the disease. The first is based on studies confirming excessive acidification of

lysosomes in the p.Arg498Trp variant of MPSPS. Lysosomal enzymes do not function properly in an excessively acidic environment and cannot break down GAGs, which accumulate in lysosomes, causing a number of severe symptoms [Kondo et al. 2017]. The second hypothesis concerns the p.Arg200Pro MPSPS variant and focuses on the function of the *VPS33A* gene protein product *(*VPS33A protein). This protein is a component of two large, multifunctional complexes: the homotypic fusion and protein sorting complex (HOPS) and the class C core-endosomal vacuolar binding complex (CORVET). Both of these complexes are involved in the transport of proteins to lysosomes, as well as in autophagy and endocytosis processes [Wartosch et al. 2015; Sofronova et al. 2022]. Reduced levels of VPS33A protein have been demonstrated in both MPSPS variants described, but in both cases the protein retained its biochemical activity, indicating that the symptoms are caused by a reduced amount of protein due to its excessive degradation in the proteasome, rather than a lack of activity. [Pavlova et al. 2022; Terawaki et al. 2025]. Further studies have indicated that the pathomechanism of MPSPS may be caused by impaired endosomal transport, which is caused by reduced levels of the VPS33A protein, and thus GAGs are not effectively delivered to lysosomes for degradation [Cyske et al. 2024a – **Article No. 2**; Cyske et al. 2025b – **Article No. 3**; Terawaki et al. 2025]. Such studies have already been conducted on cellular (MPS I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, IX) and mouse models of MPS I and have indicated significant disturbances in vesicular transport, as well as an improvement in these parameters after the use of genistein [Gaffke et al. 2023]. If the presented hypothesis were true, there should be significant changes in the levels of proteins involved in vesicular transport in MPSPS, as well as in selected cellular organelles.

Due to the lack of knowledge about the exact pathomechanism, very limited knowledge about cellular disorders in MPSPS, and the need to develop an effective drug, it is justified to investigate which cellular processes may be impaired in MPSPS, which may lead to understanding the exact mechanism of disease development and potential drugs for MPSPS.

Therefore, the aim of my doctoral dissertation was:

1) **To determine which cellular processes are impaired in MPSPS and, consequently, to understand the pathomechanism of the disease**
2) **To test two natural compounds, ambroxol and genistein, as potential drugs for patients with MPSPS**

In my work, I used skin cells – fibroblasts - taken from the only Polish patient with MPSPS with the homozygous variant c.599G>C (p.Arg200Pro) of the *VPS33A* gene. I was unable to obtain more MPSPS cell lines due to the extremely small number of patients (only a few dozen worldwide), the great distance and geographical isolation of most of them (patients mainly live in the regions of Yakutia and Siberia), and the extremely short life expectancy of patients (most die before the age of 2). These and many other problems related to rare diseases are discussed in more detail in the article by Cyske et al. 2025 [Cyske

et al. 2025a - **Article No. 1**]. As a control, I chose skin cells taken from a healthy person matching the age and gender of the patient. In some of the experiments, cell lines taken from patients with Sanfilippo syndrome were also used as a comparison to the already described and better understood cellular disorders in MPS.

1) Identifying which cellular processes are disrupted in MPSPS, and thus understanding the pathomechanism of the disease

To test the hypothesis of impaired endosomal GAG transport resulting from reduced VPS33A protein levels and inefficient delivery to lysosomes, I first determined the level of GAGs in MPSPS cells with the p.Arg200Pro variant of the *VPS33A* gene in control cells and in a patient suffering from Sanfilippo syndrome (MPS III). Experiments using the dot-blotting method showed significantly elevated levels of heparan sulfate (HS) in MPSPS cells compared to control cells. I also visualized the intracellular localization of HS, which proved to be extremely interesting, as the image obtained after staining MPSPS cells differed significantly from that obtained from MPS III cells. Namely, in MPS III, HS was visible in the form of multiple clusters; according to previous literature data, it can be assumed that these are HS aggregates in lysosomes, while in MPSPS, HS was evenly distributed throughout the cell. This image may indicate impaired transport and delivery of GAG to lysosomes.

Previous studies, using both the p.Arg200Pro and p.Arg498Trp variants, indicated a reduced amount of VPS33A protein in MPSPS [Pavlova et al. 2022; Terawaki et al. 2025]. I therefore examined the level of this protein in fibroblasts taken from a Polish patient in comparison with control cells. I found a drastic reduction of more than 80% in the level of VPS33A protein in MPSPS cells compared to control cells. Using fluorescence microscopy, I also demonstrated a reduced fluorescence intensity of this protein. The VPS16 protein, like VPS33A, is one of the subunits of the HOPS and CORVET complexes. These proteins interact closely with each other, which is why I also determined this protein. Similar to the previous experiment determining the level of VPS33A protein, I also found a reduced level of VPS16 protein in MPSPS.

Electron microscopy allows for a broad view of the overall morphology of the cell. For the previously described types/subtypes of MPS, this image is characterized by "onion skin" and "zebra body" structures, which are specific images that can be used to identify lysosomes in which undegraded GAGs are accumulated [Piotrowska et al. 2006]. For a complete view of MPSPS cells, I performed the same test, which showed a significantly increased number of vesicles with altered structure compared to control cells. However, if the theory of impaired vesicular transport is true, it is uncertain whether the vesicles visible in the microscopic image are lysosomes, as in other types of MPS, or endosomes. To answer this question, I stained the lysosomes using LysoTracker dye. The experiments showed a significantly stronger signal in MPSPS cells than in control cells. This may indicate an increased

number of lysosomes, but previous experiments have shown that lysosomes in MPSPS are excessively acidified [Kondo et al. 2017; Pavlova et al. 2022], and it is known that LysoTracker dye has a higher affinity for organelles with lower pH. Therefore, I asked whether lysosomes are more numerous or merely excessively acidified. To obtain the answer, I used the LAMP2 protein, a lysosomal membrane protein and one of the most common markers in lysosome research. Both the level of this protein and the intensity of fluorescence were elevated, on the basis of which it can be concluded that lysosomes are more numerous in MPSPS, regardless of their greater acidification.

Considering the "scattered" image of HS obtained from fluorescence microscopy analyses, I asked whether GAGs are effectively delivered to lysosomes for degradation. Therefore, I determined the colocalization of lysosomes with HS in MPSPS and MPS III and showed that in MPS III, colocalization was at a significantly higher level than in control cells, but in MPSPS, there were no differences. This may indicate that GAGs are not delivered to lysosomes and, although lysosomal enzymes function properly, GAGs cannot be broken down. Another experiment showing the colocalization of HS with LAMP1, another lysosomal membrane protein, proved to be extremely interesting, as the result was the opposite. Namely, HS/LAMP1 colocalization was significantly increased in MPSPS compared to control cells, while in MPS III it was decreased. This might seem contradictory to the previous experiment, but there are studies in the literature describing that the LAMP1 protein is also found in early and late endosomes [Cook et al. 2004]. Therefore, it is likely that GAGs are trapped in endosomes and are not transported to lysosomes for degradation.

Since the VPS33A protein is involved in the maturation of endosomes and their transport to lysosomes [Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022], then, according to the tested hypothesis, the level of endosomal markers should be elevated in MPSPS cells. The marker of early endosomes is the EEA1 protein, which is essential for the sorting of early and late endosomes, as well as for sorting at the early endosome stage [Yap and Winckler 2022]. I demonstrated elevated levels of this protein and fluorescence intensity in MPSPS cells compared to control cells, using the western-blotting method and fluorescence microscopy, respectively. In this case, the result also confirms the hypothesis and is consistent with experiments conducted by another team on cells from a patient with the same mutation [Pavlova et al. 2022]. Following this line of reasoning, I checked the level of Rab7 protein, which can be considered a marker of late endosomes, as it is essential for the transport of late endosomes and their delivery to lysosomes. This experiment also showed an increased level of this protein, and microscopic analyses confirmed the increased fluorescence intensity of this protein.

Returning to the above assumption that HS accumulates in endosomes and cannot be transported to lysosomes, I determined the colocalization of HS with the EEA1 protein. Based on the results I obtained, it can be concluded that there is a significantly increased colocalization of HS/EEA1 in MPSPS cells compared to control cells, which confirms the hypothesis. However, it is important to note the level of

HS/EEA1 colocalization in MPS III, which was even higher than in MPSPS, while studies reporting a secondary vesicular transport defect in MPS have already been conducted [Gaffke et al. 2023].

2) Testing of two natural compounds, ambroxol and genistein, as potential drugs for patients with MPSPS

MPSPS is currently an incurable disease, and due to the extremely small number of patients and their very short life expectancy, developing an effective treatment seems to be a huge challenge for modern science. A potential treatment for MPSPS would have to focus on correcting the underlying molecular defects, but it would also have to be safe for long-term use.

I selected two natural compounds that could potentially become drugs for MPSPS: ambroxol (4-((2-amino-3,5-dibromophenyl)methylamino)cyclohexan-1-ol) and genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopiran-4-on). This is because both have been described as inhibitors of protein degradation – endoplasmic reticulum-associated degradation (ERAD) and proteasomal degradation, respectively. Both compounds also have anti-inflammatory and antioxidant properties and induce autophagy [Dhanve et al. 2023; Cyske et al. 2024b; Ponugoti et al. 2024; Borah et al. 2025; Goswami et al. 2025; Pierzynowska et al. 2025], but ambroxol also alleviates endoplasmic stress and inhibits ERAD degradation [Bendikov-Bar et al. 2011; Dhanve et al. 2023], while genistein inhibits proteasomal degradation by modulating protein ubiquitination [Pierzynowska et al. 2020]. As required for drugs that must be taken for life, both compounds have been proven safe for long-term use [Kim et al. 2013; Ghosh et al. 2021; Ponugoti et al. 2024]. Based on the above data, I selected ambroxol and genistein as potential drugs for MPSPS and, in the future, also for other diseases associated with protein instability and endosomal transport disorders. Taking into account other mechanisms of proteolysis inhibition by ambroxol and genistein, I determined whether the use of combination therapy would be more effective than the use of each compound separately.

The first and one of the most important parameters in the search for new drugs is safety. Therefore, this experiment was the first one I performed for ambroxol and genistein as potential drugs for MPSPS, even though both compounds have already been used in medicine for many years. Based on previous literature data, I chose concentrations of 100 μM for ambroxol and 50 μM for genistein, which effectively inhibit proteolysis [Bendikov-Bar et al. 2011; Pierzynowska et al. 2020]. Based on a cell viability test (MTT), I demonstrated that both compounds are safe for MPSPS cells at the concentrations indicated above.

The main problem in MPSPS turned out to be reduced levels of the VPS33A protein, which leads to inefficient vesicular transport and GAG entrapment in endosomes. A potential drug should therefore increase the level of this protein. My research showed a 4-fold reduction in VPS33A protein levels in

MPSPS cells compared to control cells. However, the administration of ambroxol, genistein, or a combination of both resulted in an increase in the level of this protein. The use of a mixture of both compounds proved to be the most effective, and the level of VPS33A protein reached approximately 80% of the control value. I obtained a comparable result for the VPS16 protein, which is closely related to the VPS33A protein. Both of these results indicate that ambroxol and genistein increase the levels of VPS33A and VPS16 proteins by reducing the activity of ERAD (ambroxol) and proteasome (genistein) [Bendikov-Bar et al. 2011; Pierzynowska et al. 2020].

The experiments I conducted confirmed significantly elevated (approximately 3-fold) HS levels in MPSPS cells compared to control cells [Cyske Z et al. 2025b – **Article no. 3**]. However, the use of ambroxol, genistein, and their mixture reduced HS levels, even to the level observed in control cells. To determine whether HS degradation occurs via the lysosomal pathway, I used chloroquine (an inhibitor that blocks the fusion of autophagosomes with lysosomes). The experiments confirmed that after the use of chloroquine at a concentration of 10 μM, very high HS levels can be observed, regardless of the presence of ambroxol and genistein. This result indicates that GAG degradation in MPSPS cells after the use of ambroxol and genistein occurs via the lysosomal pathway.

Since lysosomal disorders occur in MPSPS, I re-determined their number and fluorescence intensity using the LysoTracker dye, but this time I also determined the effect of ambroxol and genistein on these parameters. I showed that the number of lysosomes and the intensity of their fluorescence is significantly increased in MPSPS compared to control cells, and treatment with ambroxol, genistein, and their mixture effectively reduced the number of lysosomes, while the use of a mixture of both compounds most effectively reduced the intensity of fluorescence in MPSPS.

To confirm the normalization of GAG transport to lysosomes, I determined the colocalization of HS with lysosomes (LysoTracker dye). The experiments again showed a significantly reduced level of their colocalization in MPSPS cells compared to control cells, but also an increase in this parameter after the use of ambroxol, genistein, and their mixture, with the use of a mixture of both compounds being the most effective. When examining the colocalization of HS with LAMP2, a lysosomal membrane protein, a reduced level of colocalization can be observed in MPSPS compared to the control, but also an increase to a level comparable to the control after the use of a mixture of ambroxol and genistein. Since my research suggested that in MPSPS, GAGs are trapped in endosomes due to defective endosomal transport [Cyske Z et al. 2025b – **Article no. 3**], I re-determined the level of EEA1 protein and its colocalization with HS, which showed that the level of EEA1 protein is significantly elevated in MPSPS compared to control cells, but the use of ambroxol, genistein, and their mixture resulted in normalization to a level indistinguishable from the control. This result indicates an improvement in vesicular transport efficiency in MPSPS after treatment. This conclusion is further confirmed by the determination of HS colocalization with LAMP1 protein (as mentioned earlier, this protein is found in both lysosomes and

endosomes) [Cook et al. 2024]. This experiment showed, as before, increased HS/LAMP1 colocalization in MPSPS cells compared to the control, but also normalization of this level after the use of a mixture of ambroxol and genistein. Once again, the results confirmed the hypothesis of defective endosomal transport of GAG and an improvement in its efficiency after the use of ambroxol, genistein, and in particular their mixture.

In addition to the level of EEA1 protein (a marker of early endosomes), I also determined the level of late endosome markers – Rab5 and Rab7 proteins. The Rab5 protein is involved in endosome maturation [Nagano et al. 2019], while Rab7 is essential for endosomal transport and the delivery of macromolecules to lysosomes [Marwaha et al. 2017; Mulligan and Winckler 2023]. The levels of both marker proteins for late endosomes were elevated in MPSPS compared to the control, and the use of a mixture of ambroxol and genistein resulted in the normalization of this parameter to the control level (in the case of Rab7) or an improvement in the parameter (in the case of Rab5).

Experiments conducted by other teams [Terawaki et al. 2025] indicated that autophagy is not impaired in the p.Arg498Trp MPSPS variant. However, the results of my research clearly show that this process is impaired in the p.Arg200Pro MPSPS variant [Cyske Z et al. 2025b – **Article no. 3**]. Both ambroxol and genistein are known inducers of autophagy, and I determined their effects on the stimulation of this process in the p.Arg200Pro variant of MPSPS. I determined the level of LC3-II protein (a marker of autophagy) and found that both ambroxol and genistein significantly increase the level of this protein, which indicates effective stimulation of autophagy, and this process is most effective after the use of a mixture of both compounds. The degradation of accumulated protein material is extremely important in the context of potential MPSPS therapy, therefore the induction of autophagy by ambroxol and genistein is extremely helpful in the context of developing a potential therapy using these compounds.

The results of my research described in this chapter were presented in a manuscript article that was submitted to the editors of the journal *Mammalian Genome* and was in peer review at the time of submission of this doctoral dissertation [Cyske et al. – **Article no. 4**].

Conclusions

This dissertation provides experimental evidence supporting the hypothesis of impaired endosomal transport caused by reduced levels of the VPS33A protein, which underlies the molecular mechanism of MPSPS. Therapy with ambroxol and genistein, which I propose as potentially effective in the treatment of MPSPS, results in increased levels of the VPS33A protein, improving almost all impaired parameters or even normalizing them to levels indistinguishable from control. The proposed therapy has the potential to be useful in the future in the treatment of other diseases associated with protein instability and impaired endosomal transport.

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The rare disease burden: a multidimensional challenge

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Although there no single, widely accepted definition of a "rare disease," this group of disorders includes conditions that affect only a small fraction of the population. A large number of rare diseases is caused by defined molecular defects, predominantly the occurrence of pathogenic variant(s) of genes. Thus, they are classified as "genetic diseases," among which there are many neurodegenerative disorders. Despite a low incidence of each such disease, majority of them are severe and no effective treatment is available. This creates a battery of problems for millions of people suffering from these disease as well as to their relatives and caregivers. However, the set of problems is larger; therefore, in this narrative review we summarize and discuss various kinds of problems caused by rare disease, including severe symptoms of patients, everyday problems of patients and caregivers, loneliness and social exclusion, diagnostic difficulties, unavailability of effective therapies and economic difficulties in introducing orphan drugs, and a complexity of studies on rare diseases due to low availability of biological material and complicated pathomechanisms. The global picture of the complex problems caused by rare diseases is presented.

KEYWORDS

rare diseases, molecular causes of diseases, diagnostics, treatment, multidisciplinary research, multiple challenges

Introduction

There is no single, specific, and commonly accepted definition of a rare disease, as each country/region of the world has established its own criteria for classifying a disease as rare or ultra-rare (Hughes et al., 2005; Richter et al., 2015; Kölker et al., 2022; Song et al., 2013). For example, according to the World Health Organization (WHO), a rare disease is one that affects no more than 1 in 2,000 people (The Lancet Global Health, 2024). In European Union, the definition is similar, but expressed as less than 5 in 10,000; however, in United States the definition describes the total number of patients, rather than a frequency, and it is expressed as less than 200,000 in the population of the country (which corresponds to about 1 in 1,500 people). Regardless of the specific definition, it is estimated that nearly 10,000 rare diseases have been identified to date, affecting approximately 6% of the global population - around 400 million people. (last accessed

on 28 February 2025[1]). Taken globally, these numbers indicate that the term "rare" is no longer correct. Hence, it is very important to constantly seek and discover innovative therapeutic methods, effective in eliminating or at least reducing the number of "rare uncurable diseases," even despite the high costs of introducing the drug to the market.

When considering etiology, it is estimated that about 80% of rare diseases are of genetic origin, although for some of them the specific genetic cause has not yet been identified (last accessed on 28 February 2025[1]). This means that for most of them, specific pathogenic variant(s) of a specific gene or a broader genetic defect (like aneuploidy, large deletions of chromosomes, chromosomal translocations, and others) are known. Nevertheless, the molecular mechanisms underlying monogenic diseases are often complex, as the dysfunction of a single gene, and resulting defect in one protein, affects a complex network of biochemical reactions and their cellular consequences. In fact, there is no specific treatment available for vast majority (over 95%) of rare diseases.

Because of the genetic character, symptoms of most of rare diseases are expressed in a childhood. Another problematic characteristics is that neurodegenerative disorders occur in some 70% of rare diseases. Neurodegenerative diseases are currently the seventh leading cause of death worldwide (Hughes et al., 2005).

An mentioned above, the challenges associated with 'rare diseases' are complex and require systematic actions improve the quality of life for both patients and their caregivers. To overview this broad topic, and to indicate common issues specific to most "rare diseases," this article will overview and discuss the problems affecting people with these diseases from several perspectives, including (i) problems faced by patients and their parents/caregivers (as mentioned earlier, most patients are children); (ii) problems with making a faster and accurate diagnosis, i.e., problems faced by physicians diagnosing patients, which is often crucial to ensure maximal possible quality of life; (iii) problems with developing and introducing to the market a therapy available to every patient, which consist of problems faced by researchers working on understanding the disease pathomechanism, finding molecular targets of potential drugs, and developing potential therapies, as well as of introducing specific drugs to the market due to economic constrains and barriers.

Methods

This is a narrative review, presenting opinions and experiences of the authors. Literature search included analysis of the PubMed database (last accessed on 28 February 2025[2]), using the term "rare disease." However, since this search gave a huge number of 392,129 records, a strong selection of papers, strictly related to the subject of this review and cited here, was performed. Moreover, some other Internet sources, related to "rare disease" were considered, including Global Genes – Allies in Rare Disease (last accessed on 28 February 2025[1]), American Academy of Pediatrics (last accessed on 28 February 2025[3]), Rapsody Online (last accessed on 28 February 2025[4]), and the European Union Rare Disease Patients Solidarity Project (last accessed on 28 February 2025[5]).

Challenges of patients

Patients suffering from rare diseases face numerous challenges, significantly more than those diagnosed for more common disorders. The primary issue is that due to usually complicated molecular mechanisms and complex changes at cellular and organismal levels, the symptoms are severe and often worsen over time. Therefore, increasing suffering each year occurs in many diseases from this group and, in some cases, there is a need for round-the-clock care.

A classic example is children with mucopolysaccharidosis (MPS),a group of inherited metabolic diseases caused by defects in genes coding for enzymes responsible for degradation of glycosaminoglycans which, in turn, accumulate in the cells. MPS patients experience a wide variety of symptoms due to the vast heterogeneity of the disease, including skeletal deformities, intellectual disabilities, respiratory issues, and sleep disturbances. Over time, these symptoms intensify, and additional complications emerge, further hindering daily functioning (Rintz et al., 2024). For many years it was believed that the only cause of MPS is the accumulation of GAGs in lysosomes, but over the years it has been shown that this is not the only problem in the pathogenesis of the disease. It has been proven that in addition to the accumulation of GAGs, the problem is also the reduced efficiency of cellular processes. It is therefore possible that it is not the appearance of GAGs alone, but the reduced efficiency of key molecular processes taking place in cells that is responsible for the pathogenesis of the disease (Pshezhetsky, 2015; Tessitore et al., 2009). In contrast, in patients with Huntington disease, symptoms appear much later, but just as with other rare diseases, they eventually prevent independent functioning, and over time, lead to exclusion from professional

1 https://globalgenes.org/rare-disease-facts/

2 https://pubmed.ncbi.nlm.nih.gov/

3 https://www.aap.org/en/practice-management/medical-home

4 http://rapsodyonline.eurordis.org/

5 https://health.ec.europa.eu/social-determinants/overview/commission-communication-solidarity-health-reducing-health-inequalities-eu_en

and social life (Doss et al., 2025). An example might be spinocerebellar ataxia (SCA), a disease that is extremely diverse, and its onset can manifest in both small children and adults. Recent studies have shown that one of the causes of this disease may be a mutation in the *FGF14* gene (Hirschfeld AS et al., 2025).

Research aimed at developing a cure for any disease is possible due to the constantly developing diagnostic/molecular methods; without this progress it would not be possible. For example, sequencing methods allow finding different variants of diseases or allow identifying a specific disease in patients who often remain undiagnosed for many years.

Children and adolescents with rare diseases encounter difficulties in adapting to the traditional education system. Doctor visits, hospitalizations, and health restrictions affect or prevent school attendance. This often leads to exclusion from peer groups. As a result, there are difficulties in building social relationships and maintaining motivation to learn (Song et al., 2013; Rintz et al., 2024).

People with rare diseases can feel isolated, both socially and emotionally. Because these diseases are little known, society can lack understanding and empathy for the patient's suffering. Patients with rare disease often struggle to maintain social connectionswith healthy individuals, as their daily lives differ significantly. As a result, they can feel isolated, and their health challenges can be overlooked by those around them (Lopes et al., 2018; Ramalle-Gómara et al., 2015).

Children growing up in families where one member has a rare disease may also experience difficulties. On the one hand, siblings may feel neglected because parents focus most of their attention on the child affected by illness. This can lead to feelings of rejection and lack of emotional support. On the other hand, siblings may feel pressured to be "good," "understanding" or "helpful," often at the expense of expressing their own emotions or needs. They may become jealous of their parents' attention or feel less important (Flores et al., 2022; Desser, 2013; Spencer-Tansley et al., 2022). Rare diseases, especially those with visible symptoms, can lead to stigmatization. In the case of diseases that cause changes in appearance (e.g., body deformities), patients may experience negative social reactions, such as avoidance, ridicule, or worse treatment. Stigmatization can also affect people with diseases that affect the patient's behavior (e.g., neurological disorders), which can lead to rejection by peers, colleagues, or even family members. Adults with rare diseases can face many challenges in securing and maintaining employment. Their health conditions can limit their career options, and in some cases, their illness can require frequent breaks from work, making employers reluctant to hire them. Additionally, a lack of understanding from coworkers can lead to isolation in the workplace, which can lead to low self-esteem and fear of rejection (Spencer-Tansley et al., 2022).

Access to specialist doctors, who can provide an accurate diagnosis, is another significant issue for patients. As a result, many remain undiagnosed for an extended period. This problem may also be linked to the insufficient number of physicians specializing in rare diseases. The issue is highly complex, as the vast number of rare diseases necessitates extensive research, which can be both time-consuming and costly. Accurate and timely diagnosis is essential for initiating treatment and mitigating the impact of the disease, even if no specific cure is currently available. Unfortunately, even after a proper diagnosis is made, the problem does not disappear. Patients often begin searching for the best possible treatment, which is not always available to them for various reasons, including a lack of specific treatment or high costs or therapies offered (Lopes et al., 2018). Prenatal testing of genetic defects is also an option, but most standard tests do not detect all mutations; they tend to focus on the most common ones. More specific tests are required for an accurate prenatal diagnosis of rare diseases, and prospective parents usually do not undergo such tests without a clear reason, such as a previous child with the disease or information of being a carrier (Ramalle-Gómara et al., 2015; Flores et al., 2022; Desser, 2013).

The most obvious and most important effect of the disease is the enormous suffering of the patient, not only because of the pain, but also for psychological reasons. The patients, if not developing severe cognitive deficits, are aware that they may not receive effective treatment and, consequently, that they will not recover. This can lead to a serious deterioration of their mental health and, over time, to the development of depression (Spencer-Tansley et al., 2022; Uhlenbusch et al., 2019). It have been shown that patients with rare diseases and their caregivers experience enormous, everyday anxiety, stress, anger or frustration, not only due to the symptoms of the disease, but also due to other aspects of life. The deteriorating mental health was associated with the fear of how the disease may affect their work and studies, relationships with partners, peers, friends or relatives (Spencer-Tansley et al., 2022). These studies have shown that 36% of patients and 19% of caregivers had suicidal thoughts.

Because of the rarity of these diseases, individuals affected by them often struggle to find others facing similar challenges. The lack of support groups that offer emotional and practical support makes it difficult to share experiences and understand the problems associated with the disease. A community of patients can be crucial in getting through difficult times, and its absence can lead to feelings of loneliness. Obviously, there are many global and local organizations that specialize in helping patients in various areas of their lives, financial, psychological, medical help, but also support in everyday functioning (shopping, cleaning, medications). Moreover, foundations can be helpful, as in addition to providing daily help to patients, they organize many conferences where patients and their parents/guardians can learn about the disease that has affected the family. This is often the only source of information, due to the limited

number of scientific publications and especially popular science articles. Another help for patients is the possibility of contact with other patients, with foundations and associations via the Internet, and also obtaining information on reliable and verified websites (like[6,7]) where one can find useful and interesting information. Patients and/or their families can also learn about the symptoms, course of the disease of interest or causes of the disease. A very interesting initiative was launched in 2008. Every year in February, a rare disease day is organized. Gradually, the event gained a global rank and is now organized in over 100 countries. The formal date of the Rare Disease Day is 29th February (as this is the rarest day in a calendar), but in years when 29th February does not occur, the Day is celebrated on 28th February.

Another important aspect involves the challenges faced by doctors caring for patients with rare diseases. Patients seeking help reported problems with difficulty in making a diagnosis, which resulted from insufficient knowledge of physicians about rare diseases. Most patients, even after making an accurate diagnosis and starting treatment, continue to live with great anxiety related to difficulties in finding prescribed medications (Richardson et al., 2024). In some instances, healthcare providers failed to take the patient's symptoms and concerns seriously, occasionally viewing them as a mere "curiosity" (Spencer-Tansley et al., 2022; Richardson et al., 2024). A significant stress factor turned out to be the lack of understanding of the disease on the part of society and the need to constantly explain and explain the disease to others. A huge problem may be the exclusion of a sick child from social life. Due to insufficient knowledge or misunderstanding of the problem, the child may be rejected by peers (Spencer-Tansley et al., 2022), and may even be accused of excessive laziness (Lopes et al., 2018). In such a case, a quick reaction on the part of caregivers/teachers may be of great importance. However, for this to be possible, the caregivers/teachers themselves should be properly prepared and trained for this.

A major challenge, sometimes even preventing treatment, may be the physical or geographical distance. Patients are often scattered all over the world, while clinical trials and specialists with the best possible knowledge of a given disease entity can be located in only one, often very distant research centre. Distance, costs related to travel and accommodation at the place of treatment, the patient's health, work and often many other factors can prevent treatment. Furthermore, even if treatment exists for a given disease, it is often associated with frequent visits to a specific hospital that has the medicine, necessitating travel not only for the patient but also for their caregiver (Cacoub et al., 2025).

6 www.orpha.net

7 www.omim.org

Challenges of parents/families/caregivers

The illness of a child is always a tremendous tragedy for the parent, and additional challenges arise when a rare disease is diagnosed, for which there is often no effective treatment (Spencer-Tansley et al., 2022). In such cases, most parents attempt to find therapies on their own that might save their child's life. Even when a treatment for a specific disease is developed, it is often prohibitively expensive, and parents do not always have sufficient financial resources (Song et al., 2013). An additional complication is the fact that, in most cases, the child will need to take medication for many years. This brings about another serious issue: due to frequent doctor visits or rehabilitation sessions, parents are often forced to reduce or even eventually give up their paid employment. As a result, their income decreases, making it increasingly difficult to finance their child's long-term treatment. In fact, a need for complex care on both patients and parents have been recognized, and presented employing a comprehensive analysis of the case of one, selected rare disease (though the problems are definitely common for most rare diseases) (Cyske et al., 2022).

An additional and serious problem may also be the possibility of the same rare disease occurring in the younger siblings of the patient. In such a case, parents should start diagnostics as soon as possible, because introducing therapy before the first symptoms appears to give agood chance that they will not develop at all, if a specific therapy is available. Nevertheless, even if no cure can be proposed now, a complex care may result in a significantly better quality of life of both patients and their parents. According to studies conducted in Great Britain, the mental health of parents of sick children deteriorates due to the stress associated with constant worry about the health and wellbeing of their children (Spencer-Tansley et al., 2022). Additionally, parents may also have problems with obtaining sufficient information about their child's disease and possible methods of treatment (Desser, 2013). Again, this highlights the need for early diagnosis and further medical treatment (even if symptomatic or palliative) which is, however, often difficult (Wiśniewska et al., 2022).

Many people are unaware of the existence of rare diseases, which leads to misunderstandings and stereotypes. The lack of public knowledge about the specifics of these diseases can cause people affected by them to be treated differently, which increases their sense of exclusion, and results in the pressure to parents. Educating the public about these diseases is key to reducing stigma and supporting patients and their families in integrating into society. The role of patient organizations, as well as support and advocacy groups is not to be underestimated, as their activities appear to be crucial to ensue quite normal existence of patients and their families (Taruscio and Gahl, 2024).

Families, especially parents, may have to take on the role of full-time caregivers, which is associated with many

difficulties. Caring for someone with a rare disease requires a huge investment of time and energy, especially since patients need regular doctor visits, therapy and specialist treatment. This affects the daily life of the family and especially the professional life of the parents. As a result, one of the parents, most often the mother, has to limit his or her professional life, leading to loss of income and additional financial burden. As a result of the constant responsibilities associated with caring for a sick family member, parents may have difficulty balancing their personal lives with their caring responsibilities. This can lead to neglect of the relationship between partners, which increases the risk of conflict and even the breakdown of marriages. Financial and emotional problems, as well as a chronic lack of time, which means that parents lack the strength to nurture their relationship, which leads to a sense of loneliness, the loss of relationships and often the breakdown of the relationship. In many countries, social care systems may not be adequately prepared to support people with rare diseases. The lack of adequate health, psychological and social services can make patients feel neglected and unable to access appropriate care. Furthermore, access to specialist care is often limited, especially in less developed regions, which increases inequalities in access to treatment.

Challenges of physicians

A major challenge in diagnosing rare diseases is forming the correct hypothesis about the type of disease, identifying the right research centre, and, most importantly, finding a doctor capable of making an accurate diagnosis and providing effective treatment. Unfortunately, when a medical facility or research center lacks specialization in a specific disease, the chances of achieving an accurate diagnosis are significantly reduced. This is mainly due to the fact that there are a huge number of rare diseases - at least several thousand - making it difficult for any single physician to be familiar with all of them. Studies conducted in Spain and Peru showed that doctors have limited knowledge of rare diseases (Lopes et al., 2018; Ramalle-Gómara et al., 2015; Flores et al., 2022). The same studies have shown that physicians are concerned about the insufficient education of future doctors on the subject of rare diseases (Lopes et al., 2018; Flores et al., 2022). On the other hand, surveys conducted among Norwegian and Spanish doctors have shown that they give much higher importance in financing diseases that are more common in society than diseases from the rare group (Desser, 2013). This is understandable to some extent, because it is obvious that a much larger part of the population suffers from such diseases, but does it mean that one of these conditions is more important, more significant, more painful for the patient?

The issue of dividing funds between rare and common diseases is certainly complicated by the fact that the treatment of people with rare diseases is less common, but the treatment is much more expensive, while the opposite may be true for more common diseases (Flores et al., 2022; Cacoub et al., 2025). The lack of interest in treating rare diseases may be the result of insufficient knowledge about the effects of this group of diseases. Another huge problem may be the failure to adapt diagnostic tests to the needs or age of the patient. Such difficulties have been reported by doctors dealing with Krabbe's disease, which manifests itself in problems with the central nervous system. The first symptoms usually appear in the first year of life, so a doctor using most standard diagnostic tests will not be able to diagnose the disease (Richardson et al., 2024). This will consequently lead to a delay in diagnosis and loss of valuable time. Advanced screening methods, like multi-omics and AI-driven analyses, can expand genetic disease detection, especially in underserved areas. Early diagnosis enables timely intervention and identifies cases with mild or atypical symptoms. Combining broader screening with genotype-phenotype insights helps clinicians diagnose, assess severity, and personalize treatment more effectively (Gallagher, 2024).

Challenges of researchers

Researchers studying rare diseases face many challenges. Unfortunately, the world of science is very closely linked to money, and obtaining funding for such research is very difficult and often involves rejection. Additionally, due to the difficult economic situation, institutions financing research often limit the overall budget for grants which makes the possibility of receiving it incredibly small. Even if a project falls within the scopeof possible funding, most reviewers often prioritize proposals perceived to have "greater impact" - typically studies involving larger numbers of cell lines, animals, or patients, and focusing on more prevalent conditions, such as common diseases. Research aimed at developing a drug is extremely time-consuming, because it begins with research on cells (*in vitro*), and only after a positive conclusion of this phase of experiments can one move on to research to determine the effect of a potential drug on the entire organism, i.e., animal research. Only such long-term, time-consuming and expensive research allows for further research, including clinical trials. However, each of these stages has its limitations. At the very beginning, scientists face the availability of cell lines for *in vitro* research. As mentioned, most rare diseases affect children, thus parents first face the difficulty of finding a specialist who will make an accurate diagnosis. Then they fight for the best possible therapy, in the meantime struggling with many other problems, including financial, social, and psychological ones. The last thing they will think about, if at all, will be depositing cells in cell banks, from which scientists can buy them in order to conduct research and potentially develop a therapy. The next stage of research, animal experiments, also require a lot of time, knowledge, and

money. This involves the need to obtain many consents to carry out procedures on animals, including the consent of the Local Bioethics Committee. The person submitting such consent must have knowledge and experience in working on animals. Finding an institution that will decide to finance research on rare diseases and will have sufficient funds can also be a huge difficulty.

Finding an institution that will decide to fund research on rare diseases and will have sufficient financial resources can also be a huge obstacle. Scientists often work with many foundations and associations that organize all kinds of help for patients, including trying to fund research that may lead to the creation of drugs. Most countries have research funding centers in various fields, but they prefer research that has a larger audience, which means that getting funding for a rare disease is extremely difficult. Fortunately, in recent years, more and more institutions/projects have been established that fund research on rare diseases (e.g., ERDERA[8]; The NORD[9]). Because they focus mainly on rare diseases, there is a greater chance of receiving a grant. Moreover, "N = 1 Collaborative"[10] is an organization that brings together patients, their families, scientists, doctors, physiotherapists, founders and many others who work together for a better future for patients. The organization focuses on providing patients with rare diseases with access to individualized treatment, tailored to the needs of a specific patient at a specific moment in the development of the disease[10].

Innovative gene editing methods (like CRISPR/Cas9) may offer a great hope for patients, as they enable the manipulation of a given organism's gene. The use of this method in the treatment of patients was first approved in 2023 in the UK for the therapy of sickle cell anemia and thalassaemia (Cetin B et al., 2025). Another similar and equally promising discovery of recent years concerns antisense oligonucleotide therapy. Antisense oligonucleotides (ASOs) are short oligonucleotides that can affect the modification of gene expression. These molecules show potential in the therapy of genetic diseases (Lauffer MC et al., 2024).

Clinical trials

The process of introducing a new drug to patients begins long before clinical trials begin. The necessary first stage is research on cell models to determine the effect of the potential drug on a cell function, cellular viability and safety. Then the research moves on to the next phase, i.e., studies aimed at determining the effect of the drug on the entire organism, i.e., animal research. Only after obtaining positive results from these experiments can clinical trials involving patients begin. Unfortunately, pharmaceutical companies tend to prioritize funding forresearch with a broader scope, i.e., research on drugs that affect a larger number of people in the world, rather than on rare diseases. This approach allows them to maximize profits. However, as a result of such company policies, patients suffering from rare diseases have very limited, and sometimes even impossible, access to treatment. Nevertheless, this is not a malice or reluctance of pharmaceutical companies, but rather a sole economic constraint. One should take into consideration that developing a drug for a rare disease, so called "orphan drug," is as costly as in the case of any other disease, however, number of patients which can potentially buy it is very low. Therefore, an orphan drug must be very expensive, not necessarily because of a high costs of its production, but rather because low number of potential buyers who might purchase such a product. This makes a business related to developing orphan drug very risky economically for pharmaceutical companies. One potential solution would be to develop one drug for many diseases (Pierzynowska et al., 2020). However, it is clear that it would not always be possible, thus, the problem awaits also other solutions.

Clinical trials offer hope for patients, but strict eligibility criteria often prevent many from participating, leaving them without treatment options. The situation has also been made more difficult by the introduction of many restrictions aimed at ensuring patient safety and demonstrating the highest possible effectiveness of the drug being introduced (Haffner et al., 2002; Haffner, 2006). It is obvious that such studies are absolutely necessary to ensure patient safety, but unfortunately the introduction of such restrictions caused pharmaceutical companies to decide to discontinue research on orphan drugs, because it was not profitable for them due to the small patient population (Wästfelt et al., 2006). However, in order to encourage pharmaceutical companies to develop drugs for rare diseases, the Orphan Drug Act was introduced, which gives a company the right to market exclusivity for 7 years from the introduction of the drug, tax relief and exemption from fees (Melnikova, 2012; Meekings et al., 2012). It can therefore be observed that the introduction of such facilities encouraged companies to conduct clinical trials on drugs for rare diseases, because since the entry into force of the Act, over 400 drugs for rare diseases have been approved, while only 10 before the introduction of the Act (Melnikova, 2012; Kumar Kakkar and Dahiya, 2014).

Costs of treatments

Pharmaceutical companies that develop treatments for a rare disease to the market usually has exclusivity for it, enabling them to set prices with minimal competition. In addition, the patient has no other alternative if they want to start treatment, and they also have no opportunity to negotiate. In fact, the price they have

8 http://erdera.org

9 http://rarediseases.org

10 https://www.n1collaborative.org/

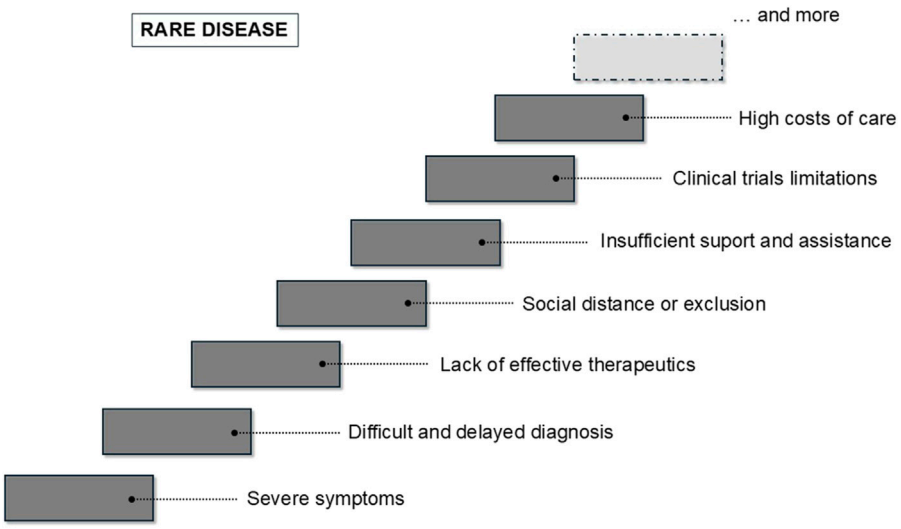

FIGURE 1
Problems related to rare disease, experienced by patients, parents/families/caregivers, physicians, and researchers.

to pay must cover the cost of introducing the drug to the market, i.e., the cost of all tests, starting with *in vitro* tests (Kumar Kakkar and Dahiya, 2014). That is why the costs of treating rare diseases are so huge, as explained in the preceding section (Simoens, 2011).

Looking at examples of several diseases separately, the cost of annual treatment of cystic fibrosis is 300,000 dollars (EvaluatePharma, 2017), Gaucher disease - 400,000 dollars per year (O'Sullivan et al., 2013), paroxysmal nocturnal hemoglobinuria - 500,000 dollars per year (Meekings et al., 2012). It have been estimated that the average direct cost of introducing a new drug may amount to about 1.4 billion dollars, while taking into account the capitalization of costs, the total amount may be about 2.6 billion dollars (Haffner et al., 2008). However, other estimates indicated considerably lower amounts, i.e. 757 million dollars (capitalized costs) for introducing a cancer drug to the market (DiMasi et al., 2016). In this light, it is worth mentioning the estimation showing that a drug registered for a rare disease is about 8 times more expensive than a drug for a more common disease (Simoens, 2011). Obviously, it also depends on whether more drugs or only one are registered for a given disease. Several drugs are currently registered for renal cell carcinoma and cystic fibrosis, thus the price may also be correspondingly lower (Prasad and Mailankody, 2017).

Differences in prices may also result from different policies of countries approving the treatment (Simoens, 2011). Nevertheless, generally prices of orphan drugs are extremely high which in most cases precludes their purchasing by individual patients. The only possibility is to count on reimbursement from either health insurance companies (if appropriate policy is active, which is usually problematic due to relatively high costs of policies covering costs of treatment of rare diseases) or governmental agencies; the latter possibility differs significantly from country to country and even in the same country the rules are changes frequently (Pierzynowska et al., 2020; Simoens, 2011; O'Sullivan et al., 2013; Dear et al., 2006).

Social assistance

As mentioned earlier in this article, many rare diseases are progressive, gradually limiting or eventually eliminating the patient's ability to live independently. Patients' families often do not have sufficient knowledge and/or skills to fully help the patient, which can lead to even greater harm. In such cases, specialist help is necessary in many ways. It is necessary for the doctor to select the appropriate, personalized therapy for the needs of the patient, for the physiotherapist to be trained to work with patients suffering from various disorders, and for the psychologist to know whether and when to introduce appropriate therapy. As mentioned earlier, very often, in addition to physical problems, patients also have mental problems resulting from misdiagnosis or lack thereof, isolation, pain or misunderstanding by others (Spencer-Tansley et al., 2022; Uhlenbusch et al., 2019). In the United States, nursing homes for people called medical homes have been established, which are to provide basic healthcare, education and support for the patient and their entire family (last accessed on 26 February 2025[3]). Similarly, the European Union has developed the Rare Disease Patient Solidarity Project (RAPSODY), a program that enables better access to care,

information and social support for people with diabetes (Bavisetty et al., 2013;[4] last accessed on 28 February 2025).

Despite the actions described above, social assistance for patients and parents/caregivers suffering from rare diseases while very necessary is still severely underdeveloped, especially in some countries. On the other hand, such assistance is usually crucial to keeping the quality of life of patients at the acceptable level, and to help parents/caregivers to avoid social distancing or exclusion.

Discussion

This article overviews major problems caused by rare diseases. These include not only dramatic problems of patients themselves related to severe symptoms of these diseases, but also problems of parents/caregivers, physicians, and researchers working in this field. Specific problems related to unusual conditions of clinical trials with rare diseases, extremely high costs of treatment, and necessary social assistance are also crucial. The summary of these problems is presented in Figure 1. This provides a picture of how complicated the problem of rare disease is and how complex care is required to provide patients and their families the minimal acceptable level of the quality of life.

On the other hand, the problem is global rather than minor, as there are some 400,000 million people suffering from rare disease, making the problems important not only medically and socially, but also economically (both patients and caregivers are often excluded from professional activities, causing serious economic losses). Ninety-five percent of patients with rare diseases lack available treatments, and without a fresh approach to therapy development, this is unlikely to change. Addressing this issue requires more than just financial support - it demands a shift in strategy and mindset to bridge the gap between clinical care, research, and innovation. Rare diseases pose a significant challenge to both healthcare systems and social policies. In response to these challenges, the European Un-ion has developed the Rare Disease Patients Solidarity Project (last accessed on 28 February 2025[5]). The project focuses on improving access to diagnostics and treatment, developing and spreading knowledge about rare diseases, supporting scientific research. Facilitating access to modern diagnostic methods and therapies, including medicines and special nutritional products, educating society and medical professionals about rare diseases, and financing and coordinating research on new methods of treatment and diagnostics are the main goals of this project.

Author contributions

Conceptualization, ZC; methodology, ZC; writing – original draft preparation, ZC and ER-A; writing – review and editing, ZC, ER-A, ER, LG, and KP; visualization, ER; supervision, ZC; project administration, ZC; funding acquisition, ZC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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polegał na:

- przygotowaniu ryciny przedstawionej w pracy przeglądowej;
- udziale w napisaniu pracy przeglądowej;
- udziale w dyskusji z recenzentami.

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Mucopolysaccharidosis-Plus syndrome: is this a type of mucopolysaccharidosis or a separate kind of metabolic disease?

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Review

Mucopolysaccharidosis-Plus Syndrome: Is This a Type of Mucopolysaccharidosis or a Separate Kind of Metabolic Disease?

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Abstract: Several years ago, dozens of cases were described in patients with symptoms very similar to mucopolysaccharidosis (MPS). This new disease entity was described as mucopolysaccharidosis-plus syndrome (MPSPS). The name of the disease indicates that in addition to the typical symptoms of conventional MPS, patients develop other features such as congenital heart defects and kidney and hematopoietic system disorders. The symptoms are highly advanced, and patients usually do not survive past the second year of life. MPSPS is inherited in an autosomal recessive manner and is caused by a homozygous-specific mutation in the gene encoding the VPS33A protein. To date, it has been described in 41 patients. Patients with MPSPS exhibited excessive excretion of glycosaminoglycans (GAGs) in the urine and exceptionally high levels of heparan sulfate in the plasma, but the accumulation of substrates is not caused by a decrease in the activity of any lysosomal enzymes. Here, we discuss the pathomechanisms and symptoms of MPSPS, comparing them to those of MPS. Moreover, we asked the question whether MPSPS should be classified as a type of MPS or a separate disease, as contrary to 'classical' MPS types, despite GAG accumulation, no defects in lysosomal enzymes responsible for degradation of these compounds could be detected in MPSPS. The molecular mechanism of the appearance of GAG accumulation in MPSPS is suggested on the basis of results available in the literature.

Keywords: mucopolysaccharidosis-plus syndrome; mucopolysaccharidosis; glycosaminoglycans; vesicular trafficking

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1. Introduction—Mucopolysaccharidosis

Mucopolysaccharidosis (MPS), belonging to the group of lysosomal storage diseases (LSD), is a hereditary, progressive disease caused by the complete lack or low residual activity of lysosomal enzymes responsible for the degradation of unbranched sugar chains—glycosaminoglycans (GAGs). The undegraded GAGs gradually accumulate in the lysosomes, slowly impairing their functions [1]. The activity of the aforementioned enzymes is closely interconnected, with each enzyme starting its function only after the previous one has completed its catalytical reaction. Therefore, in the absence of the activity of even one enzyme, the entire pathway ceases to function correctly. There are 13 types/subtypes of MPS, classified based on the inactive or highly deficient enzyme and the accumulating GAG(s) [2].

MPS is inherited in an autosomal recessive manner except for MPS II (Hunter syndrome), which is X-linked. Depending on the type, the disease occurs with varying frequency. MPS I is the most common variant, occurring in about 1 in 88,000 live births, while MPS VII occurs in 1 in 2,111,000 live births. MPS IX is the rarest type, with only four cases described worldwide to date [3]. There are many symptoms characteristic of all patients with MPS, but there are also symptoms typical for specific types/subtypes. Although the mutations leading to the development of various types of MPS involve different genes, they result in one outcome: the accumulation of GAG(s) in lysosomes [4]. The most commonly

used method for the preliminary diagnosis of MPS is the measurement of GAGs in a urine sample. Subsequent stages of the diagnostic process include determining the activity of lysosomal enzymes and then searching for specific mutations in the identified gene [5].

For many years, the assistance for patients affected by MPS was limited to palliative care. A possible therapy turned out to be a bone marrow transplantation [6]. However, this therapy is only effective if the disease is diagnosed very quickly, that is, before the first symptoms appear. It is not possible to reverse existing neurological defects, such as cognitive functions, hyperactivity, and emotional disturbances. The initiation of the work on enzyme replacement therapy (ERT), which involves the intravenous administration of the active form of the deficient enzyme, led to a departure from the routine use of bone marrow transplants. The benefits of ERT for patients with MPS include improved joint mobility, and consequently, improved mobility; improved respiratory function, reduced liver size, and a significant decrease in the amount of GAG(s) excreted in the urine [7–10]. However, ERT cannot eliminate all symptoms affecting patients with MPS, and most importantly, the enzyme used in the therapy cannot cross the blood–brain barrier, making the treatment ineffective for types with central nervous system symptoms. Due to the need to treat neuronopathic types of MPS, an alternative form of therapy has been proposed: substrate reduction therapy (SRT) [4]. This therapy focuses on trying to reduce the efficiency of GAG synthesis (given the reduced efficiency of their degradation), which restores the balance between their production and decay.

2. Mucopolysaccharidosis-Plus Syndrome—General Features

At the beginning of 2010, an LSD leading to death before the age of two years was described in the Republic of Sakha (Yakutia, Russia). It was diagnosed as an 'undifferentiated hereditary metabolic disease' [11]. In 2017, after the clinical picture was described and the cause of the disease was identified, the new disease was named mucopolysaccharidosis-plus syndrome (MPSPS). The name of the disease indicates that, in addition to the typical symptoms of conventional MPS, patients developed other features such as congenital heart defects, kidney dysfunctions, and hematopoietic system disorders. MPSPS is inherited in an autosomal recessive manner and was initially reported to be caused by a homozygous-specific mutation (c.1492C>T; p.Arg498Trp) in the gene encoding the VPS33A protein. To date, it has been described in 39 out of 41 patients reported in the literature. It is estimated that the incidence of MPSPS in the Yakut population is 1 in 12,100 births [12,13], but it is much rarer in other populations. Patients with MPSPS exhibited excessive excretion of GAGs in the urine and exceptionally high levels of heparan sulfate in the plasma. These results indicated that dysfunction of the VPS33A protein leads to GAG accumulation and causes a phenotype similar to MPS. However, the accumulation of substrates is not caused by a decrease in the activity of any known lysosomal enzymes.

Recently, the first two patients with MPSPS outside Yakutia and Turkey were diagnosed. These patients exhibited symptoms similar to, but somewhat milder than, those previously described in MPSPS patients. One of them was a young adult of Southern European/Mediterranean origin [14], while the second was a 12-year-old patient from Poland [15]. It is clear that the life spans of these two patients were significantly longer than those of previously described patients with the c.1492C>T (p.Arg498Trp) mutation. In both cases of the juvenile MPSPS patients, genetic analyses (whole-exome sequencing) indicated the presence of a homozygous pathogenic variant of the *VPS33A* gene (c.599G>C; p.Arg200Pro). Apart from symptoms similar to those described previously in other MPSPS patients, the Polish patient revealed some additional features, like recurrent joint effusion, peripheral edemas, and visceral obesity, while being of normal growth. Therefore, one might suggest that the c.599G>C variant may be responsible for the milder phenotype than that caused by the c.1492C>T mutation.

3. Excretion of Glycosaminoglycans and Mutations in the *VPS33A* Gene

As in all types/subtypes of MPS, patients suffering from MPSPS also exhibit increased excretion of GAGs, mainly an excess of heparan sulfate, dermatan sulfate, and chondroitin sulfate in urine and plasma [13–16]. In one patient, the presence of keratan sulfate was also detected [16]. Studies have also shown increased amounts of sialooligosaccharides and sialic acid in the urine of patients with MPSPS [15–17].

The *VPS33A* gene is located on chromosome 12q24.31 and contains 13 exons. The product of this gene, the VPS33A protein, consists of 596 amino acid residues and has a molecular weight of approximately 67 kDa. It is composed of four domains: 1, 2, 3a, and 3b [18,19]. Different domains within the VPS33A protein are responsible for its various functions. It is predicted that domain 3a may interact with the SNARE complex [18,20]. Domain 3b binds to the VPS16 protein (a subunit of the HOPS complex) [19]. The c.1492C>T (p.Arg498Trp) mutation in the *VPS33A* gene, described in most MPSPS patients, is located in domain 2 of the VPS33A protein. Currently, domains 1 and 2 of VPS33A are those of unknown functions [11]. However, it was demonstrated that the c.1492C>T mutation in the *VPS33A* gene affects GAG metabolism [13]. Arginine residue at position 498 is highly conserved across many species. In silico analysis indicated that the p.Arg498Trp mutation is disease-causing and decreases the stability of the protein.

Patients with MPSPS showed excessive GAG excretion in urine and high levels of heparan sulfate in plasma. These results suggested that reduced VPS33A protein activity may lead to GAG accumulation and cause phenotypes similar to MPS I (Hurler syndrome). However, the accumulation of GAGs was not due to the reduced activity of any known lysosomal enzymes involved in GAG degradation. The mutation also did not affect the localization of these enzymes and their substrates (GAGs), cathepsin D processing, or lipid transport. The c.1492C>T mutation did not affect endocytic and autophagic pathways, as examination of autophagy revealed no disturbances in this process in fibroblasts derived from patients. A study of the binding of the VPS33A mutant variant (p.Arg498Trp) to its known partners, VPS16 (HOPS and CORVET) and STX17, showed that these interactions were unchanged. In cells derived from patients, slight excessive acidification of lysosomes was observed [11]. On the other hand, the conclusion about unaffected intracellular trafficking in MPSPS cells came from only a couple of experiments, namely, colocalization of heparan sulfate with lysosomal enzymes and membranes, and on normal levels of filipin [13].

Only two MPSPS patients have been described with a mutation ((c.599G>C; p.Arg200Pro) in the *VPS33A* gene) other than the previously known (c.1492C>T) and considered for some time as the only one occurring in MPSPS patients [14,15]. As mentioned above, the c.599G>C mutation presents with a milder phenotype than that observed in other patients, and the affected individuals live significantly longer than those with the c.1492C>T mutation [14,15]. Molecular analyses suggested that the deficiency of the enzymatic activity of the product of the c.599G>C variant of *VPS33A* mostly results from decreased stability of the protein, which is more prone to proteasomal degradation than the wild-type variant [14].

4. Why Glycosaminoglycans Accumulate in MPSPS—A Hypothesis

Mucopolysaccharidoses (MPS) are diseases caused by mutations in genes encoding lysosomal enzymes involved in the degradation of GAGs. Recent studies indicated that changes in the expression of many genes may cause secondary and tertiary cellular dysfunctions, influencing the course of these diseases. The changes included sequences coding for both proteins and regulatory RNAs [21–23]. It was shown that apart from other changes, the regulation of vesicle trafficking is impaired in cells from patients suffering from various types of MPS due to changes in the levels of key proteins involved in this process [24]. Therefore, one might ask whether vesicular transport of GAGs might be impaired in MPSPS, leading to their improper localization and thus inefficient degradation.

We assume that although colocalization of GAGs with lysosomal enzymes in MPSPS cells was reported [13], the colocalization does not necessarily determine the intra-

lysosomal localization of GAGs, which could also be present in the cytoplasm. Moreover, normal levels of filipin (demonstrated previously in MPSPS [13]) suggested unaffected lipid trafficking indeed; however, specific GAGs can be transported through different pathways, dependent on either caveolin or clathrin [25,26]. Therefore, as suggested previously [24], the potential defective transport of GAGs in MPSPS cells might lead to their accumulation. Such a proposal might be corroborated by the already demonstrated impairment of intracellular glycosphingolipid trafficking. Taking this into consideration, our hypothesis is that the mutation in the *VPS33A* gene, through resulting in impaired VPS33A protein function or stability, impairs vesicular transport so that GAGs cannot be properly transported to lysosomes and degraded. The scheme of the putative MPSPS pathomechanism, based on the accumulation of GAGs, is presented in Figure 1.

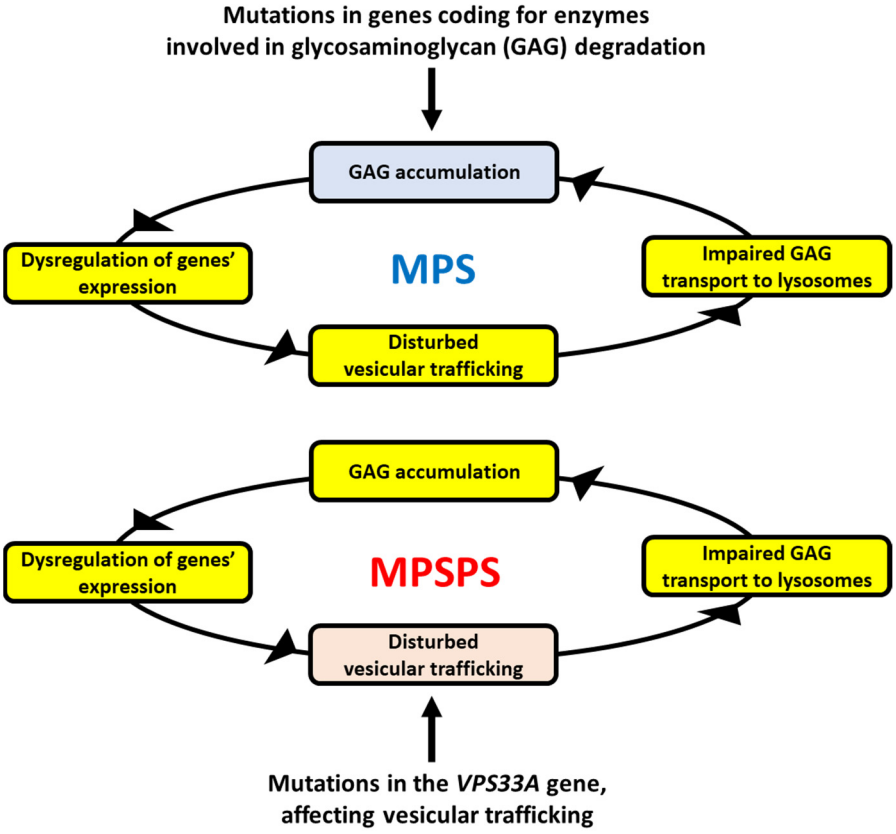

Figure 1. The hypothesis of the mechanisms of GAG accumulation in MPSPS. In classical MPS types (upper panel), GAG(s) accumulate(s) due to mutation(s) in one of genes coding for enzymes involved in degradation of this/these compound(s). GAG storage causes various secondary effects, among others, dysregulation of expression of many genes, resulting in pathological changes in different cellular processes. Disturbed vesicular trafficking is among them, leading to impaired transportation of GAGs into lysosomes. This makes GAG accumulation even more pronounced (as GAGs cannot be effectively degraded outside of lysosomes), which drives a spiral of above-described reactions within a positive feedback loop, enhancing the pathological processes. In MPSPS (lower panel), a similar spiral of reactions resulting in GAG accumulation occurs. However, it is initiated by impaired vesicular transport of GAGs (due to deficiency in VPS33A activity, which is required in this process) rather than dysfunction of an enzyme involved in GAG degradation. Nevertheless, the final effect (GAG accumulation) is similar in both classical MPS and MPSPS.

As mentioned above, an excess of different GAGs, especially heparan sulfate, dermatan sulfate, and chondroitin sulfate, occurs in the urine and plasma of MPSPS patients [13–16]. Since all GAGs are degraded by lysosomal hydrolases, significant differences in the defects of intra-lysosomal decay of specific GAGs in MPSPS are unlikely. Thus, we assume that

the accumulation of specific, above-mentioned GAGs might be related to the mechanisms of their transport to lysosomes. Indeed, there are different pathways of transportation of GAGs into lysosomes. For example, heparan sulfate and hyaluronate are transported inside the cell by employing caveolae-dependent endocytosis, while dermatan sulfate might also be transported by the clathrin-promoted process [25,26]. Importantly, recent studies demonstrated that caveolin is significantly less abundant in MPS cells than in normal ones, apparently due to impaired *CAV1* gene expression [24]. In MPS, the primary GAG storage (arising from dysfunctional lysosomal hydrolases) causes stress conditions, which result in (among others) disrupted cell signaling and dysregulation of the expression of many genes. The dysregulated genes include those encoding caveolin and other proteins involved in caveolin-dependent endocytosis and vesicle trafficking [24]. In the case of MPS, this may lead to the accumulation of undegraded GAGs not only in lysosomes but also in the cytoplasm and outside the cells. If so, impaired transport of GAGs into lysosomes further stimulates pathological processes due to even more pronounced cellular stresses and changes in gene expression regulation. In the case of MPSPS, the disturbed GAG transport (caused by VPS33A dysfunction) could be the initial, rather than a secondary step of the spiral of the above-described events; nevertheless, causing positive feedback leading to more and more efficient GAG accumulation (Figure 1).

An alternative hypothesis is that impaired GAG degradation in MPSPS is due to increased endolysosomal acidification, demonstrated previously in patient-derived cells [13,14]. However, if this hypothesis were true, one should observe the accumulation of not only GAGs but also many other compounds, like proteins, lipids, and others, due to general dysfunctions of lysosomes, similar to the processes occurring in mucolipidosis (ML) type II and III, where lysosomal enzymes are improperly localized, thus being unable to catalyze specific reactions. Contrary to ML II and III, such defects in the degradation of very different macromolecules were not reported in MPSPS.

5. Symptoms of MPSPS in Comparison to Those Occurring in Classical MPS

All children suffering from the infant form of MPSPS developed extremely severe symptoms leading to death before the age of two years. A few years after the disease was described, it was included in the group of mucopolysaccharidoses, with the addition of 'plus'. This term refers to the fact that, in addition to the symptoms present in all other types/subtypes of MPS, additional symptoms such as kidney dysfunction, hematopoietic system disorders, and congenital heart defects also developed.

Children with MPSPS exhibit characteristic facial features, facial and limb swelling, and loose skin, similar to other types/subtypes of MPS [13–15,17,27]. Additionally, severe enlargement and dysfunction of the liver and spleen have been observed in both types/subtypes of MPS and MPSPS [11,13–15,27].

Hematopoietic system disorders are also present in MPSPS. Most patients exhibit a decrease in red blood cells and hemoglobin levels [28], as well as normocytic anemia, thrombocytopenia, neutropenia, and coagulation disorders [16,17]. The hypogammaglobulinemia seen in patients may be responsible for impaired humoral immunity and frequent recurrent infections [16]. The autopsy results of patients showed the presence of hypoplastic bone marrow [13,17].

5.1. Symptoms Detectable during Prenatal Testing

Prenatal testing is extremely important for the diagnosis of genetic diseases and the selection of appropriate treatments. Early diagnosis allows for immediate treatment, which can significantly improve the quality of life for affected individuals and may even prevent the onset of symptoms. Prenatal testing for MPS is possible, but due to the rarity of the disease, prospective parents typically do not undergo such tests without a specific reason, such as having an affected first child or knowing they are carriers.

In a patient with MPSPS, prenatal ascites were detected via ultrasound at 11 and confirmed at 24 weeks of pregnancy [15]. A similar symptom was described in a patient

with MPS VI, where prenatal symptoms began with ascites and rapidly progressed to mitral valve regurgitation and congestive heart failure, ultimately requiring surgical intervention within the first year of life [29]. Interestingly, all patients with MPSPS have congenital heart defects, such as mitral or aortic valve regurgitation, thickening of the interventricular septum, or atrial enlargement [11,14,17].

Prenatal abnormalities, including edema, fluid in the abdominal cavity, pleural effusion, and polyhydramnios, have also been diagnosed in patients with MPS VII [30]. Patients with MPS VII often suffer from severe prenatal conditions such as NIHF (non-immune hydrops fetalis). About half of patients with MPS VII die due to complications related to NIHF [31]. Similar edema symptoms have been prenatally diagnosed in patients with MPS II. A case was described where siblings were affected; the older child showed left hydronephrosis prenatally and inguinal hernia a month after birth, while the younger child underwent prenatal testing, revealing the presence of a mutation leading to MPS II development [32].

Further studies of women at risk of having children with MPSPS showed increased nasal mucosa thickness in the fetus. In another woman, an ultrasound examination of the child showed an increased nuchal fold and prenasal thickness [27].

5.2. Development

In patients with MPSPS, developmental disorders are quickly noticeable compared to healthy children. Patients with MPSPS are typically born on time, but the first symptoms of the disease appear within a few months. Most patients exhibit delayed psychomotor development with autistic symptoms [14,15]. There are also delays in learning to sit and walk: independent sitting after 13 months of age, walking after 28 months of age [15], sitting from 10 months of age, walking with assistance after 22 months of age, and babbling started at 11 months [17]. Usually, at the age of 2 years, patients can sit with support but cannot stand and speak [11]. The motor skills of patients with MPSPS are often limited due to joint stiffness and clawed fingers, resulting in frequent falls and difficulties in holding objects. Concentration and attention difficulties, poor memory, and limited cognitive abilities are also commonly described [14,17].

Similar symptoms are observed in patients with various types/subtypes of MPS. For example, patients with MPS VII show motor development issues, which is evident in unstable walking and frequent falls [30]. A typical patient with MPS VII could sit independently at seven months and begin walking at fourteen months [30]. At the age of 5 years, such a patient still has walking difficulties, a limp, and abnormal posture [30]. Patients with MPS III (Sanfilippo disease) also exhibit similar disorders as those with MPSPS. A child with Sanfilippo A began walking at 2 years, speaking at 3 years, and exhibiting general psychomotor developmental delays while being impulsive and hyperactive [33]. Similar symptoms also occur in patients with other subtypes of MPS III, where, over time, their mental health deteriorates, eventually leading to severe dementia and behavioral problems such as hyperactivity and aggression [34].

Patients with MPSPS often have a shorter stature compared to healthy children of the same age [17]. Similar observations have been made in patients with other types/subtypes of MPS. After birth, their average z-scores for body height were lower compared to reference values [35]. Children with MPS IVA stop growing at the age of 8 years, and their earlier growth is very slow. In MPS VI, growth can be variable during the first four years of life, with abrupt declines. Before the age of 5 years, these changes stabilize and are no longer as abrupt. The average z-scores for other MPS types (MPS I, II, and III) indicate that up to the age of 2 years, patients' growth is similar, and the average z-scores for body height are higher than in reference charts [36].

5.3. Central Nervous System

Patients with MPSPS exhibit severe central nervous system disorders similar to those seen in other types/subtypes of MPS with neurological symptoms (MPS I, II, III, and VII).

Neurological examinations show neurodevelopmental delay, and MRI brain scans reveal delayed myelination, brain calcifications, retinal hypopigmentation, cerebellar abnormalities, and, in some cases, global brain atrophy [13,16,17]. However, studies have shown that after treatment initiation and stabilization of the patient's condition, neurological abilities may gradually improve [17]. Some patients also exhibit nystagmus, which subsides over time in some cases [16,17].

Studies on a mouse model of MPS IIIC revealed similar disorders to those seen in MPSPS, including significantly reduced levels of proteins associated with myelination, such as myelin protein, myelin glycoprotein, and oligodendrocyte myelin glycoprotein. Structural studies showed disorganization of myelin sheaths and loss of myelin thickness in the brains of MPS IIIC animals [37]. In patients with MPS IIID, these disorders were less severe, namely, they exhibited mild cortical and cerebellar atrophy [38]. Patients with MPS I showed similar disorders to those with MPSPS, including severe white matter loss, brain atrophy, and, in some cases, hydrocephalus [39]. MRI studies showed that almost all patients with Sanfilippo disease exhibit cortical atrophy [40].

5.4. Heart

Cardiovascular diseases are currently the leading cause of premature death in patients with MPS [41]. Problems with proper diagnosis and a lack of appropriate treatment lead to rapid disease progression and the development of many complications, including heart failure [42,43]. All patients with MPSPS were found to have severe heart function disorders, the most common being mitral valve regurgitation [13,17], as well as aortic and tricuspid valve regurgitation [11]. A patient with a milder mutation (p.Arg200Pro) also showed mitral valve stenosis, but it was significantly milder than in other MPSPS patients and did not progress with age [15]. Additionally, some MPSPS patients were noted to have thickening of the interventricular septum [14] or atrial enlargement [17]. The most common cardiovascular disorder found in patients with other types/subtypes of MPS is mitral valve regurgitation [44]. Systolic murmur, aortic valve stenosis and regurgitation, thickened ends of aortic cusps, and mild left ventricular hypertrophy were observed in MPS X patients. Further studies on the MPS II model confirmed that the most common defect in these patients (56% of those studied) is mitral valve regurgitation, followed by aortic valve defect (33%) [45].

Overall, studies with MPS patients did not show a correlation between patient age and cardiac phenotype. In some cases, a patient with a mild disease course exhibited more severe heart damage than a patient with a severe phenotype [44].

5.5. Lungs

Lung diseases are extremely severe and rapidly recognized in patients with MPSPS. Patients are very often hospitalized due to recurrent upper respiratory tract infections and pneumonia [13,17]. In almost every described case, MPSPS patients were admitted to the hospital due to upper respiratory tract infections. A notable example is a female patient hospitalized at 2.5 months, 7 months, and 10 years, each time diagnosed with severe pneumonia [15], as well as a female patient who was repeatedly hospitalized after her first year of life due to respiratory tract infections [11]. Patients also frequently experience episodes of dyspnea and apnea attacks [16].

Patients with other types/subtypes of MPS also very often have recurrent upper and lower respiratory tract infections. Additionally, patients with MPS I frequently exhibit airway obstruction during sleep, which can result in obstructive sleep apnea [46]. Newborns with MPS II have been reported to have a higher incidence of respiratory failure than healthy children [47]. In MPS IV, a reduced chest size compared to healthy children and obstructive lung disease have been noted. Diaphragm displacement associated with hepatosplenomegaly has also been described [48].

Respiratory complications become more common in all children with MPS as the disease progresses [49]. Additionally, it has been proven that pneumonia is the cause of death in more than half of the children with MPS IIIA [50,51].

5.6. Skeletal System

Skeletal system problems do not occur in all patients with MPS. In some types, these symptoms are limited to short stature, while in others, they manifest as severe deformities of almost the entire skeleton. These symptoms are most visible and frequently diagnosed in patients with MPS IVA [52].

Patients with MPSPS also exhibit certain skeletal system disorders, one of the typical features being claw-shaped hands and wider metacarpal bones [16]. Similar disorders have been described in patients with severe forms of MPS I, II, and VII, which can eventually lead to a loss of hand function [3]. Additionally, MPSPS patients have been observed to have limited joint mobility and contractures, multiple deformities in bones other than the hands, and progressive skeletal dysplasia (dysostosis multiplex) [13,16,27]. Identical symptoms have been described not only in severe types of MPS I, II, and VII but also in MPS VI, where patients exhibit skeletal dysplasia, including short stature, dysostosis multiplex, and degenerative joint disease, while in MPS X patients, disproportionate short-trunk short stature and genu valgus were noted [53,54]. Patients with MPSPS also show joint inflammation and stiffness, as well as progressively worsening swelling of the lower limbs [15,27]. One patient also exhibited deformed femoral heads, excessive synovial membrane accumulation, and joint effusions [15]. Consequently, a gradual loss of motor skills was observed [15]. In some cases, vertebral flattening and metatarsal bone widening have also been documented [14].

The most significant skeletal system problems were observed in patients with MPS IVA. These symptoms include vertebral flattening, severe dysplasia of long bones (especially femoral epiphyses), joint instability, knee valgus, and resultant gait abnormalities [52]. However, a beta-galactosidase deficiency diagnosed as MPS IVB causes a considerably milder phenotype compared to MPS IVA [55]. It was reported that patients with MPS IVB exhibit limb dysplasia, but growth disturbances are less pronounced. Additionally, in these patients, enzymatic activity may be sufficient to prevent the development of peripheral nervous system symptoms observed in MPS IVA patients [56]. MPS IVA can be divided into three subtypes based on clinical presentation, depending on symptom severity. The severe form is characterized by systemic skeletal dysplasia identified at birth, while the mild form features slight skeletal disturbances and is usually diagnosed in adulthood. Children with the severe form often do not survive beyond the second or third decade of life, mainly due to cervical instability and impaired lung function [57–59].

The symptoms described in patients with MPS IX are usually mild, making proper diagnosis and appropriate treatment extremely difficult. The first diagnosed case of this type involved a 14-year-old girl who exhibited short stature, occasional soft tissue swelling, and periarticular soft tissue masses, while joint mobility was normal [60]. The other three patients diagnosed with MPS IX were siblings aged 11–21 years. One of them, at age 4 years, showed only hip or knee pain resulting from swelling. Further studies revealed synovitis and joint involvement [61].

5.7. Kidneys

Most of the symptoms observed in patients with MPSPS overlap with those in patients with other MPS types/subtypes. The organ where changes were observed only in MPSPS is the kidneys.

The term ‘plus’ refers to symptoms not found in other MPS types/subtypes. These additional symptoms include, in particular, kidney failure and hematopoietic system disorders. All patients with MPSPS are described as having nephrotic syndrome: enlarged kidneys and significant proteinuria [13–17,28], accompanied by hypoalbuminemia, creatinemia, elevated blood creatinine levels, and calcium deficiencies [16,28]. Most patients

also show higher blood uric acid levels compared to healthy individuals [16,28]. All these data indicate kidney failure in MPSPS patients.

Histopathological studies were also conducted on some MPSPS patients. Some analyses even showed complete destruction of glomerular structures [28]. In milder cases, periglomerular fibrosis, interstitial inflammation, and the presence of foam cells in podocytes were observed [16]. Foam cells were also observed in MPS I and MPS VII; however, in the liver and spleen rather than the kidney. Some MPSPS patients also exhibit renal tubular diseases (tubulopathies) [17].

5.8. Other Symptoms

In MPSPS, several symptoms related to other systems/organs besides those mentioned above are also observed. Some patients have been noted to exhibit subclinical hypothyroidism [15,17]. Children with MPSPS have also been observed to have peripheral and retrocochlear hearing impairment [16,17]. Significant hearing impairment is also present in other MPS types/subtypes, which may be caused by middle ear infections, ossicular chain deformities, inner ear abnormalities, or auditory nerve impairment [62,63].

The summary of the main findings in MPSPS, in comparison to other MPS types, is presented in Table 1.

Table 1. The summary of the main findings of the MPSPS throughout life, with indication of the occurrence of similar symptoms in different MPS types.

Symptom(s)	Type of MPS	References
Characteristic facial features	All (including MPSPS)	[13–15,17,27]
Dysfunction of the liver and spleen	All (including MPSPS)	[11,13–15,27]
Prenatal ascites	MPS VI, MPSPS	[15,29]
Delayed psychomotor development with autistic symptoms	MPS III, MPS VII, MPSPS	[11,14,15,17,30,33,34]
Short stature	MPS IVA, MPS VI, MPS X, MPSPS	[17,35]
Delayed myelination, brain calcifications, retinal hypopigmentation, cerebellar abnormalities, and, in some cases, global brain atrophy	MPS I, MPS III, MPSPS	[13,16,17,37–40]
Mitral, aortic, and tricuspid valve regurgitation	MPS II, MPS X, MPSPS	[13,17,44,45]
Recurrent upper respiratory tract infections and pneumonia	MPS I, MPS II, MPS III, MPS IV, MPSPS	[11,13,15,17,46–48,50,51]
Claw-shaped hands and wider metacarpal bones	MPS I, MPS II, MPS VII, MPSPS	[3,16]
Dysostosis multiplex	MPS I, MPS II, MPS VI, MPS VII, MPS X, MPSPS	[13,16,27,52–55]
Enlarged kidneys, significant proteinuria	MPSPS	[13–17,28]

6. Similarities of MPSPS to Other Lysosomal Storage Diseases

Patients with MPSPS exhibited excessive accumulation of sphingolipids, β-D-galactosylsphingosine (psychosine), and the deacylated form of galactosylceramide, which also excessively accumulates in another LSD, Krabbe disease [64]. Psychosine excess is involved in the loss of oligodendrocytes and myelin damage, leading to severe disturbances in the white matter of the brain and peripheral nerves. Indeed, such disturbances have been described in patients with mutations in the *VPS33A* gene [13].

Fabry disease (FD) is a lysosomal storage disorder caused by deficiencies of the lysosomal enzyme α-galactosidase A, leading to the accumulation of globotriaosylceramide (Gb3) [65]. One of the main causes of death in FD patients is end-stage renal failure. The exact mechanism of kidney damage is not yet known, but it is hypothesized that it is caused by nephrotic syndrome resulting from the accumulation of Gb3 [28,66]. A very similar

pattern of disorders has been observed in almost all patients with MPSPS [67–69] and in patients with ML III [28].

A mutation in the VPS33B gene leads to ARC syndrome (arthrogryposis–renal dysfunction–cholestasis), which includes renal tubular dysfunction, thrombocytopenia, and cholestasis [70]. The same mutation causes the ARKID syndrome (autosomal recessive keratoderma–ichthyosis–deafness), where patients also exhibit platelet dysfunction, ichthyosis, hearing loss, and osteopenia [71].

Symptoms caused by mutations in genes of other members of the HOPS and CORVET complexes differ phenotypically from those in MPSPS patients. A homozygous mutation in the VPS16 gene leads to primary dystonia during adolescence [72]. Loss of VPS18 functionality in mice leads to severe neurodegeneration and neuron-migration issues [73]. Joint contractures, delayed psychomotor development, and craniofacial deformities have been described in patients with mutations in the VPS8 gene [74]. Leukodystrophy caused by mutations in the VPS11 gene is associated with seizures, tetraplegia, or hearing loss [75–77]. However, this phenotype differs from that described in MPSPS patients. Leukodystrophy patients have been reported to exhibit increased urinary excretion of glycosphingolipids and elevated lysosomal storage. It was proposed that a defect in one of the HOPS complex components leads to substrate accumulation in lysosomes (including GAGs) but not to decreased lysosomal enzyme activity [13].

The p.Asp251Glu mutation in the *VPS33A* gene has been used in a mouse model for Hermansky–Pudlak syndrome [78,79]. Affected mice exhibit, among other things, severe behavioral disorders and degeneration of Purkinje cells [80].

Finally, it is worth noting that that similarities occur between some symptoms of MPSPS patients and those described in a recently discovered MPS X-type (deficiency in arylsulfatase K (ARSK) [81–85]. However, little similarity can be detected between MPSPS patients and those suffering from another recently recognized disease, MPS IIIE, though one should take into consideration the fact that there are serious doubts whether the latter disorder, caused by dysfunction of arylsulfatase G (ARSG), should be classified as MPS in humans or not [86].

7. Potential Therapies for MPSPS

Currently, there is no therapy for MPSPS. Regarding different MPS types, various therapeutical options were proposed, some of them being used in clinical practice [87]. Enzyme-replacement therapy (ERT) is available for clinical use for MPS I, MPS II, MPS IVA, MPS VI, and MPS VII. This therapy is based on the administration of recombinant human enzyme (which is lacking in the cells of patients) that is specifically modified to be efficiently recognized by the cellular receptor (usually the mannose-6-phosphate receptor), allowing the direction of the therapeutic protein to lysosomes [88]. Therefore, the recombinant enzyme can replace the function of the deficient one in the lysosomes of the patient's cells. However, in MPSPS, the dysfunction concerns a non-lysosomal protein. Therefore, ERT cannot be effective, at least as long as a method for the efficient delivery of cytosolic proteins is lacking. Hematopoietic stem cell transplantation (HSCT) is another therapeutic option already in use for some MPS types [89]. Nevertheless, for reasons similar to those described for ERT, HSCT seems to be unlikely to work effectively in MPSPS. Gene therapy is an obvious potential cure for genetic diseases, and intensive work has been conducted to develop and introduce this kind of therapy to treat MPS patients [90]. However, no report has been published to date on the development of gene therapy targeting the *VPS33A* gene. Thus, even if such an effort is started, the putative introduction of gene therapy for MPSPS should take at least several years, making this kind of treatment a matter of the future rather than a promise for rapidly solving the medical problem.

Because of the problems mentioned above, it is perhaps more likely to develop an alternative therapy for MPSPS rather than focusing on ERT, HSCT, or gene therapy. One possible option would be to stimulate the autophagy process. Since the major cellular problem in this disease is extra-lysosomal accumulation of GAGs, with possible secondary

storage of other compounds, activation of a process leading to the elimination of the storage material could be beneficial for patients. In fact, a potential use of autophagy stimulators has been proposed as a putative therapeutic approach in the treatment of MPS [91]. Such a strategy appeared recently to be effective in the case of cellular and animal models of another genetic disease caused by the accumulation of macromolecules, Huntington's disease [92]. There are various potential autophagy stimulators that could be employed in long-term use (which is necessary in the case of genetic diseases), including already-tested genistein [92] or ambroxol [93], a drug previously approved for the treatment of other conditions. Obviously, extensive studies are required to test if the hypothesis about the efficacy of autophagy stimulation in the treatment of MPSPS is true.

8. Concluding Remarks

MPS are defined as diseases in which GAG is stored due to dysfunctions of lysosomal enzymes involved in the degradation of these compounds. MPSPS is a specific disease where accumulation of GAGs occurs despite normal activities of all lysosomal hydrolases and transferases involved in their decay. Thus, MPSPS does not fulfill the strict definition of MPS while being a disease revealing the common primary metabolic defect—inefficient removal of excess GAG. Despite genetic and biochemical differences, MPSPS and various MPS types share many common symptoms. On the other hand, some of them are specific to MPSPS, reflecting the etymology of this disease. Here, we propose a possible mechanism for GAG accumulation in MPSPS due to impaired vesicle trafficking and resultant improper localization of these complex carbohydrates, precluding their efficient degradation by lysosomal enzymes (Figure 1). If this hypothesis is true, it might explain the pathomechanism of MPSPS in more detail. Still, the question remains whether MPSPS is a type of MPS or should classified as a separate metabolic disease. The answer actually depends on how we define MPS. If both conditions (GAG storage and lysosomal enzyme dysfunction) must be met to call a disease MPS, then MPSPS is a separate disorder. If, however, we agree that the crucial feature of MPS is GAG storage and that the accumulation of GAG(s) is enough to include a disorder into the group of MPS, then MPSPS should be treated as another MPS type.

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HUMAN GENETICS • ORIGINAL PAPER

Cellular and molecular changes in mucopolysaccharidosis-plus syndrome caused by a homozygous c.599G > C (p.Arg200Pro) variant of the *VPS33A* gene

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Abstract

Mucopolysaccharidosis-plus syndrome (MPSPS) is an ultrarare inherited metabolic disease (a few dozen patients diagnosed to date) which is characterised by accumulation of undegraded glycosaminoglycans (GAGs). Despite GAG storage occurs also in the groups of diseases classified as mucopolysaccharidoses (MPS), contrary to MPS, no dysfunctions of lysosomal enzymes is detected in MPSPS which is caused by mutations in the *VPS33A* gene. The c.1492C > T (p.Arg498Trp) variant, associated with a severe course of the disease, was found in most MPSPS patients. There are only two patients described to date with a homozygous c.599G > C (p.Arg200Pro) variant and a milder (juvenile) form. Until now, the molecular mechanism of MPSPS remained largely unknown, especially for the juvenile form. Here, a battery of cellular and molecular assays, performed using fibroblasts derived from a patient bearing the c.599G > C (p.Arg200Pro) variant of the *VPS33A* gene, indicated specific changes in cellular vacuoles, elevated levels of the EEA1 protein (required at the stages of the fusions of early and late endosomes, and early endosome sorting), changes in Golgi apparatus morphology, decreased levels of F-actin, and increased levels of α- and β-tubulins, as well as elevated levels of the LC3-II and p62 proteins (autophagy markers). Results of experiments presented here might suggest that severely decreased levels the p.Arg200Pro variant of VSP33A could cause defective endosomal trafficking, possibly resulting in inefficient delivery of GAGs to lysosomes, and their subsequent accumulation in cells. This might induce a cascade of secondary and tertiary disorders, finally expressing as the disease symptoms.

Keywords Mucopolysaccharidosis-plus syndrome · VPS33A · The c.599G > C (p.Arg200Pro) variant of the *VPS33A* gene · Endosomal trafficking · Cytoskeleton

Abbreviations

GAG	Glycosaminoglycan
HOPS complex	Homotypic fusion and protein sorting complex
HRP	Horse radish peroxidase
HS	Heparan sulphate
LSD	Lysosomal storage diseases
MPS	Mucopolysaccharidosis
MPSPS	Mucopolysaccharidosis-plus syndrome

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Introduction

Mucopolysaccharidosis (MPS) is a group of inherited metabolic diseases, caused by mutations leading to a lack or a severe deficiency in activities of lysosomal enzymes involved in degradation of glycosaminoglycans (GAGs) (Nagpal et al. 2022). GAGs are long, non-branched polysaccharides which are degraded due to their cleavage by endohexosidases or exoglucoronidases, accompanied by removing of chemical moieties by sulfatases (Freeze et al. 2015). Decay of GAGs is a sequential process; thus, a lack of a single enzyme in the chain of reactions leads to accumulation of partially degraded molecules. As a result, GAGs accumulate in lysosomes; thus, MPS diseases are classified as lysosomal storage diseases (LSD) (Parenti et al. 2015; Trovão de Queiroz et al. 2016).

In addition to several MPS types, classified according to a lack or deficiency of specific enzyme and the kind of stored

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GAG(s) (Wiśniewska et al. 2024), several years ago, a newly identified disease has been described in which GAG accumulation is evident (Kondo et al. 2017). However, this disease, named mucopolysaccharidosis-plus syndrome (or MPSPS), is unusual when compared to "classical" MPS, as accumulation of GAGs occurs in the absence of defects in lysosomal hydrolases. The phenotype of the MPSPS patients was found to resemble Hurler syndrome, a severe variant of MPS I (Vasilev et al. 2020); however, in addition to the symptoms typical to other MPS, the patients develop other disorders such as congenital heart defects, kidney dysfunction, and hematopoietic disorders (Faraguna et al. 2022; Sofronova et al. 2022). MPSPS is an ultrarare disease, because only a few dozen patients were described to date, though the exact number of patients is somewhat enigmatic as it appears that in analyses of the prevalence of the disease some patients could be counted twice due to uncertainty whether the same or different individuals were described in different publications (Lipiński et al. 2022; Cyske et al. 2024). Most of already described MPSPS patients came from Yakutia and Turkey (Dursun et al. 2017; Kondo et al. 2017), one from the Southern European/Mediterranean region (Pavlova et al. 2022), and one from Poland (Lipiński et al. 2022).

The genetic defect found in MPSPS consists of pathogenic variants of the *VPS33A* gene. Interestingly, only two mutations leading to MPSPS were described to date. The c.1492C > T (p.Arg498Trp) variant was found in a homozygous state in patients from Yakutia and Turkey (Dursun et al. 2017; Kondo et al. 2017; Faraguna et al. 2022), and the c.599G > C (p.Arg200Pro) variant was determined, also in a homozygous configuration, in the two patients from the Mediterranean region and Poland (Pavlova et al. 2022; Cyske et al. 2024). Comparison of phenotypes of MPSPS patients indicated that the latter variant is responsible for a milder course (the juvenile form) of the disease than the former one (Pavlova et al. 2019, 2022; Lipiński et al. 2022). In this report, further in the text, we will use the names MPSPS^{R498W} (severe form) and MPSPS^{R200P} (attenuated form) for the disease caused by the presence of the c.1492C > T (p.Arg498Trp; p.R498W) and c.599G > C (p.Arg200Pro; p.R200P) variant of the *VPS33A* gene, respectively, both in the homozygous configuration.

Despite the identification of pathogenic mutations causing MPSPS^{R498W} and MPSPS^{R200P}, the molecular mechanism of the disease, including the cause of GAG accumulation, remains largely unknown. One suggestion of a putative mechanism was based on lysosomal over-acidification in MPSPS^{R498W} (Kondo et al. 2017). Another hypothesis (Pavlova et al. 2022; Cyske et al. 2024) which might explain a pathomechanism of MPSPS^{R200P} is related to the known functions of the *VPS33A* gene product (the VPS33A protein) which is involved in the formation of the homotypic fusion and protein sorting (HOPS) complex and the observations of the impaired intracellular glycosphingolipid trafficking (Solinger and Spang 2013). Obviously, both mechanisms are also plausible to be responsible for development of MPSPS, irrespective of the specific type of the nucleotide change in *VPS33A*. In fact, earlier observations suggested that Hook1, which perhaps indirectly interacts with VPS18, functions in attachment to the cytoskeleton (Richardson et al. 2004) and that the FTS/Hook/p107 (FHIP) complex interacts with and promotes endosomal clustering by the HOPS complex (Xu et al. 2008). Subsequent studies suggested that this complex might be required for endosome-to-cytoskeleton attachment, and could be involved in the control of endosome maturation and its trafficking to the lysosome, as well as lysosome fusion with endosomes and autophagosomes (Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022). Results of recent studies suggested that dysregulation of vesicle trafficking might contribute to GAG storage due to impaired delivery of these compounds to lysosomes (Gaffke et al. 2023). Thus, it was speculated that due to deficiency in the VPS33A activity, vesicular transport of GAGs might be impaired, leading to accumulation of these compounds, as they cannot be efficiently degraded outside of lysosomes (Cyske et al. 2024). This proposal might be corroborated by demonstration that the p.Arg200Pro variant of VPS33A is responsible for destabilisation of the protein, most probably through its rapid proteasomal degradation (Pavlova et al. 2022). However, since the above proposal remained hypothetical, the aim of this work was to test the presented hypothesis experimentally. We assumed that if the hypothesis is true, changes in selected cellular organelles and in the cytoskeleton should occur, and levels of crucial proteins involved in endosomal trafficking should be dysregulated in MSPSP, specifically in the MPSPS^{R200P} variant, investigated here.

Materials and methods

Cell lines

This study was carried out with the use of a cell line obtained after isolation of dermal fibroblasts from a patient with MPSPS, bearing a homozygous c.599G > C (p.Arg200Pro) variant of the *VPS33A* gene. This patient has been described previously (Lipiński et al. 2022). As a control, the line of human dermal fibroblasts taken from a healthy person, of similar age and the same sex, was used. Other controls used in this work were lines of fibroblasts derived from MPS IIIA, MPS IIIB, and MPS IIIC patients, being a complex heterozygote p.Glu447Lys/p.Arg245His in the *SGSH* gene (the line purchased from the Coriell Institute, #GM00879), a recessive homozygote p.Arg626Ter/p.Arg626Ter in the *NAGLU* gene (the line purchased from the Coriell Institute,

#GM00156), or a complex heterozygote p.Gly262Arg/pArg509Asp in the *HGSNAT* gene (the line purchased from the Coriell Institute, #GM05157), respectively. The ethical approval has been obtained from the Bio-Ethical Committee of the Children' s Memorial Health Institute (Warsaw, Poland; decision no. 23/KBE/2020). Fibroblasts were cultured in Gibco Dulbecco' s modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and antibiotic–antimycotic solution (Thermo Fisher Scientific), under standard laboratory conditions (temperature 37 °C, 5% CO_2 saturation, 95% humidity).

Electron microscopy

Cells (1×10^5) were plated onto 12-well plates and incubated overnight. Following sedimentation in a centrifuge, cells were washed with the PBS buffer three times at 10-min intervals. The next step was flooding cells with 2.5% glutaraldehyde in PBS for 10 min for fixation, followed by washing three times with PBS as described above. Then, the cells were treated by flooding with osmium tetroxide in PBS for 1 h at dark. After washing with PBS, the dehydration stage consisted with washing the cells with ethyl alcohol of increasing concentrations (30%, 50%, 70%, 90%, 100%, and again 100%), replacing a solution every 15 min. Then, immersing the cells was performed in a solution of resin and ethanol (1:1 v/v ratio) overnight. The next day, the sample was poured with clean resin twice, each lasting 2 h. After the second pouring, the plate was placed in an incubator at 60 °C. Then, sections were made using diamond knives on an ultramicrotome and viewed using a Tecani Spirit BioTWIN electron microscope.

Fluorescence microscopy

Cells (4×10^4) were plated onto 12-well plates with a coverslip placed inside and incubated overnight. Cells were treated with LysoTracker (#L7528, Termo Fisher) or MitoTracker (#M7510, Life Technologies) and CellMask™ Green Actin Tracking Stain (#A57243, Thermo Fisher) for 1 h. After the indicated time, the cells were fixed by using 2% paraformaldehyde, 0.1% Triton-X100, and DAPI mounting medium. Alternatively, after the overnight incubation, cells were fixed with 2% paraformaldehyde for 15 min, washed with PBS, and incubated with 0.1% Triton X-100 for 15 min. After washing five times with PBS and 1-h incubation with 5% BSA, primary antibodies (or they combination for colocalisation) (anti-heparan sulphate, #NBP2-23,523 Novus; anti-LAMP1, #46,843 Cell Signalling; anti-LAMP2, #49067S Cell Signalling; anti-EEA1, #3288S Cell Signalling; anti-Rab7 (D95F2), #9367 Cell Signalling; anti α-tubulin, #2125 Cell Signalling; anti β-tubulin, #2128 Cell Signalling; VPS33A, #16,896–1-AP, ProteinTech) were added, and samples were kept overnight at 4 °C. Next day, fibroblasts were washed five times with PBS and incubated in anti-rabbit/anti-mouse secondary antibody (or they combination for colocalisation) (#4413S, Cell Signalling; #4408S, Cell Signalling) for 1 h in dark at 4 °C. Then, cells were washed five times with PBS and coverslips were fixed to glass slides using a mounting medium ProLong™ Gold Antifade Mountant with DAPI (#P36935, Invitrogen). Cells were analysed using Leica CTR 4000 or Olympus IX83 fluorescence microscope with software provided by each manufacturer.

Staining of cells to determine the intracellular colocalisation of LysoTracker and HS was a combination of the two staining methods described above. Fibroblasts (4×10^4) were plated onto 12-well plates with a coverslip placed inside and incubated overnight. Following the treatment of cells with LysoTracker (#L7528, Termo Fisher) for 1 h, fixation was conducted by using 2% paraformaldehyde and 0.1% Triton-X100. After washing five times with PBS and 1-h incubation with 5% BSA, cells were incubated with primary antibodies (different combinations of following antibodies: anti-heparan sulphate, #NBP2-23,523 Novus; anti-LAMP1, #46,843 Cell Signalling; anti-EEA1, #3288S Cell Signalling) and kept for 2 h at 4 °C. Next, fibroblasts were washed five times with PBS and incubated with anti-mouse antibody (#4408S Cell Signalling) for 1 h in dark at 4 °C. Cells were then washed five times with PBS, and coverslips were fixed to glass slides using a mounting medium ProLong™ Gold Antifade Mountant with DAPI (#P36935, Invitrogen). Cells were analysed using Olympus fluorescence microscope. Colocalisation analysis was performed as described previously (Adler and Parmryd 2013) using the CellSens 4.3 software.

Western-blotting

Cells (5×10^5) were plated onto 10-cm plates and incubated overnight. Lysates of fibroblasts were prepared exactly as described previously (Rintz et al. 2023). The sample was divided into two parts. Using one part, proteins (LAMP2, rab5, Rab7, α-/β-tubulin, p62, VPS33A, VPS16) were separated using the automatic Western-blotting system—the WES system (WES—Automated Western Blots with Simple Western; ProteinSimple, San Jose, California, USA), based on capillary electrophoresis, with a 12- to 230-kDa separation module (#SM-W003), and detected (inside the capillary) using primary antibodies (anti-LAMP2, #49067S Cell Signalling; anti-Rab5, #3547 Cell Signalling; anti-Rab7 (D95F2), #9367 Cell Signalling; anti α-tubulin, #2125 Cell Signalling; anti β-tubulin, #2128 Cell Signalling; anti-SQSTM1/p62, #39,749 Cell Signalling; VPS33A, #16,896–1-AP, ProteinTech; VPS16, #17,776–1-AP, ProteinTech), and anti-mouse (#DM-002)

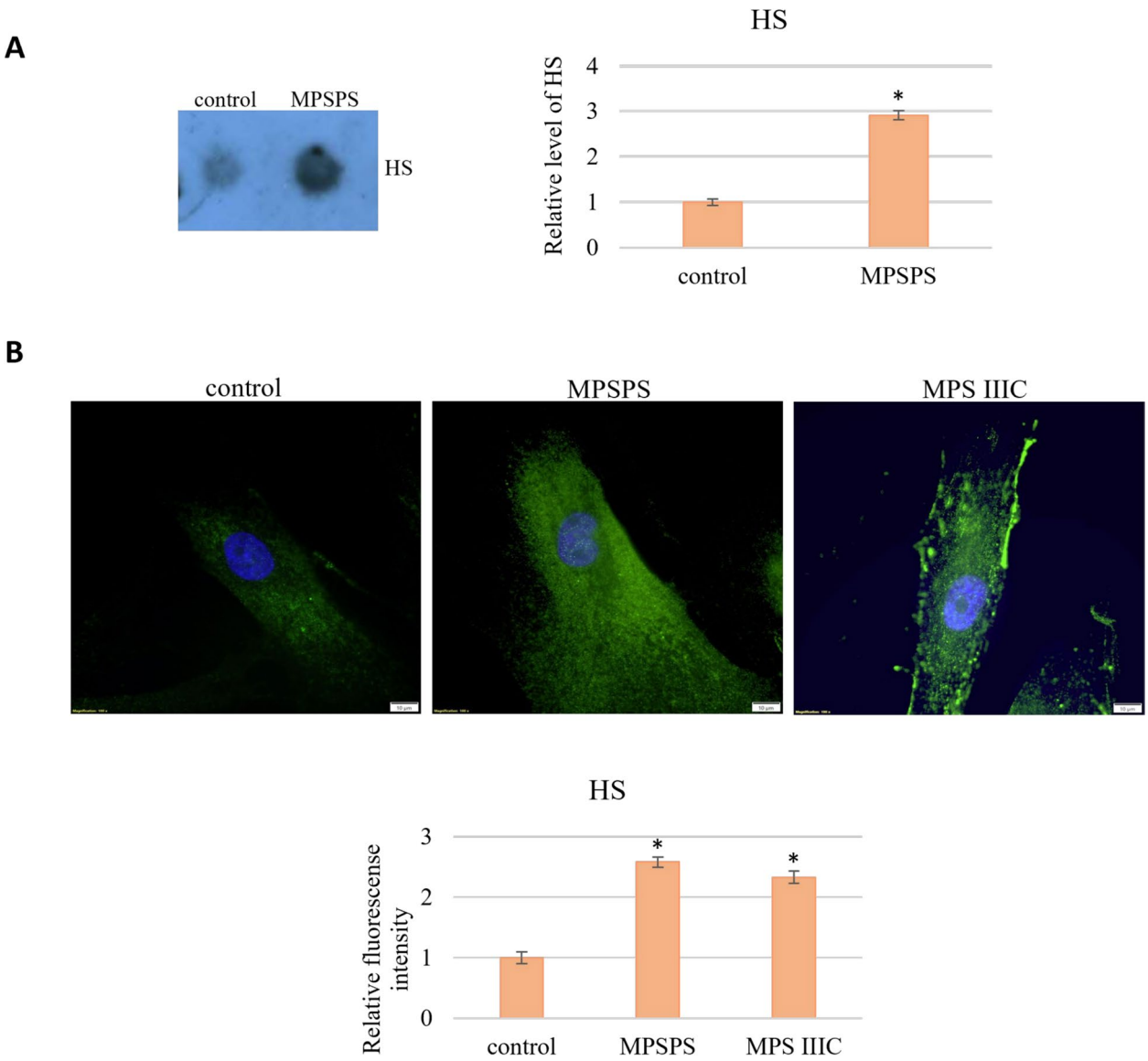


Fig. 1 Storage (**A**) and distribution (**B**) of heparan sulphate (HS) in MPSPS^{R200P} (marked as MPSPS) fibroblasts. Abundance of HS was estimated by dot blotting using lysates of MPSPS and control fibroblasts, with quantification performed using densitometry (**A**). Distribution of HS was investigated in control, MPSPS^{R200P}, and MPS IIIC fibroblasts by fluorescent microscopy (**B**; bars in microscopic images represent 10 µm). All analyses were performed in three independent biological replicates, and representative results are presented in the panels. Error bars represent the standard deviation of three independent experimental replicates (in fluorescence microscopy experiments, > 100 cells were analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

or anti-rabbit (#DM-001) detection module, according to the manufacturer' s instructions. The total-protein module (#DM-TP01) was used as a loading control. Using the second part of the sample, EEA1 and LC3 proteins were detected by traditional Western blotting after SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk in the PBST buffer and incubated with primary anti-EEA1 (#3288S, Cell Signalling) or anti-LC3 antibody (G-4, #sc-398822, Santa Cruz Biotechnology) overnight at 4 °C. Then, the membrane was rinsed two times in PBST and incubated with secondary antibody (anti-rabbit, #A0545, or anti-mouse, #AP124P; Sigma Aldrich) at room temperature for 1 h in dark. After this time, the membrane was treated with a solution of substrates for horse radish peroxidase (HRP) and exposed to the X-ray film.

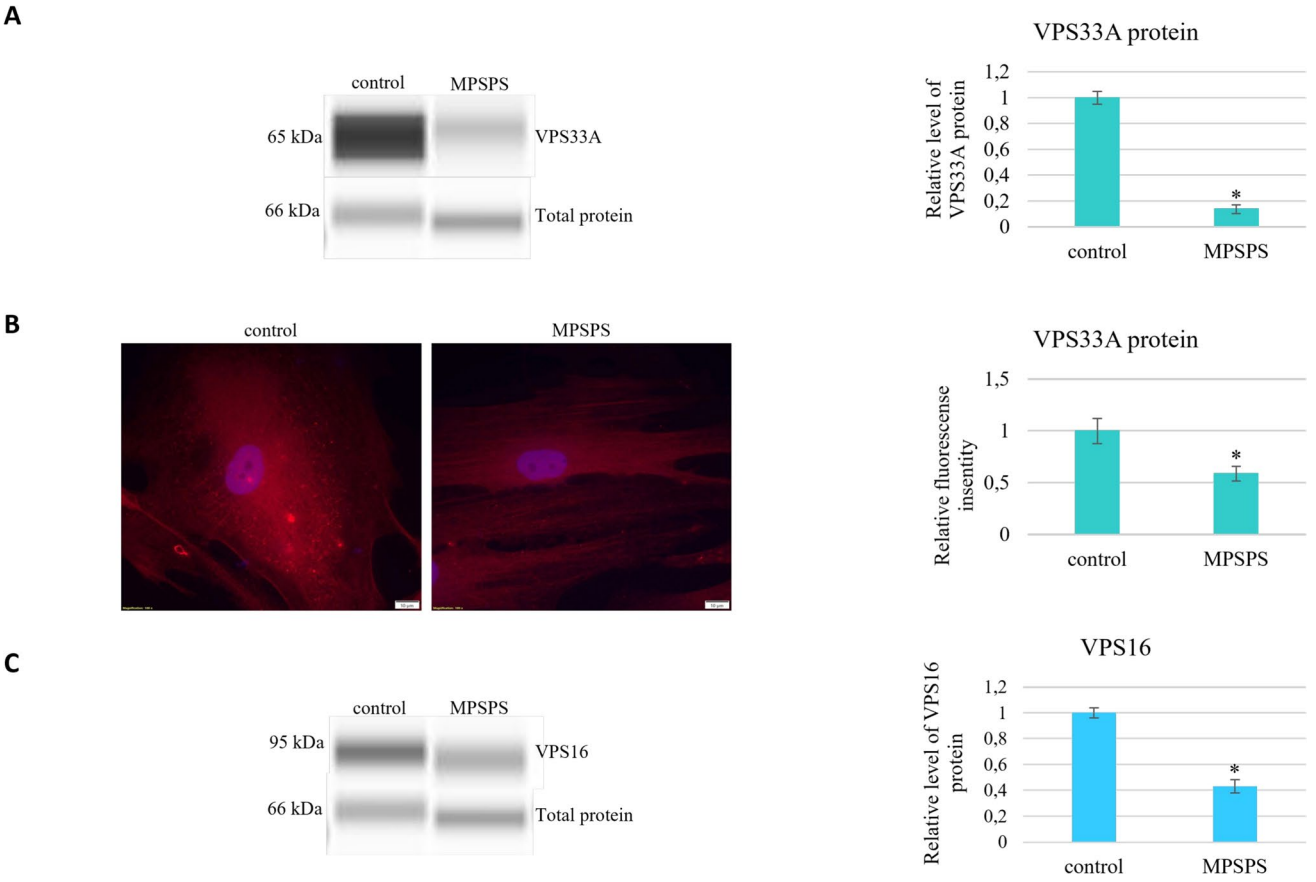


Fig. 2 Abundance of the VPS33A (**A**, **B**) and VPS16 (**C**) proteins in MPSPS^{R200P} (marked as MPSPS) and control (the wild-type variant) fibroblasts. Levels of VPS33A were estimated by Western blotting, with quantification performed using densitometry (**A**, **C**). Abundance of VPS33A was also determined by fluorescent microscopy (**B**; bars in microscopic images represent 10 µm). All analyses were performed in three independent biological replicates, and representative results are presented in the panels. Error bars represent the standard deviation of three independent experimental replicates (in fluorescence microscopy experiments, > 100 cells were analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

Determination of heparan sulphate levels by dot-blotting

The levels of heparan sulphate (HS) in cells were determined by dot blotting, as described previously (Piotrowska et al. 2006). Briefly, extracts from fibroblasts (after treatment with the lysis buffer, consisting of 0.1% Triton X-100, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris, pH 7.5) were fixed to the PVDF membrane (#IPFL00010, Millipore) in a dot-blot apparatus (Bio-Rad, Hercules, CA, USA). Following membrane blocking with the use of a non-fat dry milk in PBST buffer (5% w/v), an incubation with an anti-HS antibody (mouse, #NBP-2–23,523, Novus) was conducted at 4 °C overnight. After PBST washing, incubation of the membrane was conducted at room temperature for 1 h with a secondary anti-mouse antibody coupled with HRP (#A9044, Sigma Aldrich). The chemiluminescent HRP substrate (Merck, Darmstadt, Germany) was added and the membrane was exposed to an X-ray film. The QuantityOne software was employed to quantify the signals. The obtained values were normalised to the amount total protein, determined by staining with Ponceaus S.

Statistical analysis

Biochemical analyses were conducted by investigating samples from at least three independent experiments. Microscopic analyses were performed on the basis of analysis of at least 100 cells. Quantitative results are presented as mean values with standard deviation (SD). Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test. The differences were considered significant when $p < 0.05$.

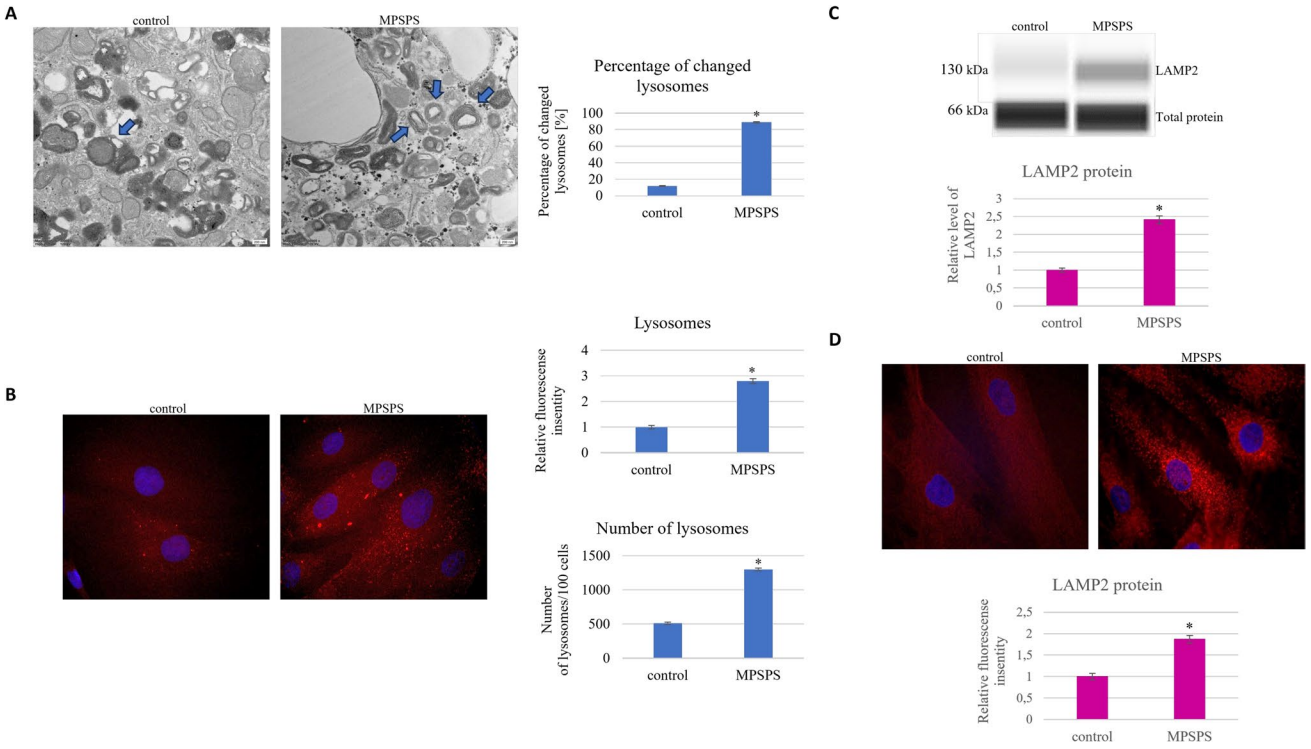


Fig. 3 Morphology of cellular vesicles (**A**), abundance of lysosomes as estimated by measurement of the LysoTracker signal (**B**), and levels of lysosome-associated membrane protein 2 (LAMP2) (**C**, **D**), in MPSPS^{R200P} cells (marked as MPSPS) compared to control cells. **A** Morphology of vesicles, with changes indicated by arrows, in MPSPS^{R200P} fibroblasts compared to control cells as assessed by electron microscopy. Analyses were made using a Tecani Spirit BioTWIN electron microscope; scale bare indicates 200 nm. **B** Fluorescence intensity and number of lysosomes in MPSPS^{R200P} cells compared to control cells as assessed by fluorescence microscopy. Fibroblasts were stained with LysoTracker Red. **C** Western-blotting analysis of the levels of the LAMP2 protein. **D** Fluorescence intensity of the LAMP2 protein in MPSPS^{R200P} cells compared to control cells. Fibroblasts in experiments shown in panels C and D were stained with the specific anti-LAMP2 antibody. Cells shown in panels B and D were analysed using a Leica CTR 4000 fluorescence microscope. In quantitative analyses, error bars represent standard deviation (SD) of three independent experimental replicates (with > 100 cells analysed in microscopic experiments). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

Results

To test the hypothesis that the molecular mechanism of MPSPS involves VPS33A deficiency-caused impairment of endosomal trafficking of GAGs and resultant inefficient delivery of these compounds to lysosomes, leading to their impaired degradation and then accumulation, we have employed fibroblasts derived from an MPSPS^{R200P} patient (called also MPSPS cells), bearing the c.599G > C (p.Arg200Pro) variant of the *VPS33A* gene and control fibroblasts, derived from a healthy person (control cells).

First, we have confirmed that there is a significant GAG storage in the MPSPS^{R200P} fibroblasts, as determined by dot blot, because previously only urinary GAG excretion was tested in this patient (Lipiński et al. 2022). Specifically, we detected accumulation of HS, one of GAGs (Fig. 1A). Interestingly, when visualising HS storage in cells, the distribution of the accumulated material detected by anti-HS antibody in MPSPS^{R200P} cells was different than in MPS III (one of classical MPS diseases), despite similar total abundance of this GAG (Fig. 1B). Namely, in MPS III, HS was visualised mostly as foci, perhaps representing lysosomal storage; while in MPSPS^{R200P}, this GAG was distributed more equally in cells but with distinct stretches of signals (Fig. 1B). Such a picture in MPSPS^{R200P} fibroblasts might correspond to disturbed trafficking and lysosomal delivery of GAGs.

Since it was reported previously that the p.Arg200Pro variant of VPS33A is characterised with low stability of the protein in cells derived from the Mediterranean patient (Pavlova et al. 2022), we tested VPS33A levels in fibroblasts of the Polish patient. We found a drastically decreased level of VPS33A in these fibroblasts relative to control cells (Fig. 2A), indicating that the c.599G > C variant of *VPS33A* encodes a protein which is either inefficiently produced or rapidly degraded. A decreased

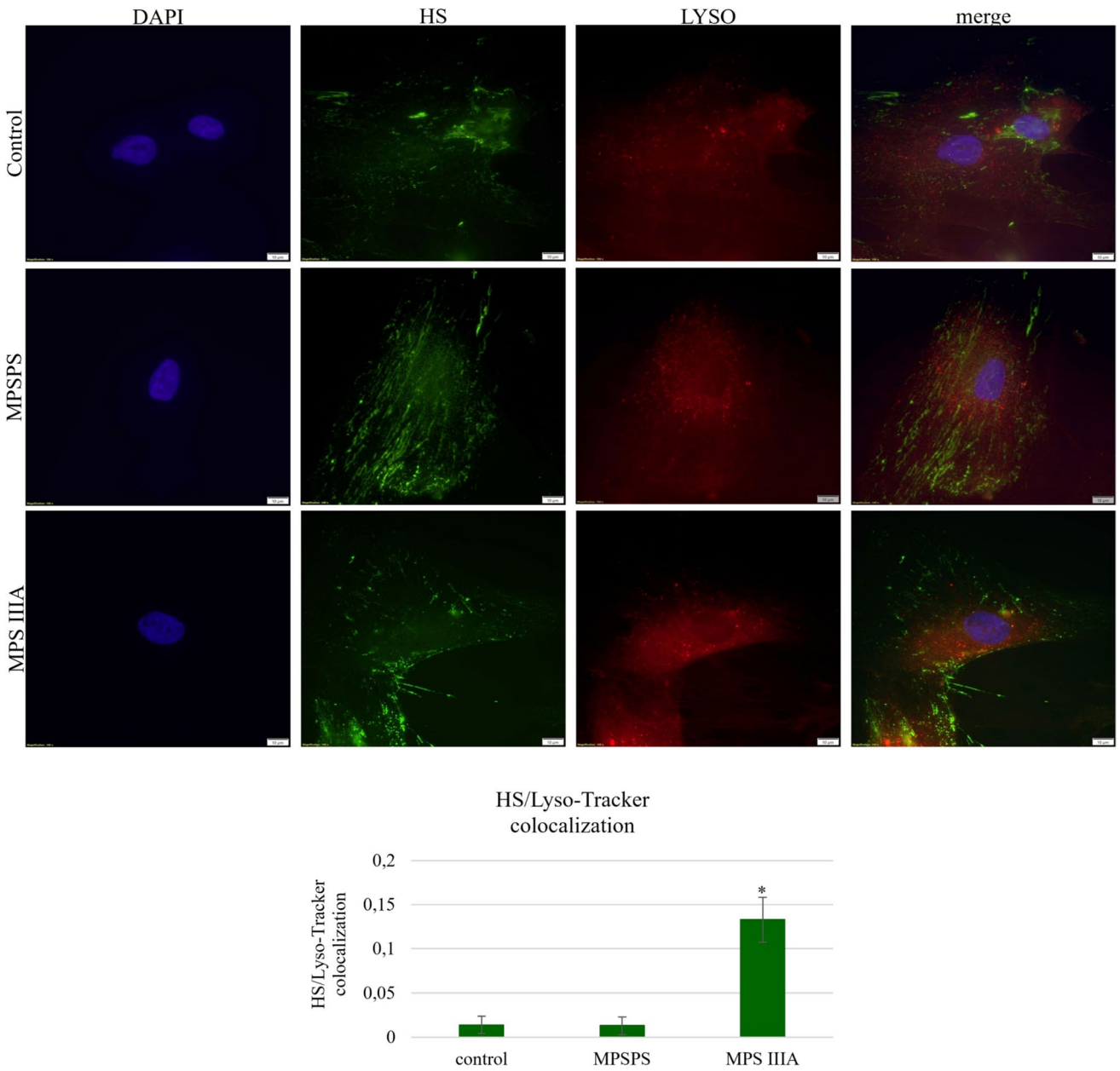


Fig. 4 Colocalisation of HS and lysosomes in MPSPS^{R200P} cells (marked as MPSPS) compared to MPS IIIA and control cells. Fibroblasts were treated with anti-HS antibody and/or stained with LysoTracker Red. Cells were analysed using a Leica CTR 4000 fluorescence microscope (bars in microscopic images represent 10 µm). In quantitative analyses of colocalisation efficiency, error bars represent standard deviation (SD) of three independent experimental replicates (with > 100 cells analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

abundance of VPS33A in MPSPS^{R200P} was also confirmed in fluorescent microscopy analysis (Fig. 2B). Interestingly, lower levels of the VPS16 protein, known to interact with VPS33A, were also detected in MPSPS^{R200P} cells (Fig. 2C).

Having confirmed specific biochemical characteristics of cells derived from the MPSPS^{R200P} patients being common to both the Mediterranean and the Polish ones, we analysed cellular and molecular changes in fibroblasts derived from the latter patient. When studying morphology of cellular vesicles, electron microscopic images of MPSPS^{R200P} cells indicated that a fraction of abnormal vesicular structures in these fibroblasts was significantly higher than in control cells (Fig. 3A). They abnormality consisted mainly of the storage material present in these organelles. This suggested the dysfunction of either

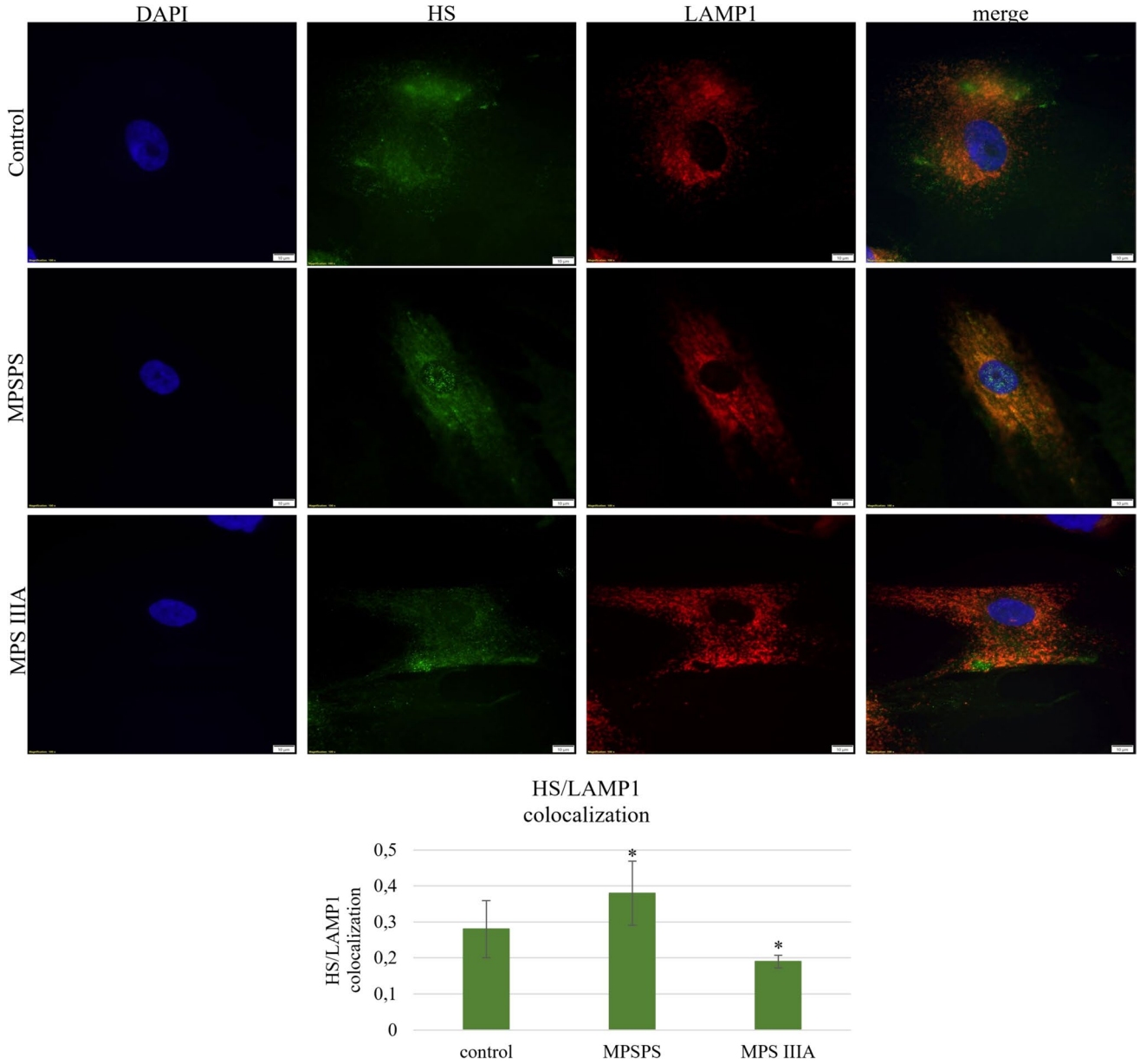


Fig. 5 Colocalisation of HS and LAMP1 in MPSPS^{R200P} cells (marked as MPSPS) compared to MPS IIIA and control cells. Fibroblasts were treated with anti-HS antibody and/or anti-LAMP1 antibody. Cells were analysed using a Leica CTR 4000 fluorescence microscope (bars in microscopic images represent 10 µm). In quantitative analyses of colocalisation efficiency, error bars represent standard deviation (SD) of three independent experimental replicates (with > 100 cells analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells, and hashtag (#) shows statistically significant difference ($p < 0.05$) between MPS IIIA and MPSPS^{R200P} cells

lysosomes or endosomes, possibly due to impaired trafficking and fusions of endosomes with lysosomes. In fact, the observed changes appeared quite different than in typical MPS cells, where lysosomes are often heavily filled with inclusions, accompanied with the presence of specific structures, like "zebra bodies" and "onion skin", which were infrequent in MPSPS^{R200P} cells (compare Fig. 3A with electron micrographs of MPS I fibroblasts taken previously in the same laboratory and presented in the previously published article by Piotrowska et al. (2006)). Fluorescent microscopy analyses with the use of LysoTracker Red indicated the stronger signal in the MPSPS^{R200P} cells relative to control ones (Fig. 3B). This might reflect that lysosomes are more abundant in MPSPS^{R200P} cells than in control fibroblasts. However, an increased acidification of lysosomes was reported previously in both MPSPS^{R200P}

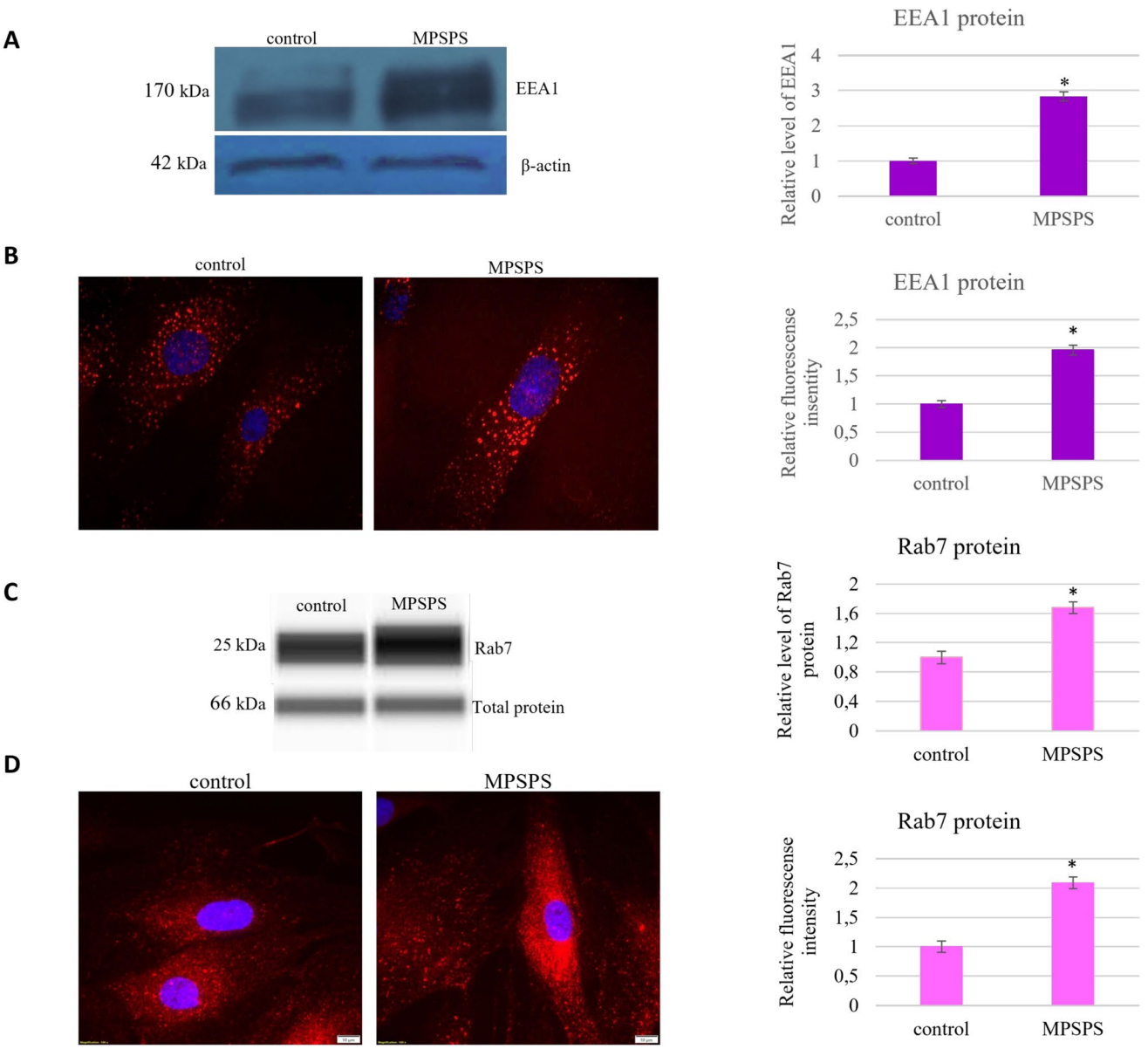
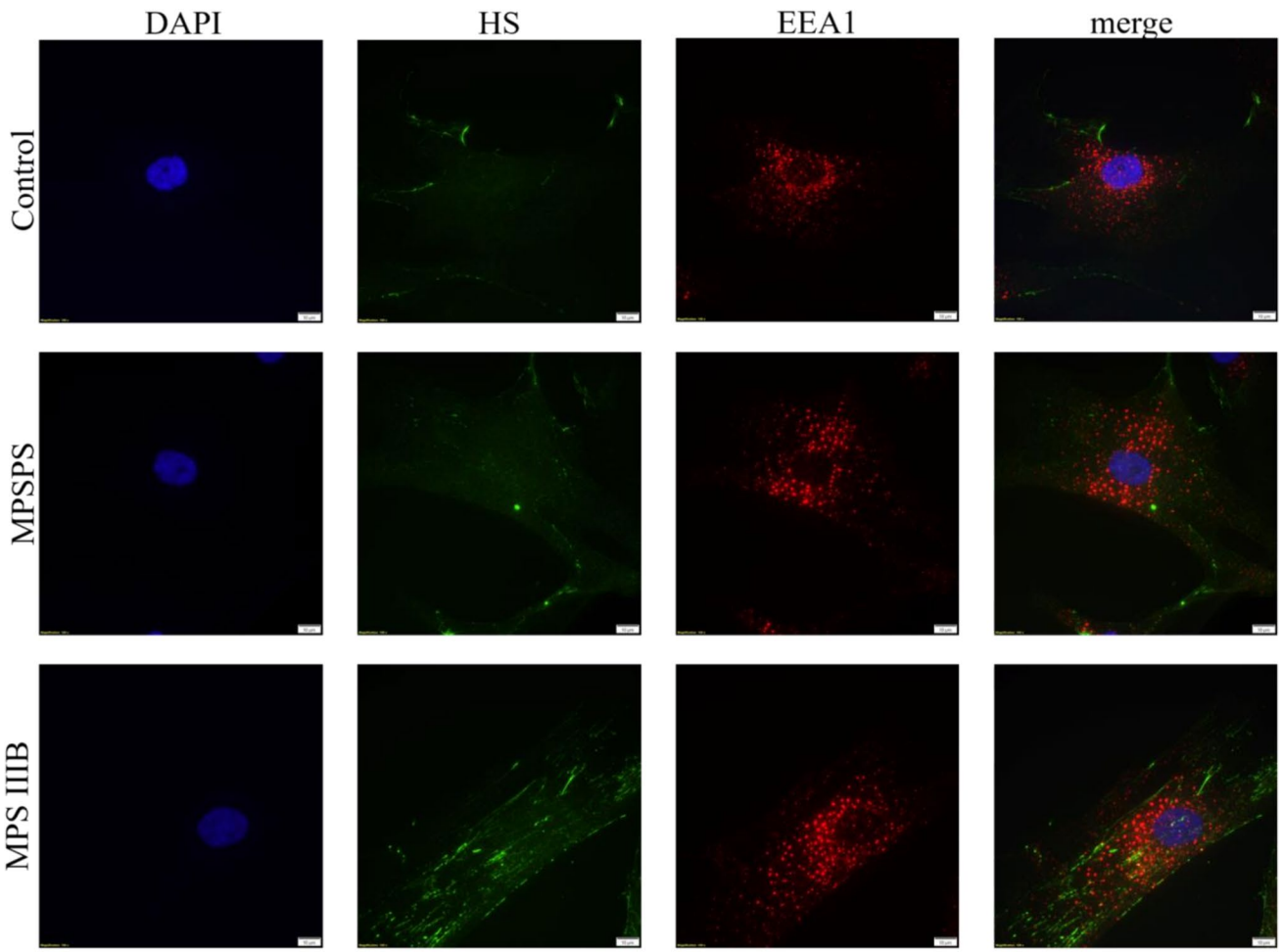
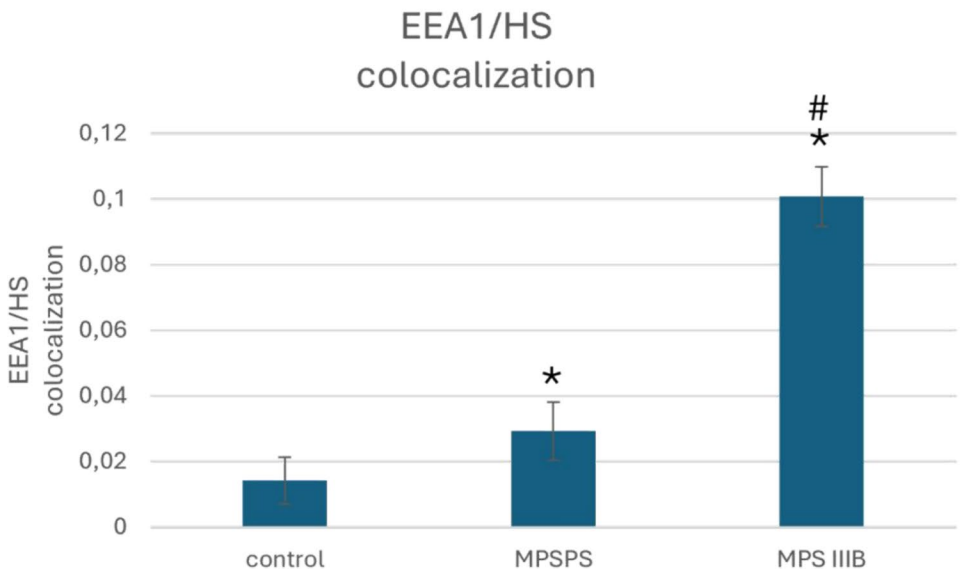


Fig. 6 Abundance of the early endosome antigen 1 (EEA1) protein (**A**, **B**) and the Rab7 protein (**C**, **D**) in MPSPS^{R200P} cells (marked as MPSPS) compared to control cells. Western-blotting analysis of the levels of EEA1 (**A**) and Rab7 (**C**) in MPSPS^{R200P} cells compared to control cells. Fluorescence intensity of EEA1 (**B**) and Rab7 (**D**) in MPSPS^{R200P} cells compared to control cells. Fibroblasts in both kinds of experiments were stained with using the specific anti-EEA1 and anti-Rab7 antibodies. Cells were analysed using a Leica CTR 4000 fluorescence microscope. All analyses were performed in three independent biological replicates, and representative results were presented in the panels. Error bars represent the standard deviation of three independent experimental replicates (in fluorescence microscopy experiments, > 100 cells were analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

(Pavlova et al. 2022) and MPSPS^{R498W} (Kondo et al. 2017), indicating that a higher affinity of LysoTracker (an acidophilic dye) to organelles with lower pH could also contribute to the strength of the observed signal. Therefore, we measured levels of LAMP2, a lysosomal membrane protein, to find that its amount appeared significantly increased in MPSPS^{R200P} cells, as estimated by both Western blotting (Fig. 3C) and fluorescent microscopy (Fig. 3D). These results corroborated the conclusion that lysosomes are more abundant in MPSPS^{R200P} cells, irrespectively of their enhanced acidification.

The results described above raised a question whether the observed storage material, which likely includes GAGs, is located in lysosomes or not. To test this, we performed a colocalisation experiment in which anti-HS antibodies were used together with (or separately from) LysoTracker Red. As indicated in Fig. 4, while HS colocalised with lysosomes considerably in MPS III, no such colocalisation was evident


DAPI
HS
EEA1
merge
Control
MPSPS
MPS IIIB


EEA1/HS
colocalization
EEA1/HS
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*
control
MPSPS
MPS IIIB

◂**Fig. 7** Colocalisation of HS and EEA1 in MPSPS^{R200P} cells (marked as MPSPS) compared to MPS IIIB and control cells. Fibroblasts were treated with anti-HS antibody and/or anti-EEA1 antibody. Cells were analysed using a Leica CTR 4000 fluorescence microscope (bars in microscopic images represent 10 µm). In quantitative analyses of colocalisation efficiency, error bars represent standard deviation (SD) of three independent experimental replicates (with > 100 cells analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells, and hashtag (#) shows statistically significant difference ($p < 0.05$) between MPS IIIB and MPSPS^{R200P} cells

in MPSPS^{R200P} fibroblasts (Fig. 4). This suggests that GAGs are not delivered efficiently to lysosomes in the case of the latter disease. Intriguingly, when localisation of HS in cells was compared to that of the LAMP1 protein, a colocalisation was even more effective in MPSPS^{R200P} than in MPS III cells (Fig. 5). This might appear ostensibly paradoxical in the light of a weak HS colocalisation with LysoTracker Red; however, LAMP1 was demonstrated to occur in considerable quantities also in early and late endosomes (Cook et al. 2004). Therefore, the explanation of these results might be that HS is trapped in endosomes and not effectively delivered to lysosomes during the vesicle trafficking. This could also explain results demonstrated in Fig. 3A, where vesicles with a storage material might represent endosomes containing accumulated substances, undelivered to lysosomes, rather than lysosomes themselves.

Since VPS33A is involved in endosome maturation and endosome trafficking to the lysosome (Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022), we assumed that if transportation of GAGs (and perhaps some other compounds) to lysosomes is impaired in MPSPS^{R200P} cells due to dysfunction of this protein (according to the tested hypothesis), one should observe accumulation of protein(s) taking part at earlier stages of endosome formation (due to a block at the later stage). The early endosome antigen 1 (EEA1) is a protein required for fusion of early and late endosomes and for sorting at the stage of the early endosome (Yap and Winckler 2022). Therefore, we have measured levels of this protein in MPSPS^{R200P} and control fibroblasts. We have observed significantly increased amounts of EEA1 in MPSPS^{R200P} cells relative to controls using both Western blotting (Fig. 6A) and fluorescent microscopy (Fig. 6B). Again, these results are compatible with the tested hypothesis, as well as with the previously reported results, demonstrating elevated levels of EEA1 in fibroblasts derived from the Mediterranean MPSPS^{R200P} patient (Pavlova et al. 2022). Moreover, levels of the Rab7 protein, which promotes late endosomal trafficking and delivery of cargo to lysosomes (Marwaha et al. 2017; Mulligan and Winckler 2023), were also elevated in MPSPS^{R200P} fibroblasts (Fig. 6C, D). Accordingly, increased levels of Rab5, a protein involved in endosome maturation through the endolysosomal trafficking pathway (Nagano et al. 2019), were also detected in MPSPS^{R200P} cells relative to control cells (Supplementary Fig. S1).

In the light of the above results, we have assessed the colocalisation of HS with the EEA1 protein to determine if GAGs are accumulated in endosomes of MPSPS^{R200P} cells more efficiently than in control fibroblasts. In fact, the colocalisation signals were significantly higher in the former cells than in the latter ones (Fig. 7) which corroborates the tested hypothesis. Interestingly, the HS-EEA1 colocalisation was drastically increased in MPS III cells (Fig. 7), which might also confirm the previous hypothesis about the secondary defect of vesicular trafficking in classical MPS types (Gaffke et al. 2023).

In the light of likely disturbed endosomal trafficking and delivery of cargo to lysosomes in MPSPS, we measured levels of the LC3-II and p62 proteins, molecular markers of the autophagy process. We found an increased level of LC3-II in MPSPS^{R200P} fibroblasts relative to control cells (Fig. 8A). While moderately elevated LC3-II levels suggest activation of autophagy, a severe increase in abundance of this protein corresponds rather to inhibition of the autophagy flux (Klionsky et al. 2021). Thus, we have also estimated levels of this protein in the presence of chloroquine, an inhibitor of the autophagy flux, in MPSPS^{R200P} to find even higher abundance of LC3-II in MPSPS^{R200P} cells (Fig. 8A). Moreover, in these cells, we observed elevated levels of the p62 protein, whose accumulation is considered an indication of impaired flux (Fig. 8B). Therefore, these results suggest that the autophagy process is affected in MPSPS^{R200P} cells, perhaps stimulated at the early stages but then blocked at the flux stage.

The VPS33A protein is involved in formation of the HOPS complex, which not only controls endosome maturation and trafficking to the lysosome but also is required for vacuolar fusion and transport from the Golgi apparatus to vacuoles (Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022). Hence, we investigated also these organelles. Electron microscopy analyses indicated that although lengths of Golgi apparatus structures were comparable in MPSPS^{R200P} and control cells, their widths were smaller in MPSPS^{R200P} (Fig. 9).

Since actin filaments (F-actin) are involved in endosomal trafficking at various steps (Capitani and Baldari 2021), we have measured levels of F-actin in MPSPS^{R200P} cells to find that this protein is less abundant in these cells than in control ones (Fig. 10A). Moreover, since the VPS33A function is also required for proper endosome-to-cytoskeleton attachment, we tested levels of α- and β-tubulins, the proteins forming microtubules that are engaged in different trafficking steps (Fourriere et al. 2020). Using Western blotting and fluorescent microscopy, we found that considerably higher levels of both α- and β-tubulins occurred in MPSPS^{R200P}

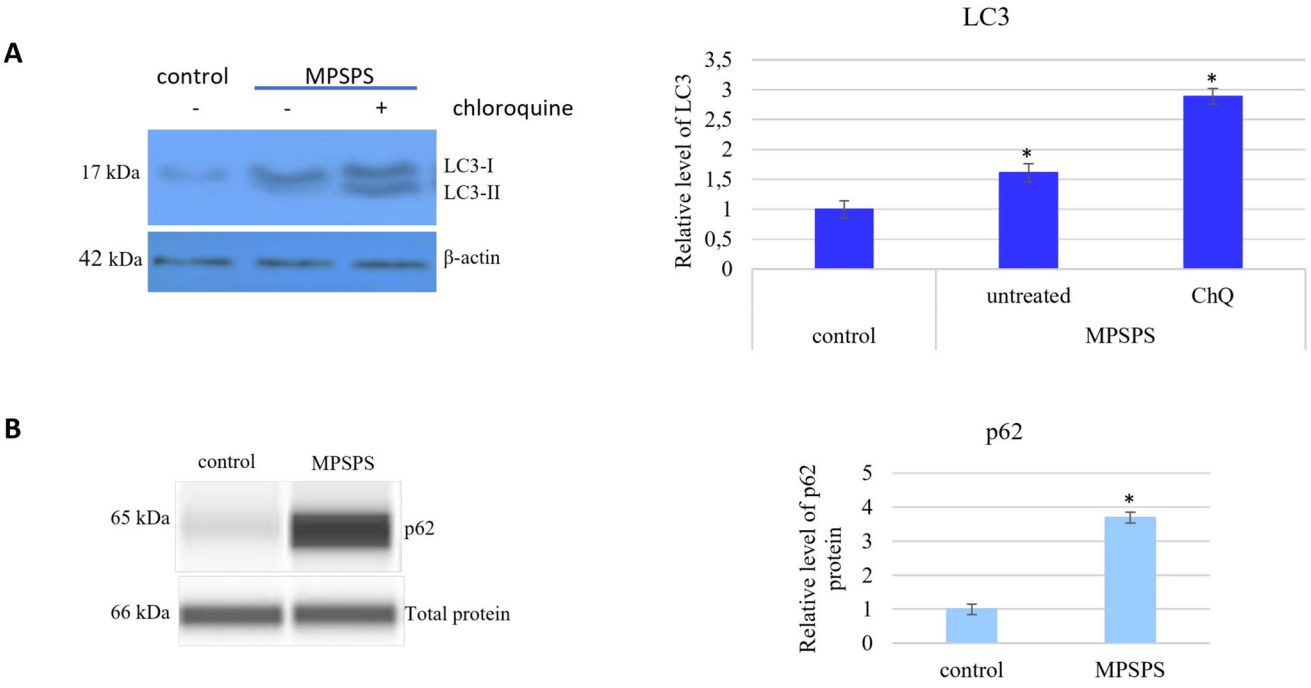

Fig. 8 Levels of the LC3-II (**A**) and p62 (**B**) proteins in MPSPS^{R200P} (marked as MPSPS) and control (the wild-type variant) fibroblasts. Where indicated, chloroquine was added to the cell culture to final concentration of 10 µM. Levels of proteins were estimated by Western blotting, with quantification performed using densitometry. All analyses were performed in three independent biological replicates, and representative results are presented in the panels. Error bars represent the standard deviation of three independent experimental replicates Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells, and hashtag (#) shows statistically significant difference ($p < 0.05$) between MPS IIIA and MPSPS^{R200P} cells

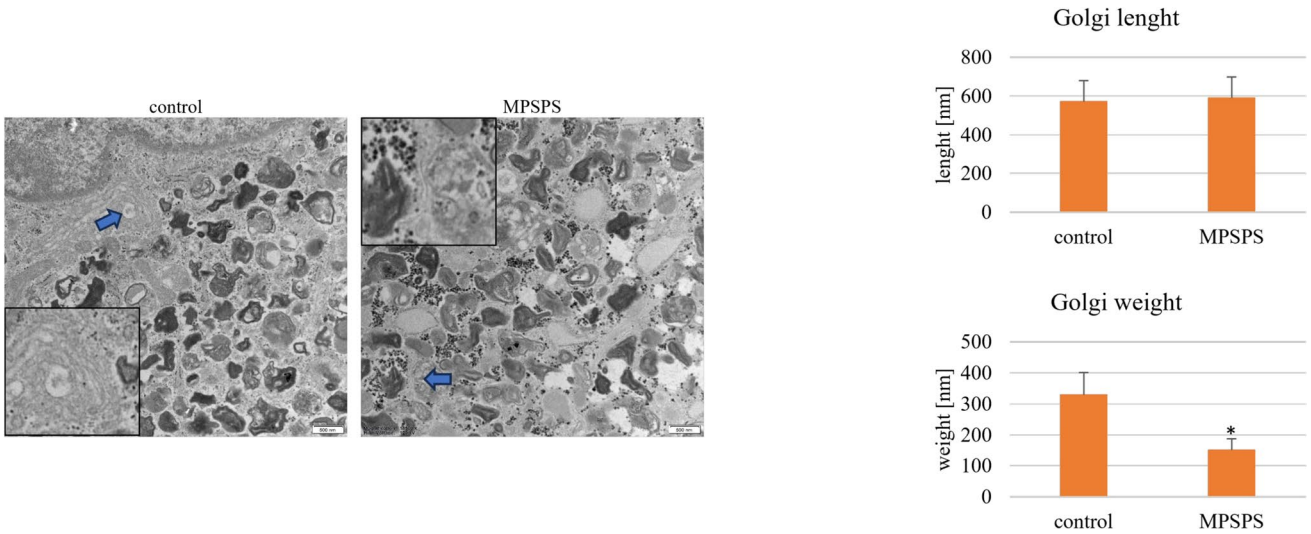

Fig. 9 Morphology of Golgi apparatus (arrow) in MPSPS^{R200P} (marked as MPSPS) fibroblasts compared to control cells. Analyses were made using a Tecani Spirit BioTWIN electron microscope. Scale bar denotes 500 nm. All analyses were performed in three independent biological replicates, and representative results are presented in the electron micrographs. Error bars represent the standard deviation (> 100 cells were analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

fibroblasts relative to the control (Fig. 10B, C). Thus, we conclude that cytoskeleton may be significantly affected in MPSPS^{R200P} cells, likely being connected to the impairment in endosomal trafficking.

Finally, we have also tested other organelles in MPSPS^{R200P} and control cells. We found that morphology of mitochondria was not significantly different between these two kinds of cells, while the investigated organelles were more abundant in MPSPS^{R200P}, perhaps reflecting a metabolic stress in the cells (Supplementary Fig. S2). When investigating morphology of nuclei, no significant differences could be detected between MPSPS^{R200P} and control fibroblasts (Supplementary Fig. S3). These results suggest that the cellular and molecular disorders observed in MPSPS^{R200P} cells are specific to processes, structures, and organelles involved in cellular trafficking rather than reflecting global changes in cell functions.

Discussion

MPSPS is an ultrarare genetic disease, with a few dozen patients described to date worldwide (Cyske et al. 2024). The course of this disease is severe (with death occurring usually before the age of 2 years) in patients bearing the homozygous c.1492C > T (p.Arg498Trp) variant of the *VPS33A* gene (Dursun et al. 2017; Kondo et al. 2017; Pavlova et al. 2019), called the MPSPS^{R498W} form in this article. However, patients with the homozygous c.599G > C (p.Arg200Pro) variant express an attenuated phenotype, corresponding to the juvenile form of the disease (Lipiński et al. 2022; Pavlova et al. 2019), called MPSPS^{R200P} here. Despite the symptoms resembling MPS and accumulation of GAGs, the mechanism of MPSPS appears to be significantly different than that of classical MPS types. As indicated above, the mutations causing MPSPS occur in the *VPS33A* gene rather than in a gene encoding a lysosomal enzyme involved in degradation of GAGs (Dursun et al. 2017; Kondo et al. 2017; Pavlova et al. 2019, 2022; Lipiński et al. 2022). Nevertheless, perhaps surprisingly, accumulation of these compounds is significant in MPSPS, leading to the proposal of including this disease to the MPS group (Kondo et al. 2017). On the other hand, the molecular mechanism of GAG accumulation and further cellular pathomechanisms remained largely unknown, which not only makes the classification of MPSPS controversial (Cyske et al. 2024) but also causes significant difficulties in developing any potential therapy.

In this light, determining the molecular mechanism of MPSPS appears to be important. Two hypotheses on the possible mechanism of GAG accumulation in MPSPS, in the presence of fully active enzymes involved in lysosomal GAG degradation, have been proposed. One of them proposes that over-acidification of lysosomes could cause inefficient hydrolysis of various compounds in these organelles, including GAGs (Kondo et al. 2017). Another proposal (Pavlova et al. 2022; Cyske et al. 2024) is based on the involvement of the VPS33A protein (which is either defective or deficient in MPSPS) in the endosomal trafficking, as demonstrated previously (Wartosch et al. 2015; Marwaha et al. 2017; Kümmel et al. 2022). According to this assumption, defective vesicular (especially endocytic) transport may facilitate accumulation of undegraded GAGs which cannot be effectively delivered to lysosomes, where they should otherwise be hydrolysed to simple sugars (Pavlova et al. 2022; Gaffke et al. 2023; Cyske et al. 2024). Since this hypothesis was proposed but not tested yet, we aimed to investigate its plausibility by examining cellular organelles, structures, and levels of selected proteins in MPSPS^{R200P} cells in comparison to control ones. One should also note that the over-acidification and the impaired trafficking hypotheses are not mutually exclusive, and both mechanisms might operate simultaneously.

Results presented in this report are generally compatible with the hypothesis assuming abnormal trafficking of GAGs, while not excluding the over-acidification hypothesis and resultant inefficient degradation of these compounds. Here, we demonstrated that lysosomes were more abundant in MPSPS^{R200P} cells, but the distribution of the storage material and lysosomal inclusions were different than in MPS III cells (represented in this analysis by MPS IIIA, MPS IIIB, and MPS IIIC fibroblasts; all classified as subtypes of Sanfilippo disease, characterised by HS accumulation, but caused by pathogenic variants of different genes—this prevented a misinterpretation of results due to putative specific effects of a particular mutation). A relatively weak colocalisation of HS with LysoTracker while considerable colocalisation with LAMP1 suggested that a storage material, observed in vacuolar structures in MPSPS^{R200P} cells, could represent a block of vacuolar trafficking at the stage of endosomes before fusion with lysosomes (this possibility was plausible as LAMP1 occurs in both lysosomes and endosomes). Importantly, the EEA1 protein, operating at the stage of early endosome, was significantly more abundant in MPSPS^{R200P} fibroblasts than in control cells (this was true for cells derived from both MPSPS^{R200P} patients described to date; compare the report by Pavlova et al. (2022)), perhaps

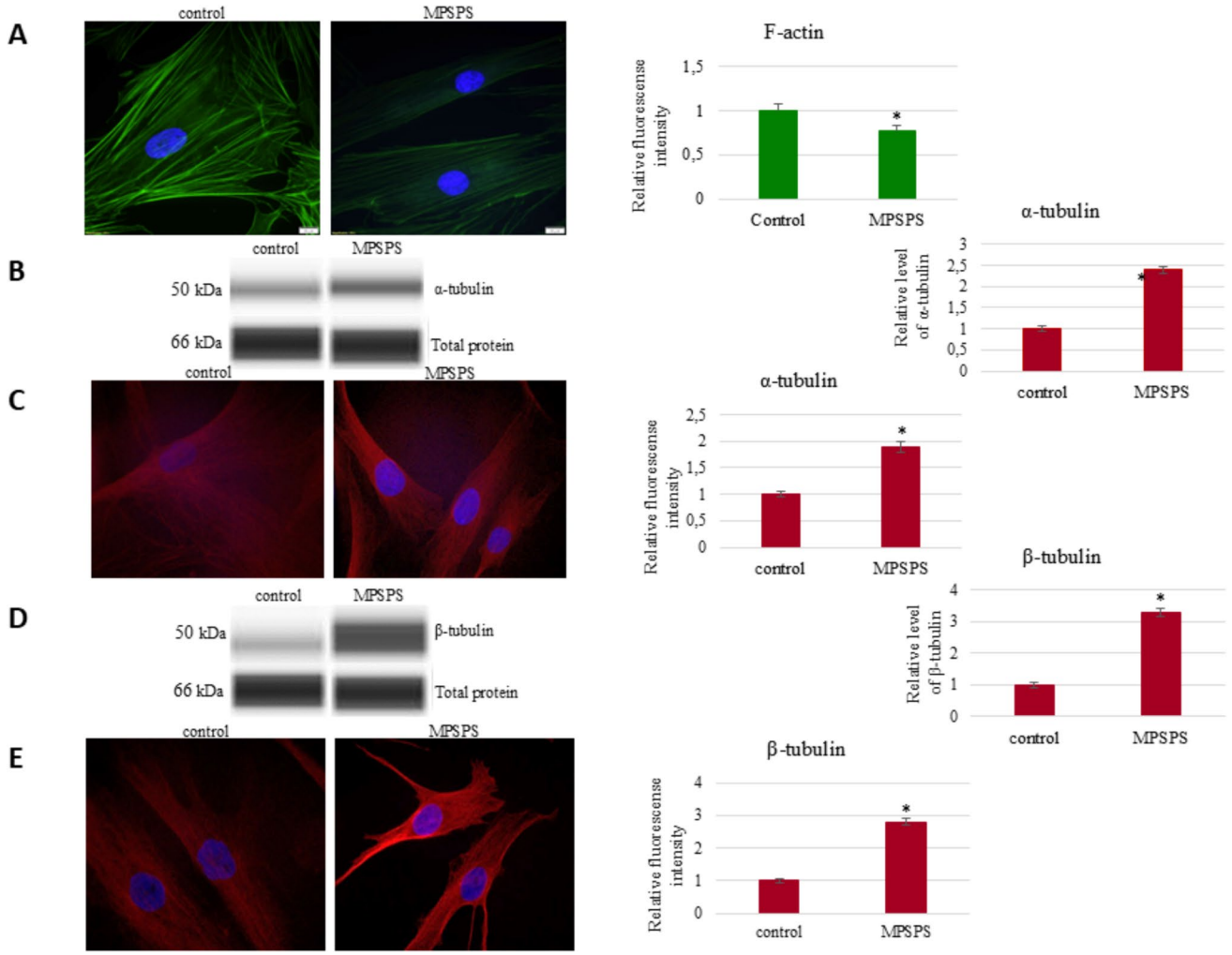

Fig. 10 Abundance of F-actin (**A**), α-tubulin (**B**, **C**), and β-tubulin (**D**, **E**) and in MPSPS^{R200P} cells (marked as MPSPS) compared to control cells. **A** Electron microscopic analysis of microtubules (arrows). **A**, **C**, **E** Fluorescence intensity of F-actin, α-tubulin, and β-tubulin, respectively, in MPSPS^{R200P} cells compared to control cells. Proteins were detected with using the specific anti-F-actin, anti-α-tubulin, and anti-β-tubulin antibodies. Cells were analysed using a Leica CTR 4000 fluorescence microscope. **B**, **D** Western-blotting analysis of the levels of α-tubulin and β-tubulin, respectively. All analyses were performed in three independent biological replicates, and representative results were presented in the panels. Error bars represent the standard deviation of three independent experimental replicates (in fluorescence microscopy experiments, > 100 cells were analysed). Asterisks (*) indicate statistically significant differences ($p < 0.05$) relative to control cells

reflecting the blockage at the stage of endosome maturation and endosome trafficking to the lysosome under conditions of VPS33A deficiency. Moreover, increased signals of HS-EEA1 colocalisation were evident in MPSPS^{R200P} cells relative to control ones, corroborating the conclusion about impaired endosomal trafficking of GAGs and their deficient delivery to lysosomes. Interestingly, under the same conditions, especially efficient colocalisation of HS and EEA1 was observed in MPS III cells. This may reflect a previous proposal (Gaffke et al. 2023) that impaired vesicular trafficking is one of secondary effects of GAG storage in classical MPS types, thus, reflecting a kind of "accumulation spiral", where the primary storage cause defects in delivery of GAGs to lysosomes, resulting in even more efficient accumulation of these compounds which in turn further impairs the trafficking, and so on. Hence, one might propose that quite a similar process occurs in MPSPS; however, the abnormal trafficking is a primarily affected process (due to VPS33A deficiency) while GAG storage is a secondary disorder. Nevertheless, the self-reinforcing "accumulation spiral" could lead to quite similar effects in both classical MPS and MPSPS; with possibly enhanced cellular disorders in the latter case due to over-acidification of lysosomes and resultant impaired hydrolysis of various compounds.

The above interpretation may be confirmed by the finding of elevated levels of Rab5 and Rab7 proteins in MPSPS^{R200P}

cells. The observed changes in morphology of Golgi apparatus, as well as changes in levels of F-actin and α- and β-tubulins (especially in the light of the elevated EEA1 levels and the interplay between endosomes and microtubules), are also compatible with the hypothesis suggesting that GAGs may accumulate in MPSPS cells because of the deficiency in the amount of the VPS33A protein which cannot support proper endosomal transport of these compounds to lysosomes. Such an explanation is also supported by previously reported defects in glycosphingolipids endocytic trafficking in cells of the Mediterranean MPSPS^{R200P} patient (Pavlova et al. 2022). Moreover, results of experiments, in which levels of the LC3-II and p62 proteins were measured in the absence and presence of an autophagy flux inhibitor, suggested an impairment of this process (which is largely dependent on lysosomal functions) in MPSPS^{R200P}.

An additional corroboration of the hypothesis tested in this work comes from results of studies on another disease caused by the mutations in the *VPS16* gene. Symptoms of this disease resemble those of MPSPS, and the product of the above mentioned gene is also involved in formation of the HOPS complex, crucial in effective endosomal trafficking (Sofou et al. 2021; Yıldız et al. 2021). Therefore, it appears likely that impaired endosomal delivery of GAGs to lysosomes, in the deficiency of activity of either VPS33A or VPS16, may cause their inefficient degradation despite activity of lysosomal hydrolases, which is the principle of GAG accumulation and further secondary and tertiary defects, leading to specific disease symptoms, as speculated recently (Gaffke et al. 2023; Cyske et al. 2024). In this light, it is interesting that we observed a decrease in the levels of VPS16 in fibroblasts derived from the MPSPS^{R200P} patient.

As mentioned previously, MPSPS^{R200P} and MPSPS^{R498W} differ in symptoms; however, results presented in this report suggest that the molecular mechanism of the disease might be common, with a difference only in the severity of disorders. As indicated, the product of the p.Arg200Pro allele is less stable than the wild-type variant; thus, the deficiency of VPS33A in this case apparently results from decreased levels of the protein in cells. If the same were true for the VPS33A protein (p.Arg498Trp) linked to MPSPS MPSPS^{R498W}, previous demonstration that this variant is able to retain some of its functions, like interactions with its known partners, VPS16 and STX17 (Kondo et al. 2017), might still be compatible with the hypothesis tested here, as low level of the protein rather than its functional defect could be responsible for the deleterious cellular effects. Increased acidification of lysosomes is also another change common for both MPSPS^{R498W} and MPSPS^{R200P}, as this cellular abnormality was reported in cells derived from patients with both relevant pathogenic variants of *VPS33A* (Kondo et al. 2017; Vasilev et al. 2020; Pavlova et al. 2022). In line with these facts, proteinuria, joint stiffness, recurrent pulmonary infections, increased GAG excretion, and neurological involvement are common for both MPSPS^{R200P} and MPSPS^{R498W} (Kondo et al. 2017; Vasilev et al. 2020; Lipiński et al. 2022; Pavlova et al. 2022). Following this line of thought, one might expect that the findings in the subcellular compartment levels would also be similar. Thus, in our opinion it is reasonable to consider the phenotype associated with the MPSPS^{R200P} variant as a milder form of MPSPS, otherwise severe in the MPSPS^{R498W} variant, while molecular mechanisms of the disease being the same in both variants.

A limitation of this study is availability of the biological material from only a single patient with the p.Arg200Pro variant. Therefore, although quite unlikely (due to compatibility of our results with some results reported previously for another patient with the same *VPS33A* variant; Pavlova et al. 2022), it is still possible that some nuances of changes in the subcellular processes might be related to individual features of the investigated cell line. Another limitation is the use of MPS III cells in control experiments, as only HS is accumulated in this disease, while DS and complex molecules other than GAGs can accumulate in MPSPS because endosomes contain many other substrates that cannot reach the enzymes in the lysosomes. However, MPS III cells were used mainly to compare GAG localisation between MPS and MPSPS cells, minimising the risk of uncertainty of the obtained results.

Conclusions

The reported results corroborate the hypothesis that the molecular mechanism of GAG accumulation in MPSPS cells is based on significantly decreased levels of the VPS33A protein, leading to impaired endosomal trafficking and ineffective delivery of GAGs to lysosomes, while over-acidification of lysosomes can be an independent and simultaneously occurring cause of the disease. The resultant impaired GAG degradation can cause the storage of these compounds and further cascade of the specific deleterious effects. This mechanism could have implications for developing possible therapeutic approaches for MPSPS, an as yet untreatable disease.

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L.G.; validation, Z.C., M.N., G.W.; writing—original draft preparation, Z.C., G.W.; writing—review and editing, Z.C., E.R., M.N., L.G., K.P., G.W.; visualisation, Z.C., E.R, M.N., L.G., K.P, G.W.; supervision, G.W.; project administration, Z.C., G.W.; funding acquisition, Z.C., G.W. All authors have read and agreed to the published version of the manuscript.

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Data availability Raw data of all experiments are available from the authors on request of qualified academic researchers.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Bio-Ethical Committee of the Children's Memorial Health Institute (Warsaw, Poland; decision no. 23/KBE/2020).

Consent to participate Informed consent was obtained from parents of the patients being donors of the biological material used in the study.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Materiały dodatkowe

SUPPLEMENTARY FIGURES

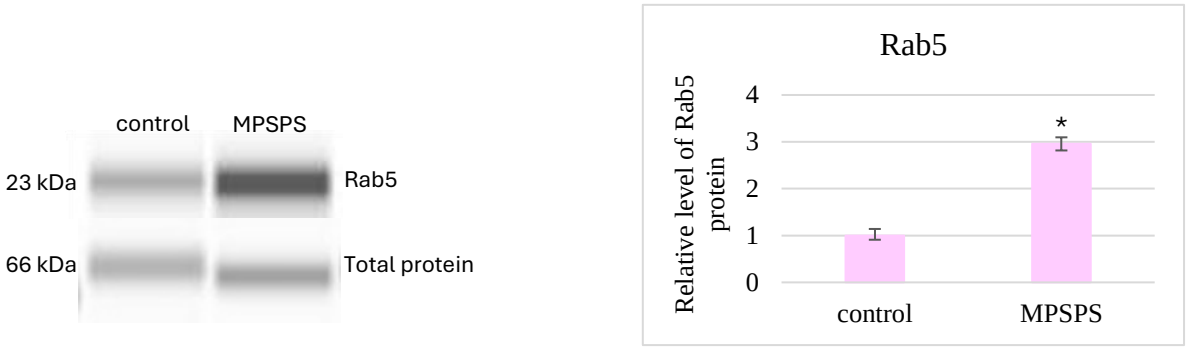

Figure S1. Abundance of the Rab5 protein in MPSPS^{R200P} (marked as MPSPS) and control (the wild-type variant) fibroblasts. Levels of Rab5 were estimated by Western blotting, with quantification performed using densitometry. The analyses were performed in three independent biological replicates, and representative results are presented in the panels. Error bars represent the standard deviation of three independent experimental replicates. Asterisk (*) indicates statistically significant differences (p<0.05) relative to control cells.

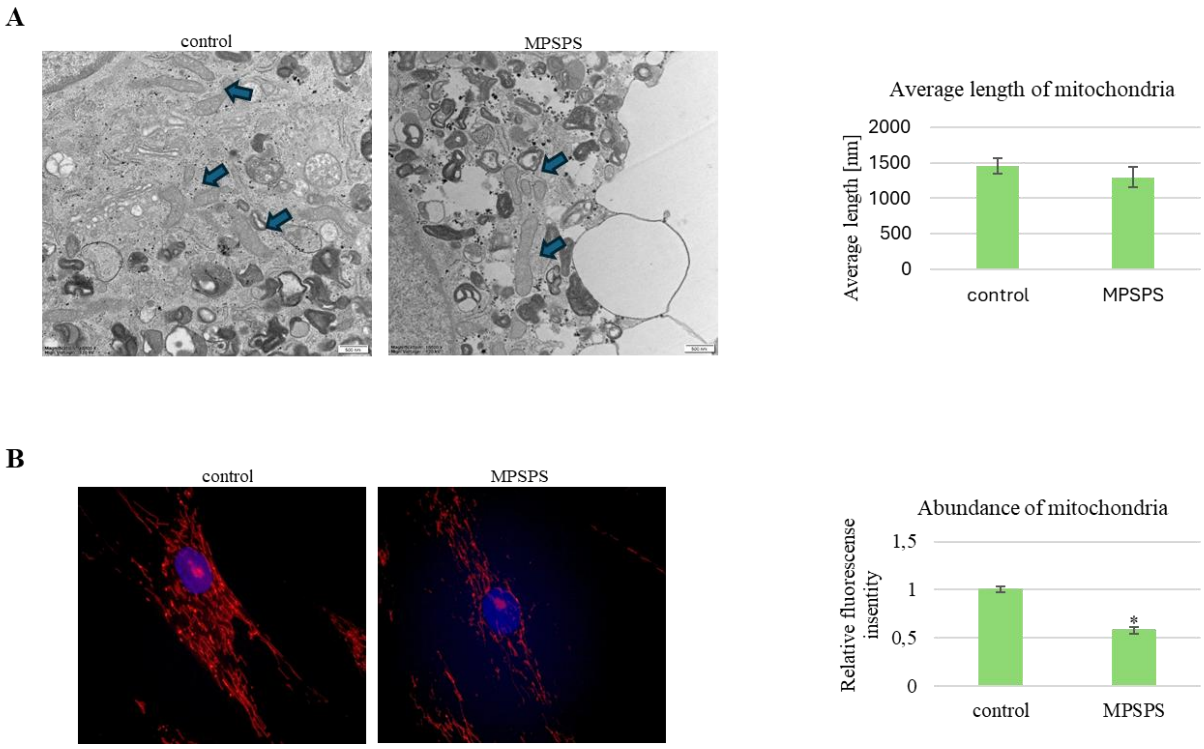

Figure S2. Mitochondrial morphology and abundance in MPSPS^{R200P} (marked as MPSPS) cells compared to control cells. Panel A shows mitochondrial morphology (arrows) in MPSPS^{R200P} fibroblasts compared to control cells. Analyses were made using a Tecani Spirit BioTWIN electron microscope. Panel B shows fluorescence intensity of mitochondria in MPSPS^{R200P} cells compared to control cells. Fibroblasts were stained with MitoTracker Red. Cells were analysed using a Leica CTR 4000 fluorescence microscope. All analyses were performed in three independent biological replicates, and representative results were presented in the panels. Error bars represent the standard deviation of three independent experimental replicates (in electron and fluorescence microscopy experiments, >100 cells were analysed). Asterisks (*) indicate statistically significant differences (p<0.05) relative to control cells.

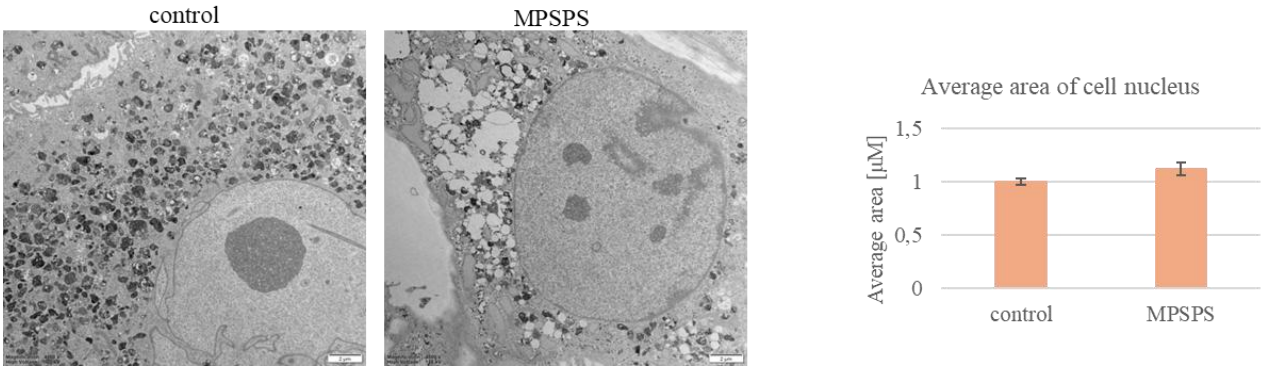

Figure S3. Morphology of nucleus in MPSPS^{R200P} (marked as MPSPS) fibroblasts compared to control cells. Analyses were made using a Tecani Spirit BioTWIN electron microscope. Scale bars denote 2 µm. The analyses were performed in three independent biological replicates, and representative results are presented in the electron micrographs. Error bars represent the standard deviation (>100 cells were analysed). No statistically significant differences (p>0.05) were detected relative to control cells.

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- napisaniu wstępnej wersji manuskryptu;
- udziale w przygotowaniu ostatecznej wersji manuskryptu;
- udziale w dyskusji z recenzentami.

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- udziale w dyskusji z recenzentami.

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polegał na:

- udziale w przeprowadzeniu eksperymentów;
- udziale w przygotowaniu rycin;
- udziale w napisaniu wstępnej wersji pracy.

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Gdańsk, 28.08.2025

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- udziale w zaplanowaniu eksperymentów;
- przygotowaniu ostatecznej wersji manuskryptu;
- dyskusji z recenzentami.

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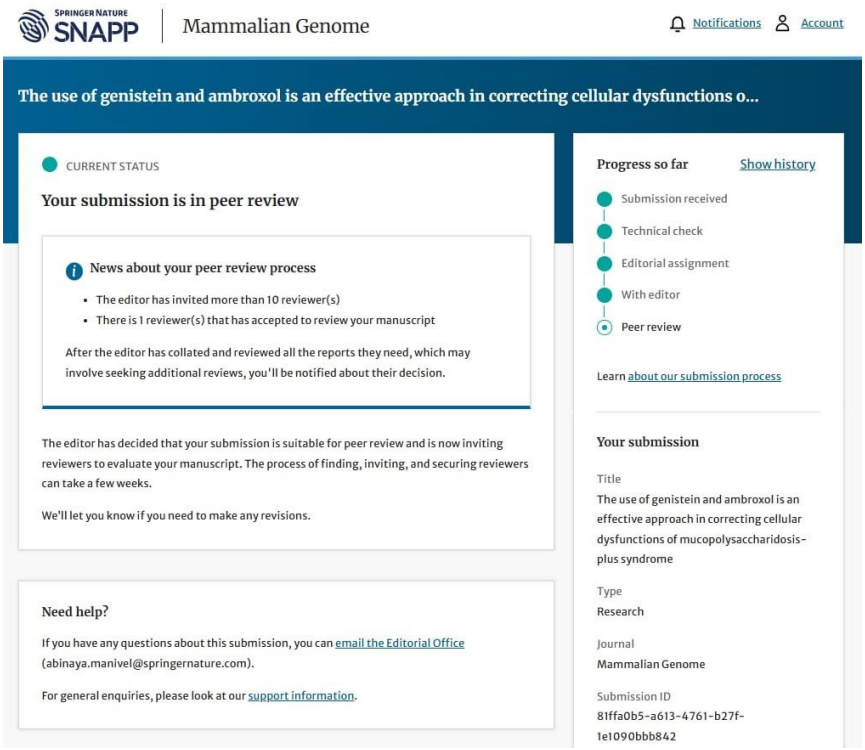

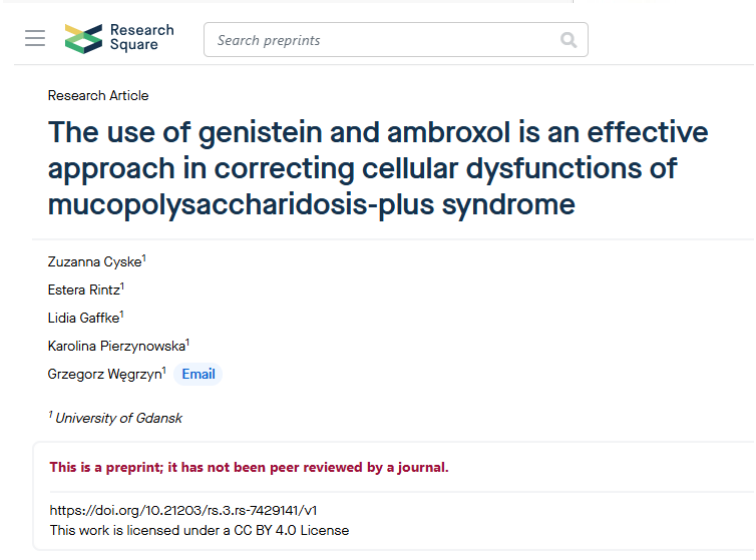

The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome

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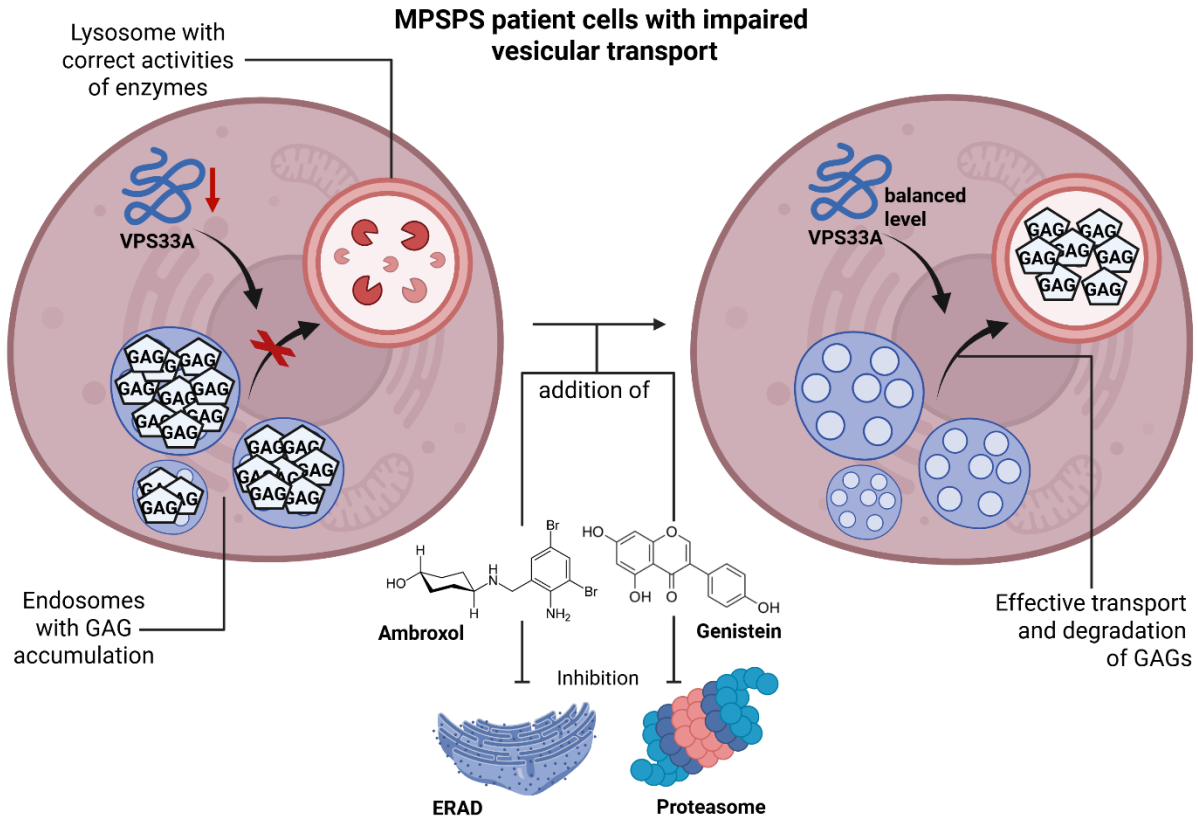
ABSTRACT

Mucopolysaccharidosis-plus syndrome (MPS plus or MPSPS) is an ultrarare inherited metabolic disease, caused by mutations in the *VPS33A* gene. Like in different types of mucopolysaccharidosis (MPS), glycosaminoglycan (GAG) storage in cells of patients is evident. However, unlike MPS, the genetic defects in MPSPS cause impairment in the VPS33A protein level rather than inactivation of lysosomal hydrolases responsible for GAG degradation. Recent works demonstrated that low abundance of mutated VPS33A causes defective endosomal trafficking, resulting in poor delivery of GAGs (and perhaps also other compounds) to lysosomes, preventing their effective turnover. Here, we tested the hypothesis that impairment of protein degradation machineries, proteasomes by genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) and endoplasmic-reticulum-associated protein degradation (ERAD) by ambroxol (4-((2-amino-3,5-dibromophenyl)methylamino) cyclohexan-1-ol), might result in elevation of levels of the mutated, partially active VPS33A and restoration of endosomal trafficking. Using MPSPS patient-derived fibroblasts, we demonstrated that treatment with genistein and ambroxol resulted in elevation of the mutant VPS33A protein level, as well as in improvement or correction of various previously reported cellular defects, including GAG levels, endosomal markers, and cytoskeleton elements. In the light of these results, and since both genistein and ambroxol were previously demonstrated to be safe when used in relatively high amounts, we propose that the use of these compounds, and especially their combination, can be considered as a potential therapeutic approach in MPSPS, which is currently an incurable disease.

KEY WORDS: Mucopolysaccharidosis-plus (MPSPS), VPS33A protein, endosomal trafficking, therapeutic approach, ambroxol, genistein


MPSPS patient cells with impaired vesicular transport
Lysosome with correct activities of enzymes
VPS33A
GAG
Endosomes with GAG accumulation
addition of
Ambroxol
Genistein
Inhibition
ERAD
Proteasome
balanced level
VPS33A
GAG
Effective transport and degradation of GAGs

1. Introduction

Mucopolysaccharidoses (MPS), belonging to lysosomal storage disorders (LSDs), are a group of inherited, progressive diseases caused by a complete deficiency or drastically reduced activity of lysosomal enzymes responsible for the degradation of glycosaminoglycans (GAGs), unbranched complex carbohydrate chains (Nagpal et al. 2022). Undegraded GAGs gradually accumulate within lysosomes, progressively impairing cellular and tissue functions and, over time, the function of the entire organism (Freeze et al. 2015). The activities of GAG-degrading enzymes are highly interdependent, with each enzyme initiating its function only after the preceding one has completed its activity. Consequently, the deficiency of even a single enzyme disrupts the entire degradation pathway. Thirteen types and subtypes of MPS are currently distinguished, classified based on the partially or completely non-functional enzyme involved (Parenti et al. 2015; Trovão de Queiroz et al. 2016; Wiśniewska et al. 2022).

The recently described mucopolysaccharidosis-plus syndrome (MPS plus or MPSPS, the latter abbreviation will be used further in this paper) is among the rarest forms of MPS. To date, only a few dozen cases of MPSPS have been reported worldwide (Faraguna et al. 2022; Lipiński et al. 2022; Cyske et al. 2024a), thus it is an ultrarare disease. Unlike other MPS diseases, MPSPS is caused by mutations in the *VPS33A* gene whose product is not directly involved in GAG degradation (Cyske et al. 2024a). In fact, accumulation of GAGs, specifically heparan sulfate (HS) and dermatan sulfate (DS), occurs in MPSPS, while no reduction in the activity of any known lysosomal enzyme has been demonstrated (Kondo et al. 2017; Dursun et al. 2017; Pavlova et al. 2019; Vasilev et al. 2020). The *VPS33A* gene product (the VPS33A protein) is a polypeptide involved in autophagy and endocytosis processes (Wartosch et al. 2015; Sofronova et al. 2022). This protein interacts with membrane-tethering protein complexes, HOPS and CORVET. Both of these complexes are involved in the regulation of membrane fluxes, necessary for lysosome biogenesis and for the processes of autophagy, endocytosis and phagocytosis (Balderhaar and Ungermann 2013; van der Beek et al. 2019).

The symptoms of MPSPS patients closely resemble those of patients with Hurler syndrome (a severe form of MPS I) (Vasilev et al. 2020). The term "plus" refers to the presence of additional symptoms beyond those commonly observed in MPS, including congenital heart defects, renal dysfunction, and hematopoietic abnormalities (Faraguna et al. 2020). Majority of described MPSPS patients came from Yakutia (Republic of Sakha) and Turkey (Kondo et al.

2017; Dursun et al. 2017), one from the Southern European/Mediterranean region (Pavlova et al. 2022), and one from Poland (Lipiński et al. 2020). Only two pathogenic variants of *VPS33A*, causing MPSPS, have been reported to date. The c.1492C>T (p.Arg498Trp) variant has been identified in the homozygous state in patients originating from Yakutia and Turkey (Kondo et al. 2017; Dursun et al. 2017; Faraguna et al. 2022), whereas the c.599G>C (p.Arg200Pro) variant has likewise been detected in a homozygous configuration in two patients from the Mediterranean region and Poland (Pavlova et al. 2022; Cyske et al. 2024a). Comparative analysis of the clinical manifestations in individuals with MPSPS indicated that the latter variant is associated with a milder, juvenile-onset form of the disease, in contrast to a severe phenotype linked to the former variant (Pavlova et al. 2019; Lipiński et al. 2020; Pavlova et al. 2022).

Studies on the biochemical mechanism of MPSPS demonstrated functional similarities between both pathogenic variants of VPS33A. The affected gene products revealed significantly decreased abundance in cells, irrespective of the presence of either Arg200Pro (Pavlova et al. 2022) or Arg498Trp (Terawaki et al. 2025) amino acid alteration. Moreover, both these VPS33A variants retained their biochemical activities (Pavlova et al. 2022; Terawaki et al. 2025), strongly suggesting that the considerably lowered levels of the protein, rather than its impaired biochemical function, is responsible for the observed pathogenic effects. Very recent reports provided the basis for understanding molecular pathomechanism of MPSPS. Namely, VPS33A deficiency may cause defects in endosomal trafficking, thus preventing effective transportation of GAGs into lysosomes, where they should be degraded (Cyske et al. 2025; Terawaki et al. 2025). In addition, over-acidification of lysosomes was noted in cells bearing the p.Arg498Trp variant of VPS33A (Kondo et al. 2017), suggesting that this defect might further impair GAG degradation. Accumulation of GAGs initiates a cascade of secondary and tertiary changes in cells, leading to further dysfunctions of tissues and organs, and development of specific symptoms (Gaffke et al. 2023; Cyske et al. 2025).

MPSPS is currently an incurable disease. The only proposal of potential therapeutic approach reported to date consists of the suggestion of the use of triclabendazole (5-chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1H-benzimidazole), a compound which is able to down regulate efficiency of transcription of genes coding core proteins which interact with GAGs to form proteoglycans (Terawaki et al. 2025). Since GAGs are attached to the core proteins, one might suspect that decreased production of these proteins should in turn result in reduction of the abundance of GAGs. Despite some efficacy of the use of triclabendazole in MPSPS was

demonstrated (Terawaki et al. 2025), we assumed that such an indirect way of reducing GAG levels can be auxiliary rather than primary treatment. Therefore, we aimed to find drug(s) which could correct primary molecular defects in MPSPS cells. Since the major problem in these cells appears to be low abundance of the mutant VPS33A protein due to its enhanced proteolytic degradation (Pavlova et al. 2022; Terawaki et al. 2025), our intention was to impair this process. However, since MPSPS is an inherited disease, any potential pharmacological treatment of patients should be lifelong. This implies that the inhibition of protein degradation in MPSPS must by relatively gentle to avoid severe adverse effects (caused by plausible impaired decay of many other proteins), especially during many years of the therapeutic procedure. In this light, we hypothesized that two compounds, genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) and ambroxol (4-((2-amino-3,5-dibromophenyl)methylamino)cyclohexan-1-ol), might be potentially effective drugs. Apart from many other potentially beneficial activities of these compounds, like antioxidative and anti-inflammatory properties, as well as autophagy stimulation (Dhanve et al. 2023; Cyske et al. 2024b; Ponugoti et al. 2024; Borah et al. 2025; Goswami et al. 2025; Pierzynowska et al. 2025), genistein was demonstrated to inhibit proteasomal degradation through modulating protein ubiquitination (Pierzynowska et al. 2020) and ambroxol was shown to reduce the endoplasmic stress and endoplasmic-reticulum-associated protein degradation (ERAD) (Bendikov-Bar et al. 2011; Dhanve et al. 2023). Moreover, both genistein (Kim et al. 2013; Ghosh et al. 2021) and ambroxol (Ponugoti et al. 2024) were demonstrated to be safe in a long-time clinical use, with ambroxol being a registered drug. Therefore, in this work we tested efficacy of genistein and ambroxol in correction of cellular defects observed previously in MPSPS. As a biological model, fibroblasts derived from an MPSPS patient, homozygous for the c.599G>C (p.Arg200Pro) variant of *VPS33A*, were used along with control fibroblasts.

2. Materials and methods

2.1. Cell lines and culture conditions

This investigation utilized a fibroblast cell line derived from dermal tissue, isolated from a patient diagnosed with MPSPS, being homozygous for the c.599G>C (p.Arg200Pro) variant of the *VPS33A* gene. This patient had been previously characterized in the literature (Lipiński et al. 2022). As a control, a dermal fibroblast line obtained from a healthy donor of matched sex and comparable age was employed. Ethical approval for the study was granted by the Bio-

Ethical Committee of the Children's Memorial Health Institute in Warsaw, Poland (approval no. 23/KBE/2020).

Cell cultures were maintained in Gibco Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and an Antibiotic-Antimycotic solution (Thermo Fisher Scientific), under standard incubation conditions (37°C, 5% CO_2, and 95% relative humidity). When indicated, genistein and/or ambroxol were added to final concentrations of 50 μM and 100 μM, respectively, as demonstrated previously for reductions of proteasomal (Pierzynowska et al. 2020) and ERAD activities (Bendikov-Bar et al. 2011) at these levels of the investigated molecules. Chloroquine was added to final concentration of 10 μM in some experiments.

2.2. Cell Viability Assay

To evaluate the effects of genistein (50 μM), ambroxol (100 μM), and their combination on cell viability, cells (3×10^3 per well) were seeded into 96-well plates and allowed to adhere overnight under standard conditions. The following day, cells were treated for 24 h with either genistein, ambroxol, both compounds simultaneously, or appropriate controls. Control groups included cells treated with an equivalent volume of DMSO (vehicle control for genistein) or untreated cells (control for ambroxol and combination treatments).

After treatment, 25 μl of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (4 mg/ml) was added to each well, followed by a 3-h incubation at 37°C. Formazan crystals formed in viable cells were solubilized in 100 μl of DMSO. Absorbance was measured at 570 nm with a reference wavelength of 660 nm using the Perkin Elmer EnSpire 2300 Multilabel Multimode Plate Reader.

Each experimental condition was performed in at least three independent biological replicates. Results were analyzed using one-way ANOVA in GraphPad Prism software.

2.3. Western-blotting

Fibroblasts (5×10^5 cells) were seeded onto 10 cm culture dishes and incubated overnight under standard conditions (see Section 2.1). The next day, cells were treated with DMSO (0.1%, as a solvent control), genistein (50 μM), ambroxol (100 μM) or their

combination. After 24-h incubation, cell lysates were prepared as previously described (Rintz et al. 2023). The lysate was divided into two fractions for protein analysis.

One fraction was used for automated Western blotting employing the WES system (Simple Western; ProteinSimple, San Jose, CA, USA), which utilizes capillary electrophoresis. Proteins (including Rab5, Rab7, VPS33A, and VPS16) were separated using the 12–230 kDa separation module (#SM-W003) and detected in-capillary using specific primary antibodies: anti-Rab5 (#3547, Cell Signaling), anti-Rab7 (D95F2, #9367, Cell Signaling), anti-VPS33A (#16896-1-AP, ProteinTech), and anti-VPS16 (#17776-1-AP, ProteinTech). Detection was performed using either the Anti-Mouse (#DM-002) or Anti-Rabbit (#DM-001) detection module, in accordance to the manufacturer's protocol. The Total Protein module (#DM-TP01) was employed as a loading control. Note that in this automated, capillary electrophoresis-based system, the same loading control module (Total Protein) was used for all samples (to detect different specific proteins).

The remaining fraction was analyzed using conventional Western blotting. Proteins EEA1 and LC3 were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% non-fat dry milk in PBST (PBS with 0.1% Tween-20). Membranes were incubated overnight at 4°C with primary antibodies: anti-EEA1 (#3288S, Cell Signaling) and anti-LC3 (G-4, #sc-398822, Santa Cruz Biotechnology). After two washes in PBST, membranes were incubated for 2 h at room temperature in the dark with HRP-conjugated secondary antibodies (anti-rabbit: #A0545; anti-mouse: #AP124P; Sigma-Aldrich). As a loading control, β-actin was detected using anti-β-actin−peroxidase antibody (mouse monoclonal**;** #A3854 Sigma-Aldrich). After blocking with 5% non-fat dry milk in PBST, membrane was incubated with anti-B-actin antibody for 1 h. Protein bands were visualized by chemiluminescence following incubation with horseradish peroxidase (HRP) substrate and exposure to X-ray film.

2.4. Detection of heparan sulphate levels by dot-blotting

Cellular levels of heparan sulfate (HS) were quantified using dot blot analysis, as previously described (Gaffke et al. 2023). Briefly, fibroblast lysates were prepared using a Tissue Protein Extraction Reagent (T-PER; #78510 Sigma). Protein extracts were applied to a PVDF membrane (#IPFL00010, Millipore) using a dot-blot apparatus (Bio-Rad, Hercules, CA, USA). Following application, membranes were blocked with 5% (w/v) non-fat dry milk in PBST (PBS with 0.1% Tween-20) and incubated overnight at 4°C with a primary anti-HS

antibody (mouse monoclonal, #NBP2-23523, Novus). After washing with PBST, membranes were incubated for 2 hour at room temperature with an HRP-conjugated anti-mouse secondary antibody (#A9044, Sigma-Aldrich). Signal detection was performed using a chemiluminescent HRP substrate (Merck, Darmstadt, Germany), followed by exposure to X-ray film. Signal intensities were quantified using QuantityOne software, and the results were normalized to total protein content, assessed by Ponceau S staining.

2.5. Fluorescence microscopy

A total of 4×10^4 cells were seeded onto 12-well culture plates containing sterile coverslips and incubated overnight under standard conditions (see Section 2.1). The next day, cells were treated with DMSO (0.1%, as a solvent control), genistein (50 μM), ambroxol (100 μM) or their combination. After 24-h incubation, cells were stained with LysoTracker (#L7528, Thermo Fisher), or CellMask™ Green Actin Tracking Stain (#A57243, Thermo Fisher) for 1 h. Subsequently, cells were fixed using 2% paraformaldehyde in the presence of 0.1% Triton X-100 and mounted with DAPI-containing medium.

Alternatively, after overnight incubation, cells were fixed with 2% paraformaldehyde for 15 min, rinsed with PBS, and permeabilized with 0.1% Triton X-100 for additional 15 min. Following five PBS washes, cells were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. Primary antibodies, either individually or in combination for colocalization studies, were then applied and incubated overnight at 4°C. The antibodies used included: anti-heparan sulfate (#NBP2-23523, Novus), anti-LAMP1 (#46843, Cell Signaling), anti-LAMP2 (#49067S, Cell Signaling), anti-EEA1 (#3288S, Cell Signaling), anti-Rab7 (D95F2, #9367, Cell Signaling), anti-Rab5 (#3547, Cell Signaling), anti-VPS16 (#17776-1-AP, ProteinTech) and anti-VPS33A (#16896-1-AP, ProteinTech). Following incubation, cells were washed five times with PBS and incubated for 2 h at 4°C in the dark with the appropriate fluorophore-conjugated secondary antibodies (anti-rabbit and/or anti-mouse; #4413S and #4408S, Cell Signaling), depending on the primary antibody combination used. After a final series of five PBS washes, coverslips were mounted onto glass slides using ProLong™ Gold Antifade Mountant with DAPI (#P36935, Invitrogen). Fluorescence imaging was performed using an Olympus IX83 microscope, with data acquisition and analysis conducted via the software provided by the manufacturer.

To assess the intracellular colocalization of HS with either LysoTracker Red or investigated proteins, a combined staining protocol was employed, integrating elements from the two previously described methods. Fibroblasts (4×10^4) were seeded onto 12-well plates containing sterile coverslips and incubated overnight under standard culture conditions (see Section 2.1). The next day, cells were treated with DMSO (0.1%, as a solvent control), genistein (50 μM), ambroxol (100 μM) or their combination. After 24-h incubation, cells were incubated with LysoTracker Red (#L7528, Thermo Fisher) for 1 h, after which they were fixed using 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. After five washes with PBS, cells were blocked with 5% bovine serum albumin (BSA) for 1 hour. Subsequently, cells were incubated for 2 h at 4°C anti-heparan sulfate (#NBP2-23523, Novus). In the case of colocalization of HS with different proteins, the above mentioned antobody was combined with anti-LAMP1 (#46843, Cell Signaling), anti-LAMP2 (#49067S, Cell Signaling), anti-EEA1 (#3288S, Cell Signaling) and anti-VPS33A (#16896-1-AP, ProteinTech). After primary antibody incubation, fibroblasts were washed five times with PBS and incubated with secondary antibodies (anti-rabbit and anti-mouse; #4413S and #4408S, Cell Signaling) for 1 h at 4°C in the dark. Following another series of five PBS washes, coverslips were mounted onto glass slides using ProLong™ Gold Antifade Mountant with DAPI (#P36935, Invitrogen). Fluorescence imaging was performed using an Olympus fluorescence microscope. Colocalization analysis was conducted using the CellSens 4.3 software, in accordance with the methodology described earlier (Adler and Parmryd 2013).

2.6. Statistical analysis

Biochemical analyses were performed using samples from a minimum of three independent experiments. Microscopy-based analyses were conducted by evaluating at least 100 individual cells per condition. Quantitative data are presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were considered statistically significant at $p<0.05$.

3. Results and discussion

3.1. Safety of genistein and ambroxol to MPSPS cells and effects of these compounds on abundance of VPS33A and VPS16 proteins

To investigate the potential usefulness of genistein and ambroxol in treatment of MPSPS, we have used fibroblasts derived from a patient being a homozygote in the *VPS33A* pathogenic variant c.599G>C (p.Arg200Pro), and a control fibroblast line, as described previously (Cyske et al. 2025). The concentrations of tested compounds (50 μM genistein and 100 μM ambroxol) were chosen on the basis of previous reports indicating partial inhibition of proteasome and ERAD activities by these small molecules (Bendikov-Bar et al. 2011; Pierzynowska et al. 2020).

For any pharmacological treatment of patients with inherited diseases, it is crucial to determine safety of tested compounds, as in such cases, a livelong therapy should be expected. Therefore, despite previously demonstrated safety of both genistein and ambroxol in clinical trials with patients suffering from other diseases (Kim et al. 2013; Ghosh et al. 2021; Ponugoti et al. 2024), we tested the viability of MPSPS cells cultured in the presence of these compounds. Using the MTT test, we confirmed that genistein at 50 μM and ambroxol at 100 μM did not significantly affect viability of investigated fibroblast lines (Figure 1A).

Since the rationale of this study was to elevate levels of the p.Arg200Pro variant of VPS33A and assess effects of such an increase in the abundance of this protein, its levels were determined in control cells and in MPSPS fibroblasts either untreated or treated with genistein and/or ambroxol. As demonstrated in Figure 1B, abundance of VPS33A was over 4-fold decreased in MPSPS cells relative to control cells, however, treatment of MPSPS fibroblasts with genistein, ambroxol or both compounds resulted in significantly increased levels of the tested protein. The most effective increase was observed for the mixture of genistein and ambroxol, although even in this case, normalization of the VPS33A level was not achieved, reaching about 75% of the control value (Figure 1B). These results were confirmed by fluorescence microscopy study, where VPS33A was detected using a specific antibody. Again, treatment with a combination of 50 μM genistein and 100 μM ambroxol was the most effective in increasing the level of VPS33A (Figure 2). These results strongly suggest that genistein and ambroxol elevate levels of the p.Arg200Pro variant of VPS33A due to reduction of activity of proteasomes or ERAD,

respectively, as such biological effects of these compounds were demonstrated previously (Bendikov-Bar et al. 2011; Pierzynowska et al. 2020).

Interestingly, decreased levels of the VPS16 protein, which interacts with VPS33A, were also observed previously in MPSPS cells (Cyske et al. 2025). We have confirmed that observation, while indicating that treatment of MPSPS fibroblasts with genistein and ambroxol results in increasing abundance of VPS16. This was demonstrated in experiments with the use of Western-blotting (Figure 1C) and fluorescence microscopy (Figure 3). Both VPS33A and VPS16 proteins are components of the HOPS and CORVET complexes, which are involved in endocytosis (Balderhaar and Ungermann 2013). Therefore, as the investigated proteins functionally interact, they might influence each other's levels.

3.2. Genistein and ambroxol improve GAG metabolism in MPSPS cells

Accumulation of GAGs is one of the most pronounced biochemical defects observed in MPSPS cells (Kondo et al. 2017). Therefore, effects of treatment of MPSPS fibroblasts with genistein and/or ambroxol on GAG levels and localization were tested. HS was investigated as an exemplary GAG known to accumulate in MPSPS (Kondo et al. 2017), and its levels were estimated by dot-blotting (Figure 4) and fluorescence microscopy (Figure 5, with quantitative analysis shown in Figure 6A). We found that while HS was significantly (about 3-4 times) more abundant in MPSPS cells than in control fibroblasts, treatment with 50 μM genistein and 100 μM ambroxol caused a considerable decrease or even normalization of levels of this GAG (Figures 4-6). However, when MPSPS cells were also treated with 10 μM chloroquine, an inhibitor of the autophagosomal-lysosomal system, high abundance of HS was still observed, irrespective of the presence or absence of genistein and ambroxol (Figure 4B). These results demonstrate that degradation of HS in MPSPS cells under conditions of partially restored abundance of VPS33A, due to actions of genistein and ambroxol, occurs through the lysosomal pathway.

Fluorescence microscopy analyses using LysoTracker Red dye, which binds specifically to lysosomes, revealed significantly higher fluorescence intensity in MPSPS cells, as well as a significantly increased number of lysosomes in MPSPS cells, compared to control cells (Figure 5, with quantitative analysis shown in Figure 6B). These observations suggest that lysosomes are affected in MPSPS. The use of genistein (50 μM), ambroxol (100 μM), and a

mixture of these compounds resulted in reduction of lysosome numbers, while the combination of both compounds was the most effective in reducing fluorescence intensity (Figure 6B).

To confirm improved transportation of GAGs into lysosomes in MPSPS cells treated with genistein and ambroxol, we have performed colocalization experiments. Using fluorescence microscopy and specific anti-HS antibody and LysoTracker Red, we found that treatment with genistein and ambroxol resulted in significantly enhanced colocalization of HS with lysosomes in MPSPS (Figure 5, with quantitative analysis shown in Figure 6C). Moreover, enhanced colocalization signals of HS and LAMP2 protein, specific for lysosomes, were detected in MPSPS fibroblasts in the presence of 50 μM genistein and 100 μM ambroxol relative to untreated MPSPS cells (Figure 7).

Previous study demonstrated that GAGs appear to be trapped in endosomes, due to dysfunctional trafficking (Cyske et al. 2025). Indeed, significantly more pronounced colocalization of HS and the endosomal protein EEA1 in MPSPS cells relative to control ones was evident (Figure 8). However, treatment of MPSPS fibroblasts with 50 μM genistein and 100 μM ambroxol normalized this parameter which was then undistinguishable from that measured in control cells (Figure 8), strongly suggesting that under such conditions the endosomal trafficking is considerably improved. This conclusion was corroborated by another experiment, in which colocalization of HS with the LAMP1 protein in MPSPS cells was determined. LAMP1 occurs in both lysosomes and in early and late endosomes (Cook et al. 2024). Hence, increased colocalization of HS and LAMP1 in MPSPS relative to control cells, together with decreasing of this parameter after treatment with genistein and amboxol (Figure 9) is compatible with the proposal that impaired endosomal trafficking in MPSPS cells, resulting in defective transportation of GAGs which are stuck in endosomes, can be restored by treatment with genistein and ambroxol which cause elevation of the VPS33A levels.

3.3. Improving levels of endosomal marker proteins in MPSPS cells by treatment with genistein and ambroxol

Previous reports pointed to impaired endosomal trafficking in MPSPS cells mostly on the basis of elevated levels of endosomal marker proteins, like EEA1, Rab7 and Rab5 (Pavlova et al. 2022; Cyske et al. 2025). EEA1 (early endosome antigen 1) participates in the processes of early endosome sorting and fusion of early and late endosomes (Yap and Winckler 2022). Rab7 takes part in late endosomal trafficking and it is required for efficient delivery of cargo to

lysosomes (Marwaha et al. 2017; Mulligan and Winckler 2023). Rab5 operates at the stage of maturation of endosomes during the trafficking (Nagano et al. 2019). Therefore, increased abundance of these proteins indicates defects in the above mentioned process and problems with delivery of cargo to lysosomes.

Using Western-blotting and fluorescence microscopy techniques, we have confirmed that elevated levels of EEA1 (Figures 8 and 10), Rab7 (Figures 11 and 12) and Rab5 (Figures 13 and 14) occur in MPSPS fibroblasts relative to control cells. Nevertheless, addition of 50 μM genistein and 100 μM ambroxol to MPSPS cell cultures either normalized (in the case of EEA1 and Rab7) or improved (in the case of Rab5) abundance of endosomal marker proteins (Figures 8 and 10-14). These results confirmed again that by elevating levels of the p.Arg200Pro variant of VPS33A, genistein and ambroxol can significantly improve endosomal trafficking which promotes effective lysosomal degradation of GAGs (and perhaps other compounds), otherwise stuck in endosomes of MPSPS cells.

3.4. Changes in the actin cytoskeleton in MPSPS cells and effects of genistein and ambroxol

Although absolute levels of β-actin did not vary significantly between MPSPS and control fibroblasts (Figure 10), it was demonstrated recently that the organization of cytoskeleton was impaired in the mutant cells, as estimated by ineffective formation of F-actin filaments (Cyske et al. 2025). Here, we confirmed this cellular deficiency, but also demonstrated that cultivation of MPSPS cells in the presence of 50 μM genistein and 100 μM ambroxol could restore normal morphology of these structures (Figure 15). Therefore, it appears that defective endosomal trafficking might indirectly result also in formation of abnormally changed actin cytoskeleton, which could be corrected by genistein and ambroxol. This explanation seems plausible as F-actin (actin filaments) could be demonstrated to be involved in various steps of endosomal trafficking (Capitani et al. 2021), thus, a considerable interplay between them in MPSPS cells appears likely.

3.5. The autophagy marker LC3-II in MPSPS cells

It was demonstrated recently that levels of the autophagy marker, the form II of the LC3 protein (called LC3-II), is elevated in MPSPS caused by the c.599G>C (p.Arg200Pro) variant of *VPS33A* (Cyske et al. 2025), while the autophagy process appeared unchanged in cells

bearing the homozygous c.1492C>T (p.Arg498Trp) variant (though it was significantly impaired in cells of *VPS33A* knock-out mice) (Terawaki et al. 2025). Since both genistein and ambroxol were reported previously as autophagy stimulators (Pierzynowska et al. 2018; Choi et al. 2018), we investigated if these compounds can modulate levels of the autophagy marker.

Using Western-blotting, we found that 50 μM genistein and 100 μM ambroxol considerably elevated levels of LC3-II in MPSPS cells bearing the c.599G>C (p.Arg200Pro) allele of *VPS33A* in a homozygous configuration, suggesting that autophagy is efficiently stimulated in these cells (Figure 16). Since lysosomal degradation is crucial for eliminating the storage material from MPSPS cells (see Section 3.2), we suggest that stimulation of autophagy by genistein and ambroxol might be an additional advantage in treatment of the disease by these compounds.

4. Conclusions

Genistein and ambroxol have been demonstrated to elevate levels of the p.Arg200Pro variant of VPS33A (most probably through reduction of proteasomal and ERAD activities) which are otherwise severely lowered in MPSPS cells. This increase in VPS33A abundance leads to improved endosomal trafficking and more effective delivery of cargo (including GAGs) to lysosomes, allowing efficient degradation of the storage material. Moreover, secondary cellular defects, like disturbed formation of actin microfilaments (F-actin), can be normalized after treatment with genistein and ambroxol. Autophagy stimulation by these compounds might be an additional advantage, leading to more efficient elimination of accumulated GAGs (and perhaps other macromolecules). Despite some limitations of this study, including the use of only a cellular model and employing fibroblasts bearing only one of two known pathogenic variants of *VSP33A* causing MPSPS (due to poor availability of biological material arising from an extremely low number of patients), results presented in this work indicate that the use of genistein and ambroxol (especially a mixture of these molecules) might be effective in eliminating cellular defects occurring in MPSPS. Therefore, we suggest that further studies focused on assessing efficacy of this therapeutical approach in *in vivo* tests with animal model(s) and in clinical trials are substantiated, especially because safety of long-term use of both genistein and ambroxol was previously confirmed in trials with patients.

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DECLARARIONS

COMPETING INTEREST

The authors have no relevant financial or non-financial interests to disclose.

THE USE OF AI

No generative AI or AI-assisted technologies were used to prepare this paper.

AUTHOR CONTRIBUTIONS

Conceptualization, ZC and GW; methodology, ZC, ER and GW; investigation, ZC, ER, LG, KP; writing–original draft preparation, ZC and GW; writing–review and editing, ZC, ER, LG, KP, GW; visualization, ZC, ER, LG; supervision, GW; project administration, ZC and GW; funding acquisition, ZC and GW. All authors contributed to the article and approved the submitted version.

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ETHICS APPROVAL

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Bio-Ethical Committee of the Children's Memorial Health Institute (Warsaw, Poland; decision no. 23/KBE/2020).

CONSENT TO PARTICIPATE

Informed consent was obtained from parents of the patients being donors of the biological material used in the study.

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FIGURE LEGENDS

Figure 1. Relative viability of MPSPS fibroblasts (A) and abundance of VPS33A (B) and VPS16 (C) proteins in these cells, either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) for 24 h. The viability of cells (A) was assessed in the MTT test, and levels of proteins (B and C) were estimated by Western-blotting (representative results are shown in upper parts of panels; total protein module was used as a loading control), with quantification performed using densitometry (lower panel). The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 100% (A) or 1 (B and C), and other values reflect these values. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbols ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 2. Abundance of the VPS33A protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using VPS33A-specific antibody (representative results are shown in upper panel; bars represent 10 μm), with quantification performed using a software provided by the microscope manufacturer (lower panel). At least 100 cells were analyzed in each experimental variant. The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 3. Abundance of the VPS16 protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM

ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using VPS33A-specific antibody (representative results are shown in upper panel; bars represent 10 μm), with quantification performed using a software provided by the microscope manufacturer (lower panel). At least 100 cells were analyzed in each experimental variant. The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 4. Levels of heparan sulfate (HS) in control and MPSPS fibroblasts either untreated (A) or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) (B); in addition, in some experiments, chloroquine (ChQ) was added to 10 μM. The HS abundance was estimated by dot-blotting (representative results are shown), with quantification performed using densitometry (panels with columns). The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean values obtained for either control cells (upper panel) or untreated MPSPS cells (lower panel) were considered as 1, and other values reflect these values. In panel B, the horizontal line represents the value obtained for control (healthy) cells, as assessed in the experiment shown in panel A. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control (A) or untreated MPSPS (B) cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 5. Levels of heparan sulfate (HS), abundance of lysosomes (LYSO), and their colocalization in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using HS-specific antibody and Lyso-Tracker. Representative results are shown; bars represent 10 μm.

692 **Figure 6.** Quantification of heparan sulfate (HS) levels (A), abundance lysosomes' (B), and
693 their colocalization (C) in control and MPSPS fibroblasts either untreated or treated with
694 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or
695 a mixture of these two compounds (G50+AMB100), estimated by fluorescence microscopy as
696 shown in Figure 5. The analyses were performed using a software provided by the microscope
697 manufacturer. At least 100 cells were analyzed in each experimental variant. The quantitative
698 results are presented as mean values from 3 independent experiments, with error bars
699 indicating SD. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) -
700 relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to
701 MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with
702 ambroxol.

703

704 **Figure 7.** Colocalization of heparan sulfate (HS) and LAMP2 in control and MPSPS
705 fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM
706 genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds
707 (G50+AMB100) as estimated by fluorescence microscopy using HS- and LAMP2-specific
708 antibodies (representative results are shown in upper panel; bars represent 10 μm), with
709 quantification performed using a software provided by the microscope manufacturer (lower
710 panel). At least 100 cells were analyzed in each experimental variant. The quantitative results
711 are presented as mean values from 3 independent experiments, with error bars indicating SD.
712 Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control
713 cells; hashtag (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with
714 genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

715

716 **Figure 8.** Abundance of the EEA1 protein and its colocalization with heparan sulfate (HS) in
717 control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for
718 genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two
719 compounds (G50+AMB100) as estimated by fluorescence microscopy using EEA1- and HS-
720 specific antibodies (representative results are shown in upper panel; bars represent 10 μm),
721 with quantification performed using a software provided by the microscope manufacturer
722 (lower panels). At least 100 cells were analyzed in each experimental variant. The quantitative
723 results are presented as mean values from 3 independent experiments, with error bars

724 indicating SD. Statistically significant differences (p<0.05) are indicated by asterisks (*) -
725 relative to control cells; hashtag (#) - to untreated MPSPS cells, dollar symbol ($) - to
726 MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with
727 ambroxol.

728

729 **Figure 9.** Colocalization of heparan sulfate (HS) and LAMP1 in control and MPSPS
730 fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM
731 genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds
732 (G50+AMB100) as estimated by fluorescence microscopy using HS- and LAMP1-specific
733 antibodies (representative results are shown in upper panel; bars represent 10 μm), with
734 quantification performed using a software provided by the microscope manufacturer (lower
735 panel). At least 100 cells were analyzed in each experimental variant. The quantitative results
736 are presented as mean values from 3 independent experiments, with error bars indicating SD.
737 Statistically significant differences (p<0.05) are indicated by asterisks (*) - relative to control
738 cells; hashtag (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with
739 genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

740

741 **Figure 10.** Abundance of the EEA1 protein in control and MPSPS fibroblasts either untreated
742 or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM
743 ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by
744 Western-blotting (representative results are shown in upper panel; β-actin was used as a
745 loading control), with quantification performed using densitometry (lower panel). The
746 quantitative results are presented as mean values from 3 independent experiments, with error
747 bars indicating SD. The mean value obtained for control cells was considered as 1, and other
748 values reflect this value. Statistically significant differences (p<0.05) are indicated by
749 asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol
750 ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with
751 ambroxol.

752

753

Figure 11. Abundance of the Rab7 protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by Western-blotting (representative results are shown in upper panel; total protein module was used as a loading control), with quantification performed using densitometry (lower panel). The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 12. Abundance of the Rab7 protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for tested compounds), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using Rab7-specific antibody (representative results are shown in upper panel; bars represent 10 μm), with quantification performed using a software provided by the microscope manufacturer (lower panel). At least 100 cells were analyzed in each experimental variant. The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 13. Abundance of the Rab5 protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for tested compounds), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by Western-blotting (representative results are shown in upper panel; total protein module was used as a loading control), with quantification performed using densitometry (lower panel). The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and

other values reflect this value. Statistically significant differences (p<0.05) are indicated by asterisks (*) - relative to control cells; hashtag (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 14. Abundance of the Rab5 protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for genistein), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using Rab5-specific antibody (representative results are shown in upper panel; bars represent 10 μm), with quantification performed using a software provided by the microscope manufacturer (lower panel). At least 100 cells were analyzed in each experimental variant. The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences (p<0.05) are indicated by asterisks (*) - relative to control cells; hashtag (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 15. Actin microfilaments (F-actin) in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for tested compounds), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by fluorescence microscopy using actin-specific antibody (representative results are shown in upper panel; bars represent 10 μm), with quantification of fluorescence signals performed using a software provided by the microscope manufacturer (lower panel). At least 100 cells were analyzed in each experimental variant. The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences (p<0.05) are indicated by asterisks (*) - relative to control cells; and hashtag (#) - to untreated MPSPS cells.

Figure 16. Abundance of the LC3-II protein in control and MPSPS fibroblasts either untreated or treated with 0.1% DMSO (solvent for tested compounds), 50 μM genistein (G50), 100 μM ambroxol (AMB100) or a mixture of these two compounds (G50+AMB100) as estimated by Western-blotting (representative results are shown in upper panel; β-actin was used as a loading control), with quantification performed using densitometry (lower panel). The quantitative results are presented as mean values from 3 independent experiments, with error bars indicating SD. The mean value obtained for control cells was considered as 1, and other values reflect this value. Statistically significant differences ($p<0.05$) are indicated by asterisks (*) - relative to control cells; hashtags (#) - to untreated MPSPS cells, dollar symbol ($) - to MPSPS cells treated with genistein; and ampersand (&) - to MPSPS cells treated with ambroxol.

Figure 1.

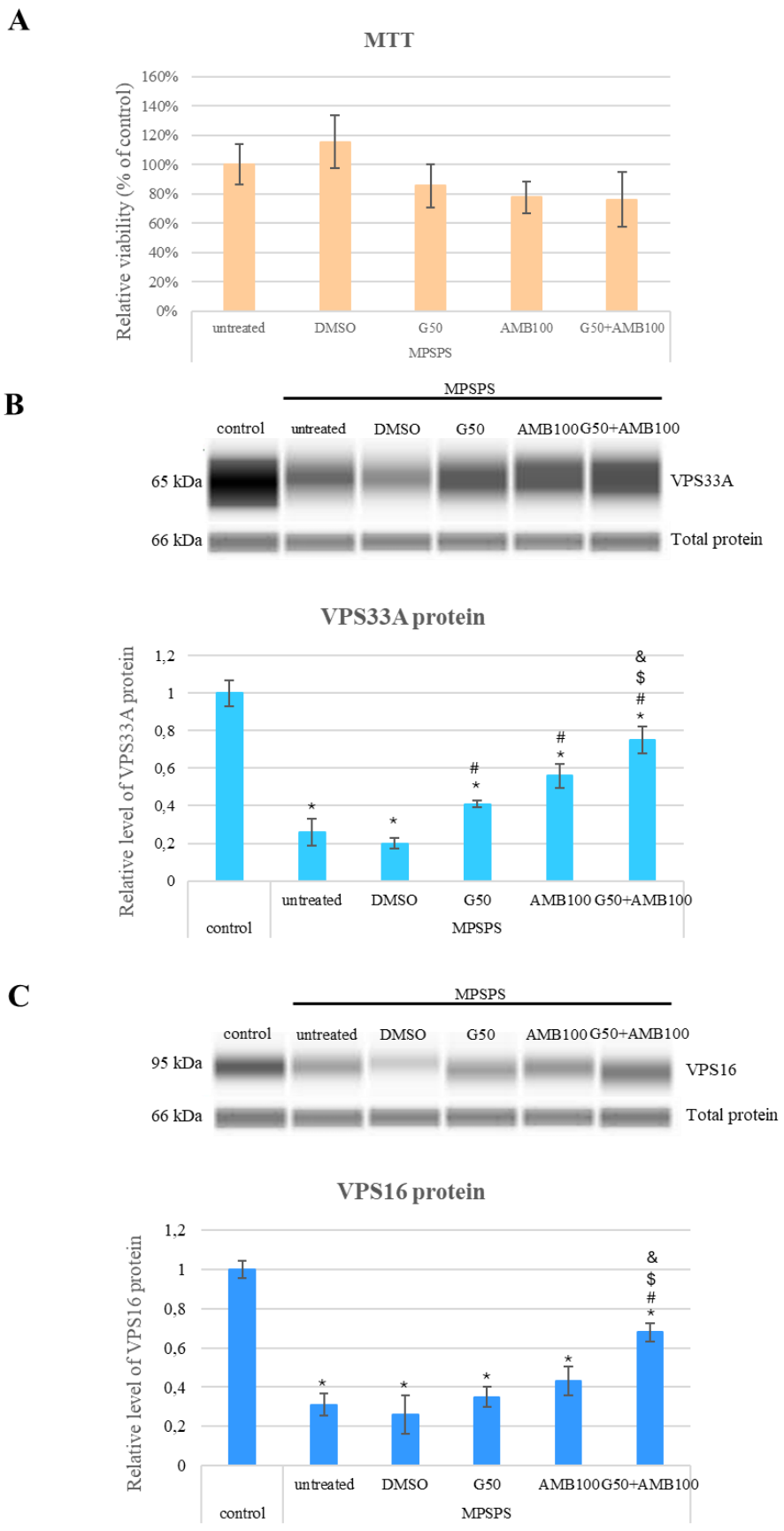

A
MTT
Relative viability (% of control)
160%
140%
120%
100%
80%
60%
40%
20%
0%
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS
B
MPSPS
control
untreated
DMSO
G50
AMB100
G50+AMB100
65 kDa
VPS33A
66 kDa
Total protein
VPS33A protein
Relative level of VPS33A protein
1,2
1
0,8
0,6
0,4
0,2
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS
C
MPSPS
control
untreated
DMSO
G50
AMB100
G50+AMB100
95 kDa
VPS16
66 kDa
Total protein
VPS16 protein
Relative level of VPS16 protein
1,2
1
0,8
0,6
0,4
0,2
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 2.

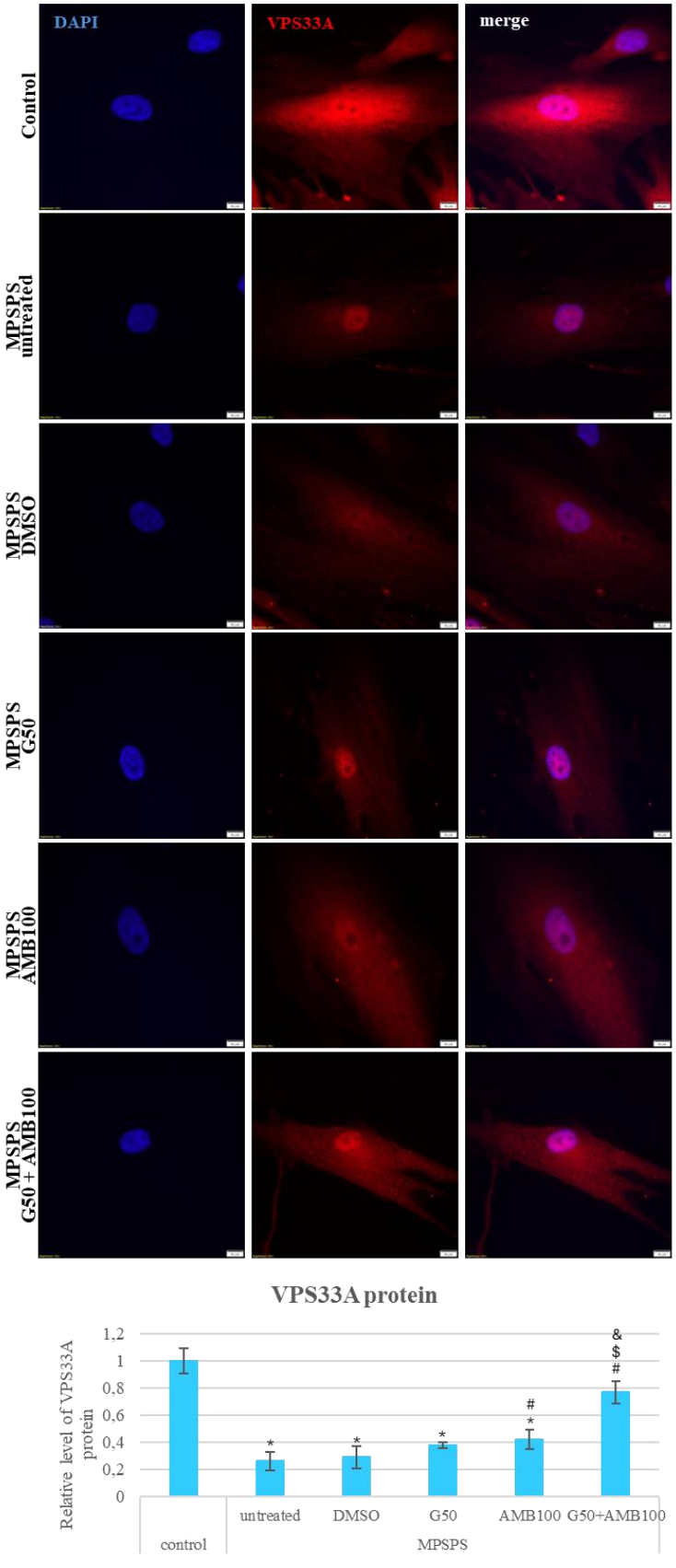

DAPI
VPS33A
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50 + AMB100
VPS33A protein
Relative level of VPS33A protein
1,2
1
0,8
0,6
0,4
0,2
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 3.

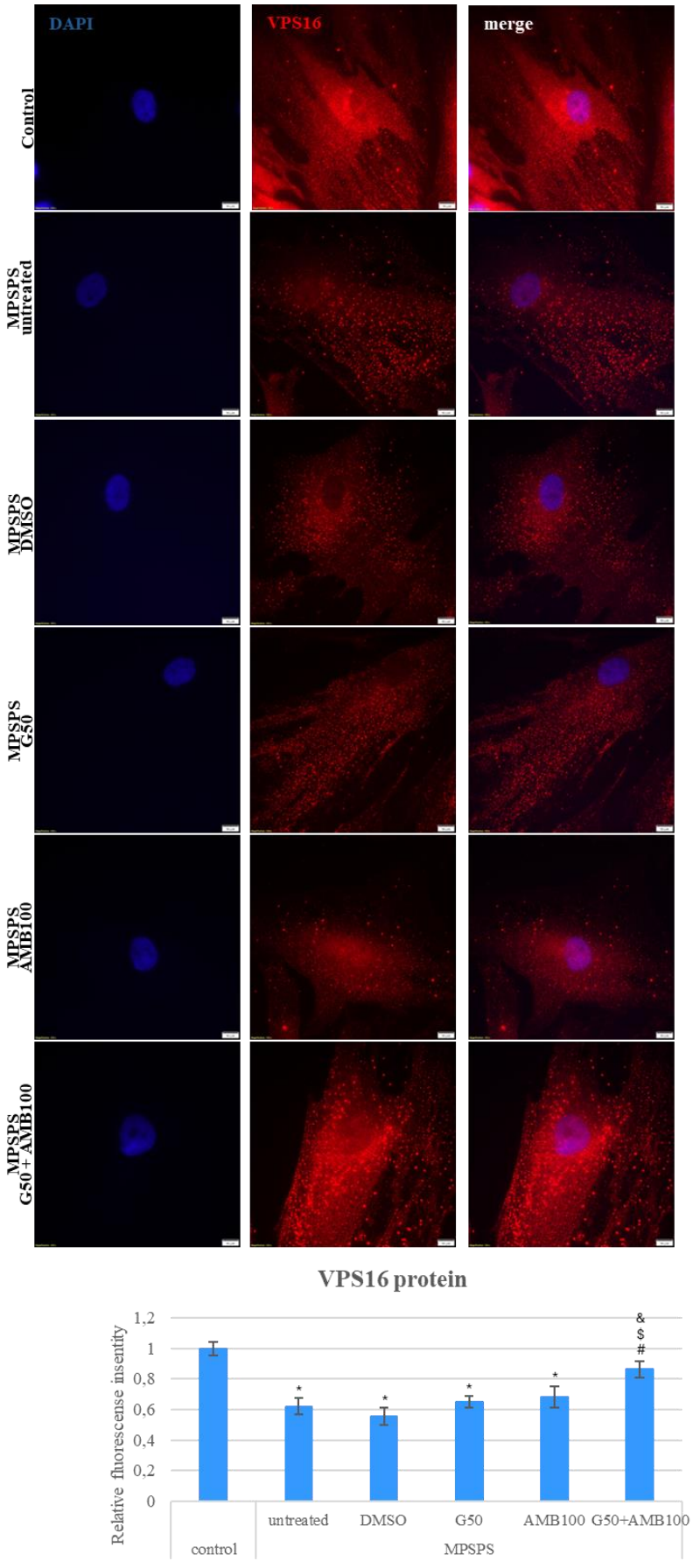
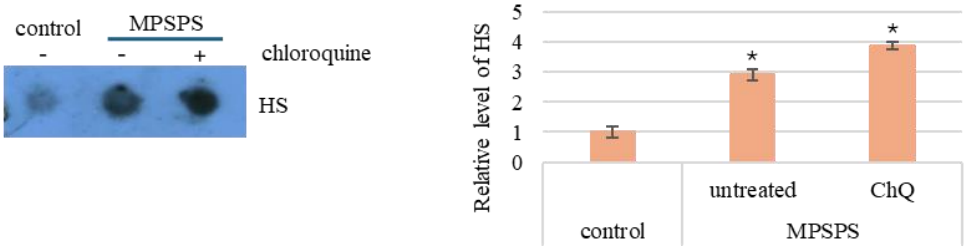

DAPI
VPS16
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
VPS16 protein
Relative fluorescense insentity
1,2
1
0,8
0,6
0,4
0,2
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS
*
&
$
#

Figure 4.

A


control
MPSPS
- - +
chloroquine
HS
HS
Relative level of HS
5
4
3
2
1
0
*
*
untreated
ChQ
control
MPSPS

B

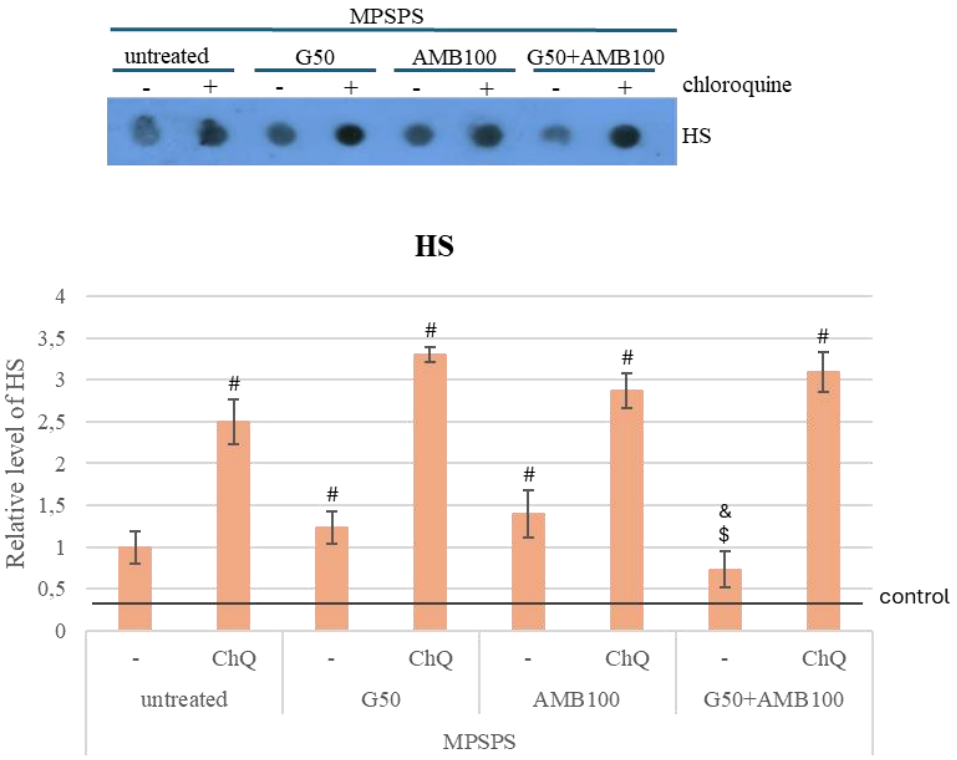

MPSPS
untreated
G50
AMB100
G50+AMB100
- + - + - + - +
chloroquine
HS
HS
Relative level of HS
4
3,5
3
2,5
2
1,5
1
0,5
0
#
#
#
#
#
&
$
control
- ChQ - ChQ - ChQ - ChQ
untreated
G50
AMB100
G50+AMB100
MPSPS

Figure 5.

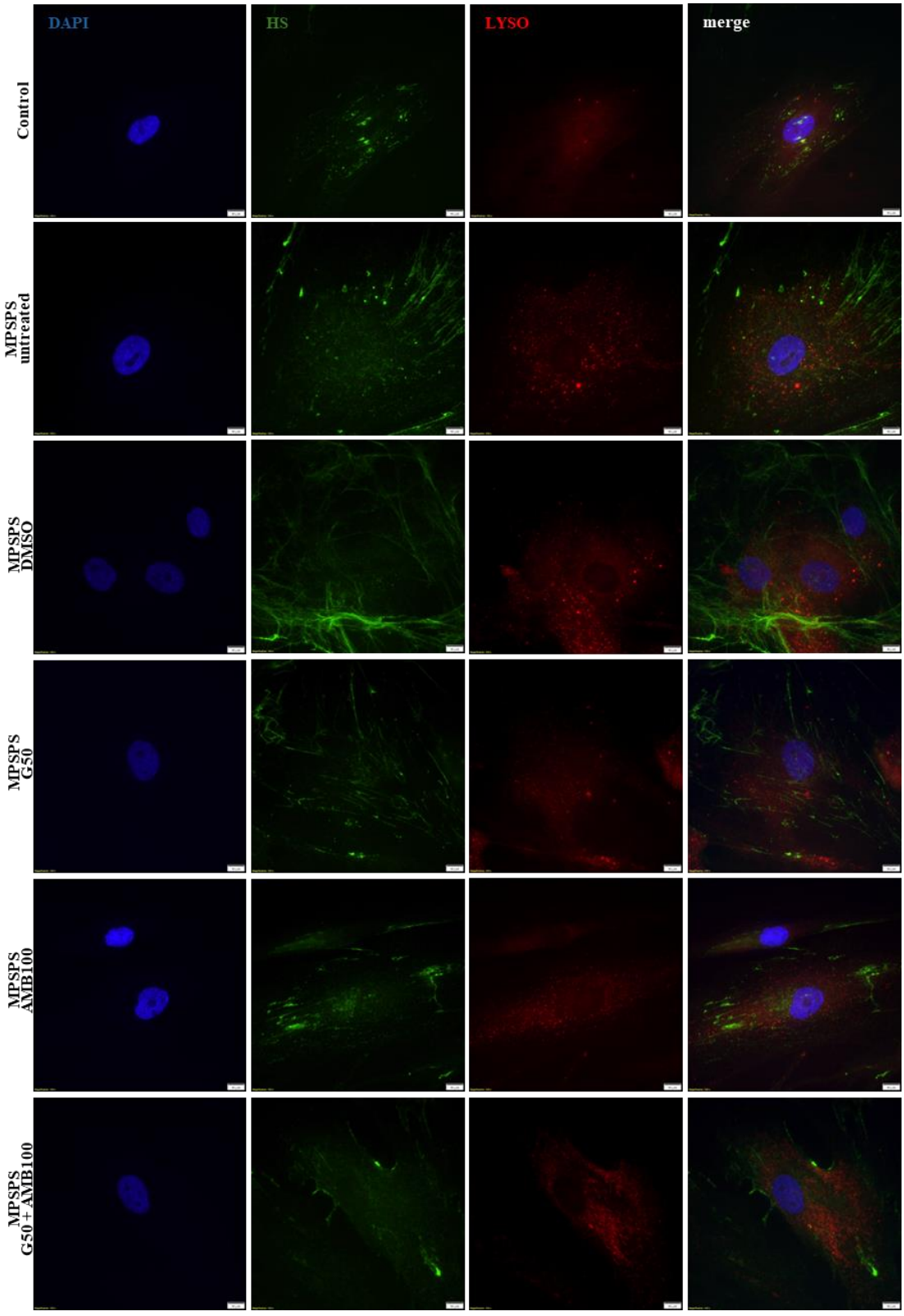
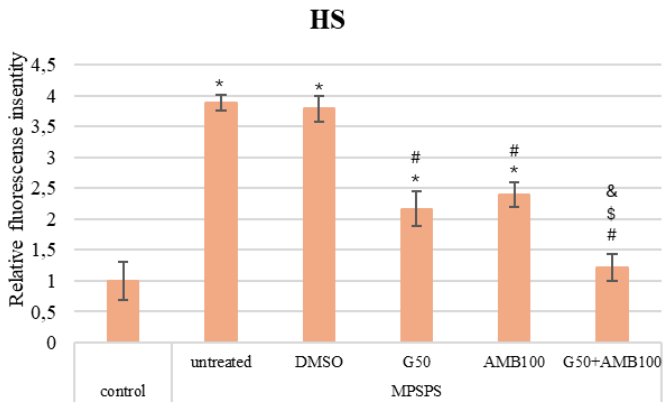
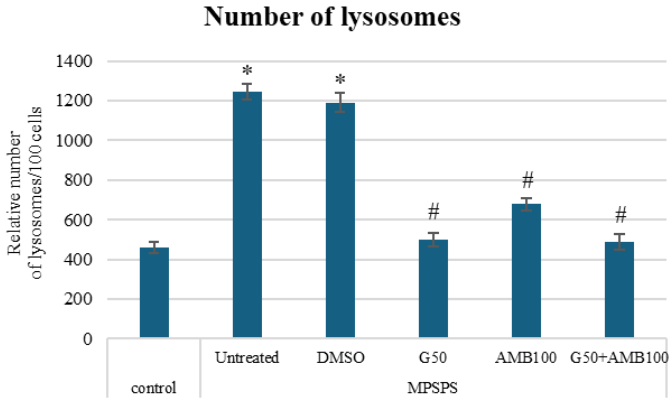

DAPI
HS
LYSO
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50 + AMB100

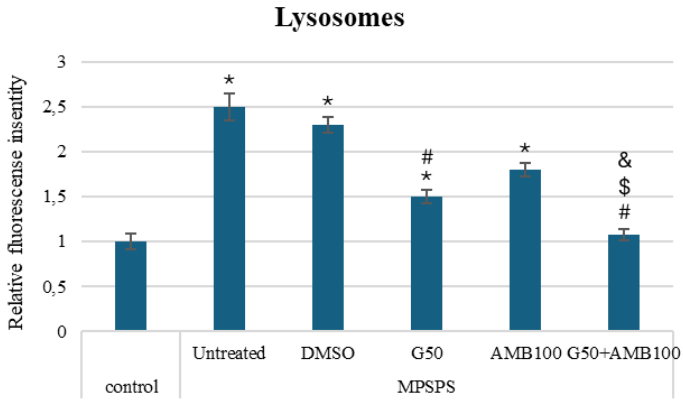
Figure 6.

A


HS
Relative fluorescense insentity
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

B


Number of lysosomes
Relative number of lysosomes/100 cells
1400
1200
1000
800
600
400
200
0
control
Untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS


Lysosomes
Relative fluorescense insentity
3
2,5
2
1,5
1
0,5
0
control
Untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

C

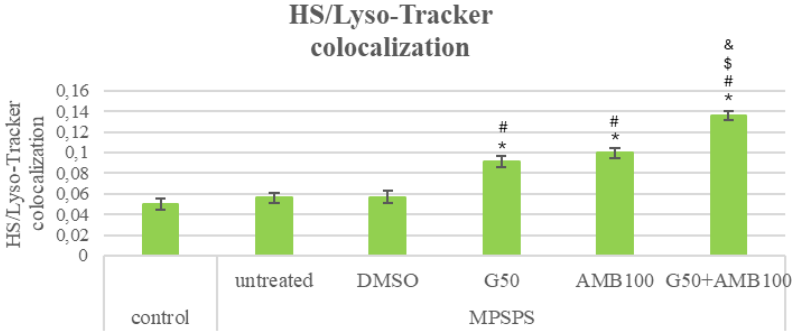

HS/Lyso-Tracker colocalization
HS/Lyso-Tracker colocalization
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 7.

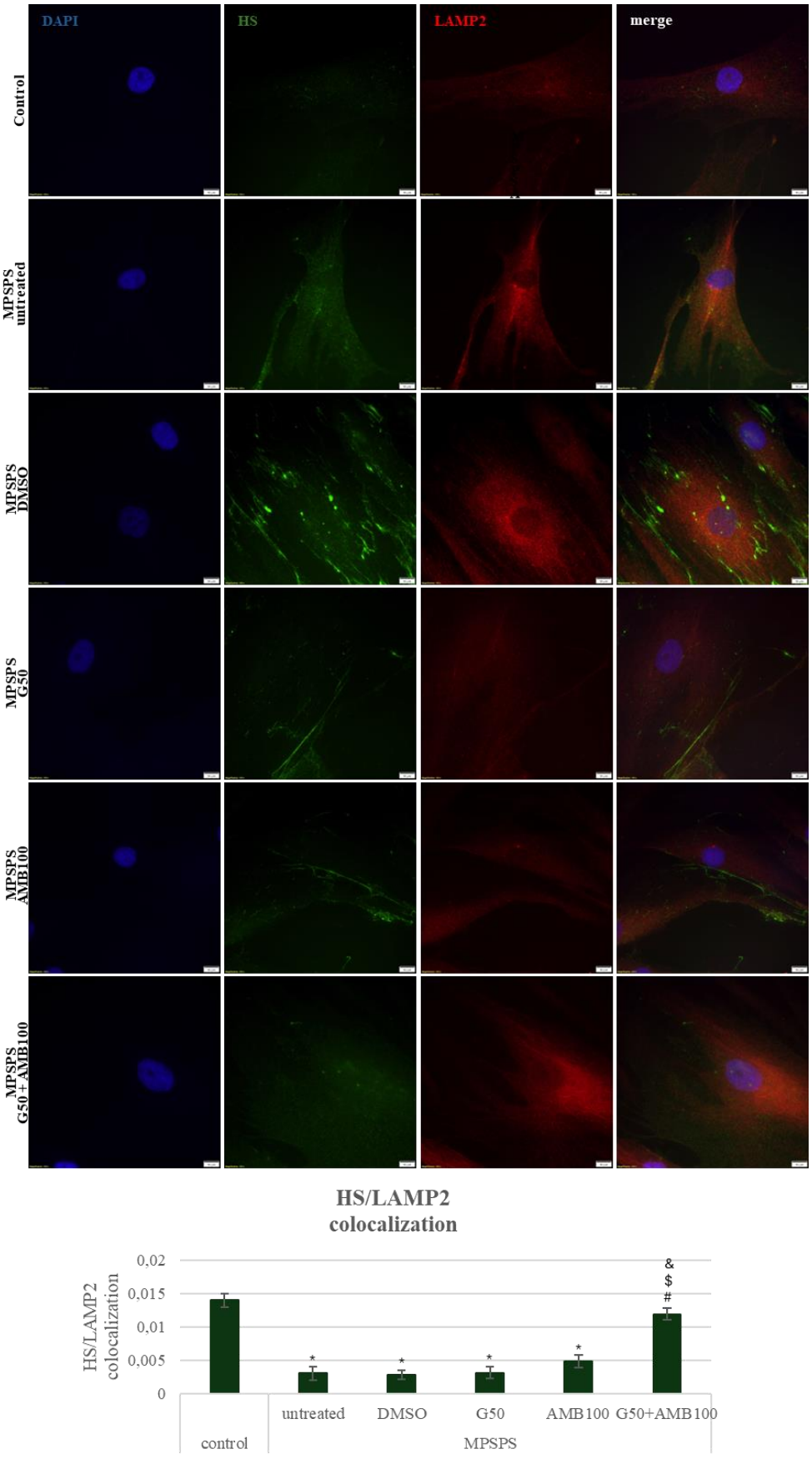

DAPI
HS
LAMP2
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
HS/LAMP2 colocalization
HS/LAMP2 colocalization
0,02
0,015
0,01
0,005
0
&
$
#
*
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 8.

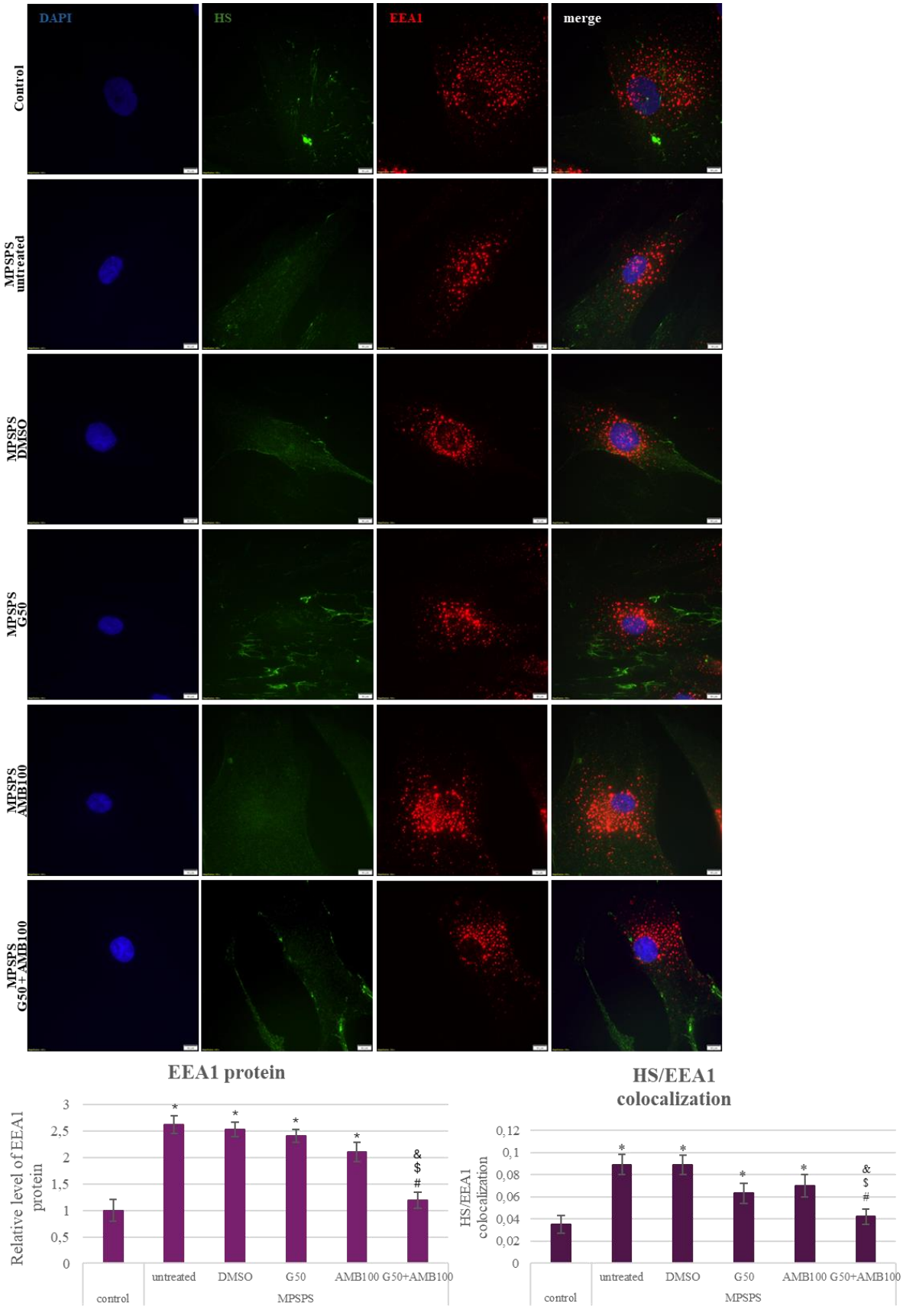

DAPI
HS
EEA1
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
EEA1 protein
Relative level of EEA1 protein
3
2,5
2
1,5
1
0,5
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS
HS/EEA1 colocalization
HS/EEA1 colocalization
0,12
0,1
0,08
0,06
0,04
0,02
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 9.

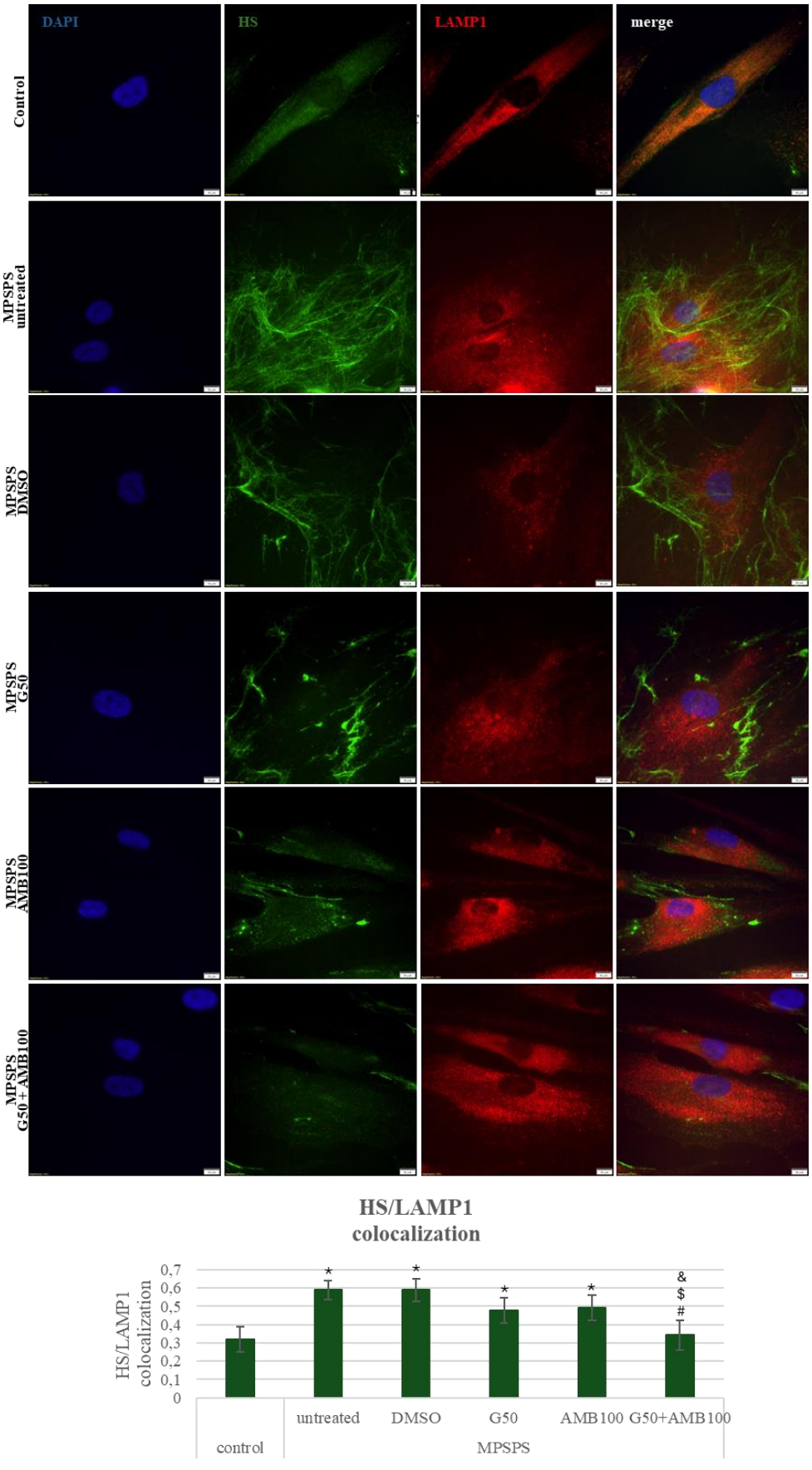

DAPI
HS
LAMP1
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
HS/LAMP1 colocalization
HS/LAMP1 colocalization
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 10.

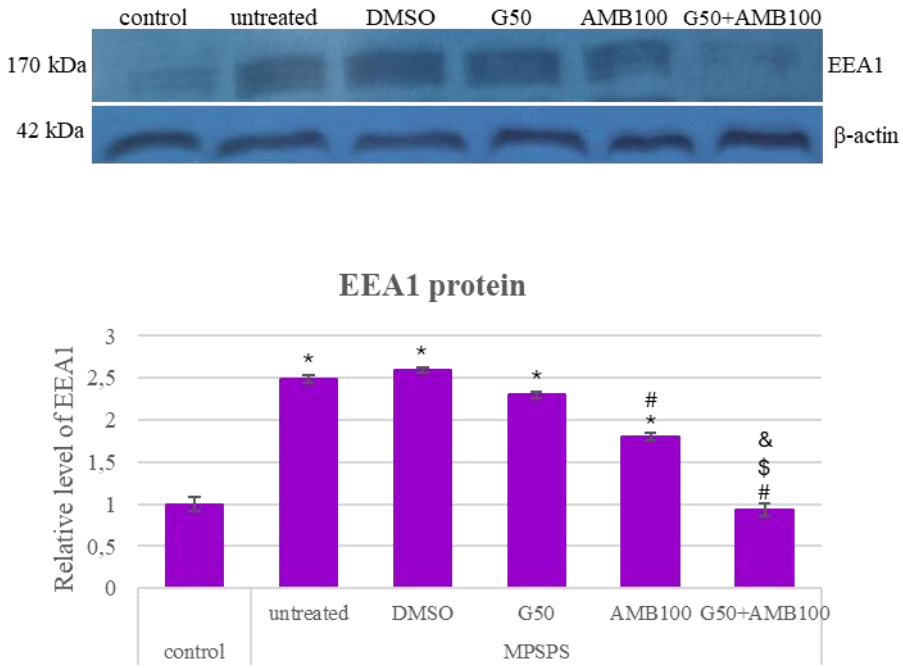

control
untreated
DMSO
G50
AMB100
G50+AMB100
170 kDa
EEA1
42 kDa
β-actin
EEA1 protein
Relative level of EEA1
3
2,5
2
1,5
1
0,5
0
*
*
*
#
*
&
$
#
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 11.

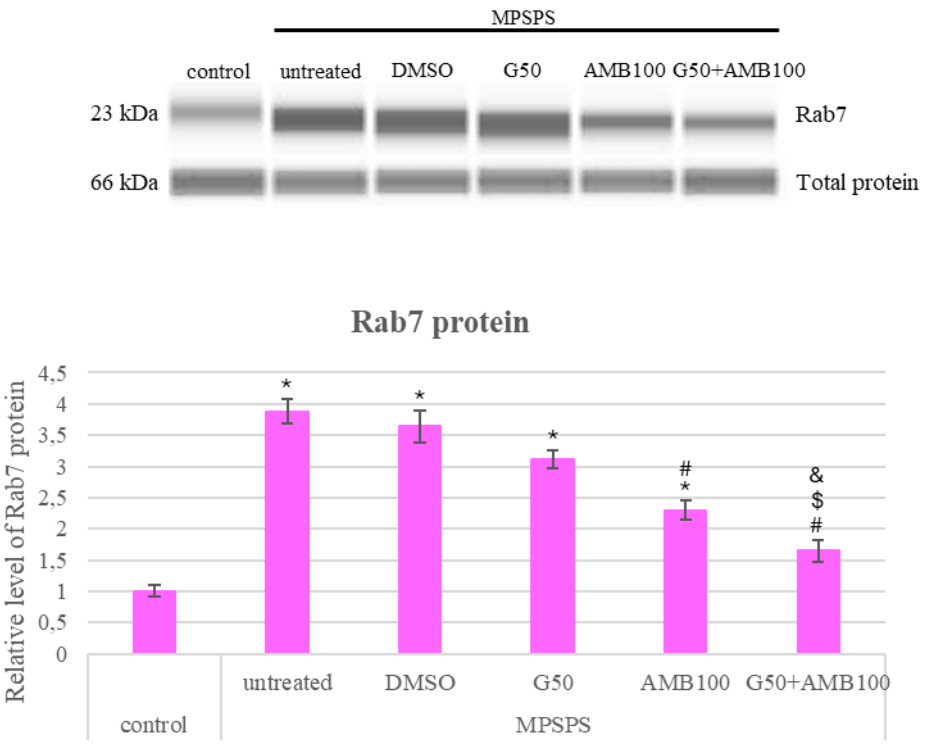

MPSPS
control
untreated
DMSO
G50
AMB100
G50+AMB100
23 kDa
Rab7
66 kDa
Total protein
Rab7 protein
Relative level of Rab7 protein
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
*
*
#
*
&
$
#
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 12.

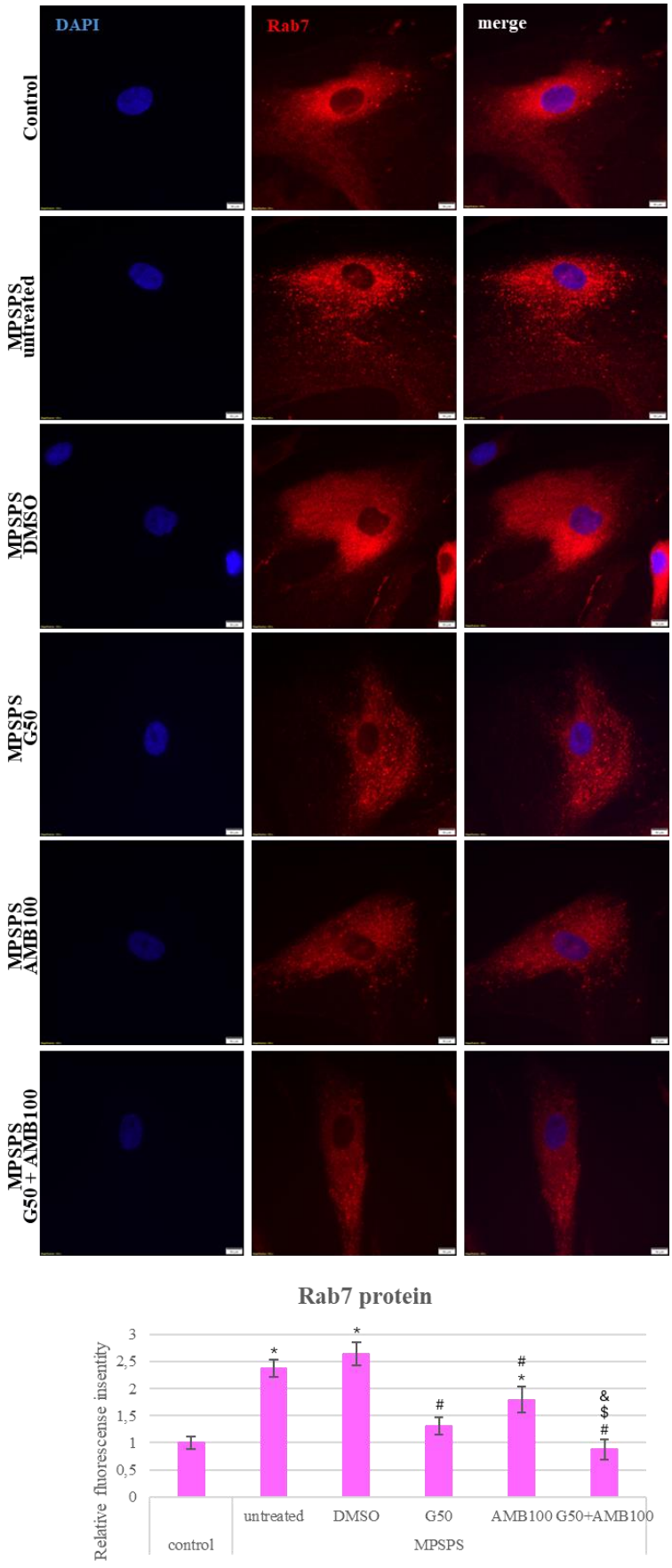

DAPI
Rab7
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
Rab7 protein
Relative fluorescense insentity
3
2,5
2
1,5
1
0,5
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 13.

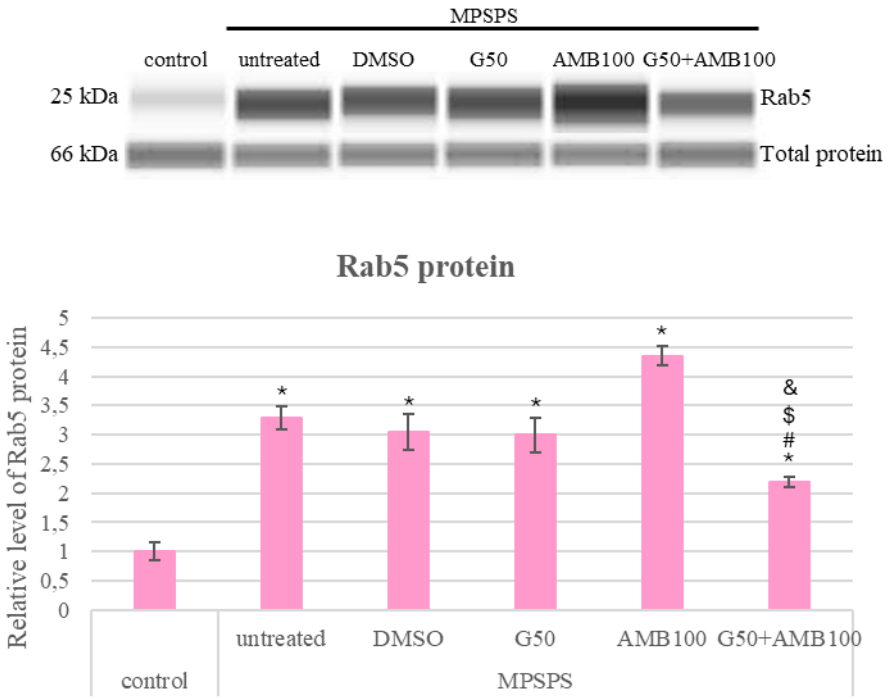

MPSPS
control
untreated
DMSO
G50
AMB100
G50+AMB100
25 kDa
Rab5
66 kDa
Total protein
Rab5 protein
Relative level of Rab5 protein
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
&
$
#
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 14.

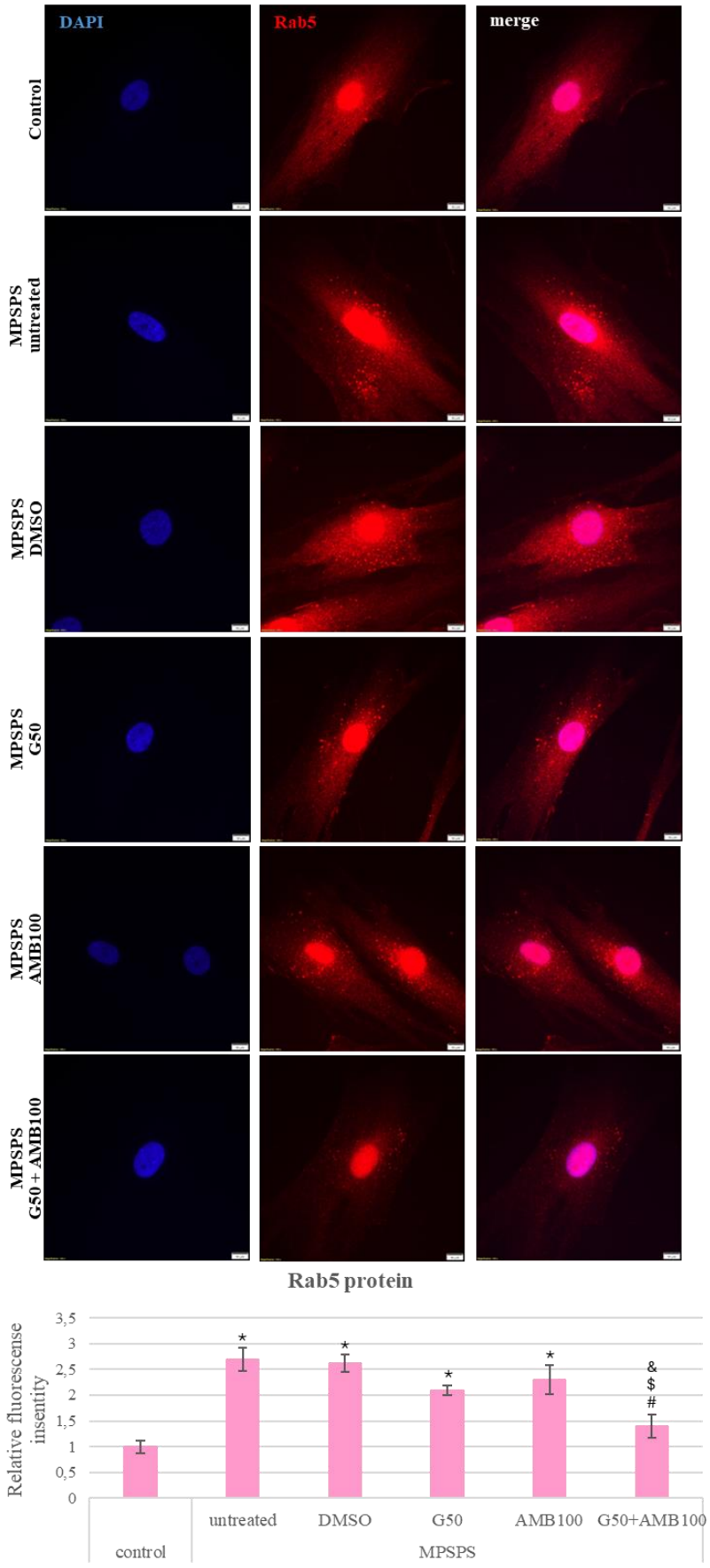

DAPI
Rab5
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50 + AMB100
Rab5 protein
Relative fluorescense insentity
3,5
3
2,5
2
1,5
1
0,5
0
*
*
*
*
&
$
#
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 15.

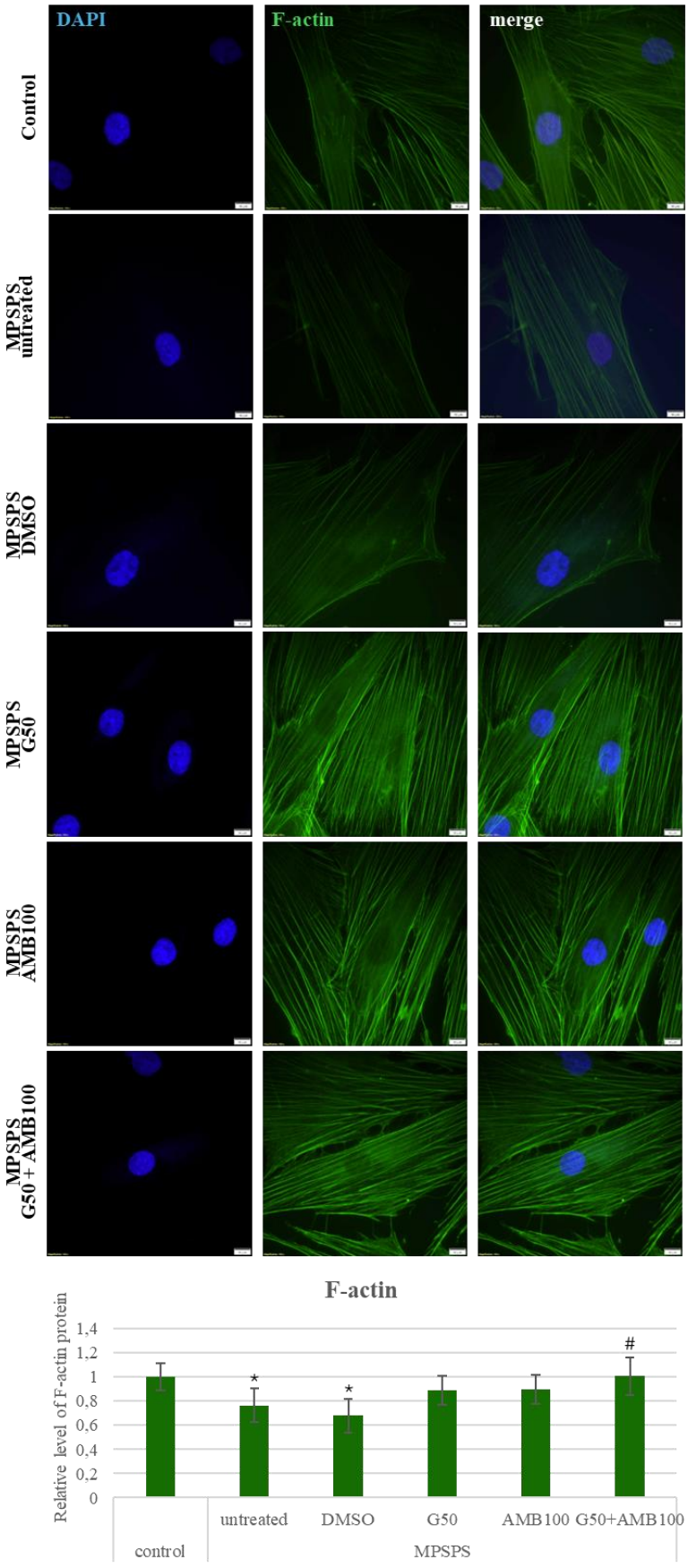
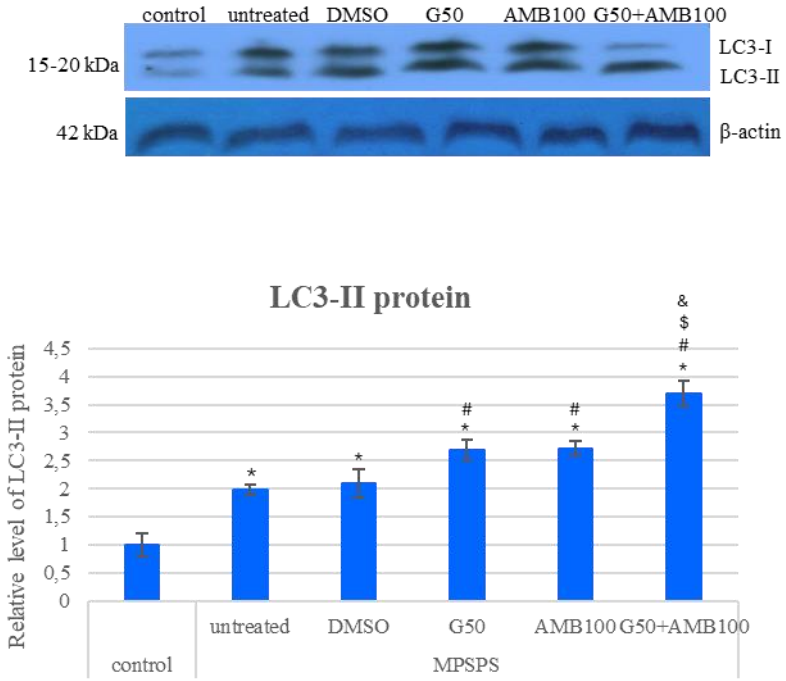

DAPI
F-actin
merge
Control
MPSPS untreated
MPSPS DMSO
MPSPS G50
MPSPS AMB100
MPSPS G50+AMB100
F-actin
Relative level of F-actin protein
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
#
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Figure 16.


control
untreated
DMSO
G50
AMB100
G50+AMB100
15-20 kDa
LC3-I
LC3-II
42 kDa
β-actin
LC3-II protein
Relative level of LC3-II protein
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
control
untreated
DMSO
G50
AMB100
G50+AMB100
MPSPS

Wkład Współautorów

Uniwersytet Gdański

mgr Zuzanna Cyske
Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

Gdańsk, 29.08.2025

Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Cyske Z, Rintz E, Gaffke L, Pierzynowska K, Węgrzyn G (2025) The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome.

polegał na:

- udziale w zaplanowaniu koncepcji badań;
- przeglądzie literatury;
- zaplanowaniu i przeprowadzeniu eksperymentów;
- przygotowaniu rycin;
- napisaniu wstępnej wersji pracy;
- udziale w przygotowaniu ostatecznej wersji manuskryptu.

Wydział Biologii
Katedra Biologii Molekularnej
Cyske
mgr Zuzanna Cyske

Uniwersytet Gdański

dr Estera Rintz
Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

Gdańsk, 3.09.2025

Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Cyske Z, Rintz E, Gaffke L, Pierzynowska K, Węgrzyn G (2025) The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome.

polegał na:

- udziale w zaplanowaniu koncepcji badań;
- udziale w przeprowadzeniu eksperymentów;
- udziale w przygotowaniu rycin;
- udziale w napisaniu wstępnej wersji publikacji.

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dr Estera Rintz

Uniwersytet Gdański

dr hab. Lidia Gaffke, prof. UG
Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

Gdańsk, 20.08.2025

Oświadczenie o wkładzie w publikację

Oświadczam, że mój wkład w publikację:

Cyske Z, Rintz E, Gaffke L, Pierzynowska K, Węgrzyn G (2025) The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome.

polegał na:

- udziale w przeprowadzeniu eksperymentów;
- udziale w przygotowaniu rycin;
- udziale w napisaniu wstępnej wersji publikacji.

Wydział Biologii
Katedra Biologii Molekularnej
dr hab. Lidia Gaffke
profesor uczelni

Uniwersytet Gdański

dr hab. Karolina Pierzynowska, prof. UG
Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

Gdańsk, 28.08.2025

Oświadczenie o wkładzie w manuskrypt

Oświadczam, że mój wkład w manuskrypt:

Cyske Z, Rintz E, Gaffke L, Pierzynowska K, Węgrzyn G (2025) The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome.

polegał na:

- udziale w przeglądzie literatury;
- udziale w napisaniu wstępnej wersji manuskryptu.

Wydział Biologii
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profesor uczelni

Uniwersytet Gdański

prof. dr hab. Grzegorz Węgrzyn
Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

Gdańsk, 28.08.2025

Oświadczenie o wkładzie w pracę

Oświadczam, że mój wkład w pracę:

Cyske Z, Rintz E, Gaffke L, Pierzynowska K, Węgrzyn G (2025) The use of genistein and ambroxol is an effective approach in correcting cellular dysfunctions of mucopolysaccharidosis-plus syndrome. Manuskrypt wysłany do redakcji *Mammalian Genome*.

polegał na:

- zaplanowaniu koncepcji badań;
- udziale w zaplanowaniu eksperymentów;
- przygotowaniu ostatecznej wersji manuskryptu.

KIEROWNIK
KATEDRY BIOLOGII MOLEKULARNEJ
prof. dr hab. Grzegorz Węgrzyn

Dorobek naukowy

Artykuły naukowe:

1. Mazur-Marzec H, Grabski M, Konkel R, Cegłowska M, **Cyske Z**, Gaffke L, Grabowski Ł, Hiskia A, Kajs M, Kaloudis T, Napiórkowska-Krzebietke A, Pierzynowska K, Rintz E, Iliakopoulou S, Walat S, Zervou SK, Zielenkiewicz M, Węgrzyn G. (2025) Genetic, metabolic and toxicological diversity within Prymnesium parvum (Haptophyte) from Polish waterbodies. Water Res. 282:123744. doi: 10.1016/j.watres.2025.123744.
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Rozdziały w książkach:

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Wystąpienia konferencyjne:

1. **Cyske Z**, Gaffke L, Rintz E, Pierzynowska K, Węgrzyn G „Zrozumieć MPS-plus: Co wiemy o molekularnych podstawach choroby?" XVIII Międzynarodowa Konferencja Chorób Rzadkich "Co nowego w chorobach rzadkich" 2025, referat wygłoszony
2. Węgrzyn G, Pierzynowska K, Podlacha M, Gaffke L, Rintz E, **Cyske Z**, Szulc A, Wiśniewska K, Żabińska M „Molecular mechanism and efficacy of 4',5,7-trihydroxyisoflavone in correction of symptoms of Huntington disease as revealed by studies with cellular and animal models" 49th FEBS Congress "Bridging continents to advance life science" 2025, referat wygłoszony
3. **Cyske Z**, Gaffke L, Rintz E, Pierzynowska K, Węgrzyn G „Understanding MPS-plus: Insights into the Molecular Basis of the Disease" 49th FEBS Congress "Bridging continents to advance life science" 2025, poster
4. Gaffke L, Szulc A, **Cyske Z**, Podlacha M, Pierzynowska K, Węgrzyn G „Mitochondrial effects of huntingtin polyglutamine expansion in a CRISPR-edited HEK model " 49th FEBS Congress "Bridging continents to advance life science" 2025, poster
5. Pierzynowska K, Wiśniewska K, Żabińska M, Szulc A, Gaffke L, Rintz E, **Cyske Z**, Podlacha M, Węgrzyn G „Disruption of ferroptosis pathways in the mouse model of mucopolysaccharidosis type I" 49th FEBS Congress "Bridging continents to advance life science" 2025, poster
6. Szulc A, Żabińska M, Wiśniewska K, Pierzynowska K, Gaffke L, Podlacha M, Rintz E, **Cyske Z,** Węgrzyn G „Transcriptomic analyses of gene expression disturbance in Huntington disease and effects of treatment with genistein" 49th FEBS Congress "Bridging continents to advance life science" 2025, poster
7. Żabińska M, Wiśniewska K, Szulc A, Gaffke L, Rintz E, **Cyske Z**, Podlacha M, Pierzynowska K, Węgrzyn G „The role of the estrogen receptor GPER in ferroptosis regulation in mucopolysaccharidosis type I" 49th FEBS Congress "Bridging continents to advance life science" 2025, poster
8. **Cyske Z**, Gaffke L, Pierzynowska K, Węgrzyn G „MPS-plus syndrome (MPSPS) - first studies on the molecular mechanism of the disease " 17th International Symposium on Mucopolysaccharidoses and Related Diseases 2024, referat wygłoszony
9. Pierzynowska K, Żabińska M, Gaffke L, **Cyske Z**, Wiśniewska K, Węgrzyn G „Aggregates of glycosaminoglycans with oxytocin and estrogen receptors as additional pathogenic factors in mucopolysaccharidoses" 17th International Symposium on Mucopolysaccharidoses and Related Diseases 2024, referat wygłoszony

10. Pierzynowska K, Podlacha M, Gaffke L, Rintz E, Wiśniewska K, **Cyske Z**, Żabińska M, Szulc A, Węgrzyn G „Autophagy stimulation corrects symptoms of Huntington disease in cellular and animal models of the disease ” EHDN and Enroll-HD Congress 2024, referat wygłoszony
11. Sibirny A, Dmytruk O, Vasylyshyn R, Fayura L, Gaffke L, Podlacha M, **Cyske Z**, Pierzynowska K, Ruchała J, Węgrzyn G, Ostrowski M, Dmytruk K „Toward anti-covid oral vaccine based on humanized Komagataella phaffii with surface displayed RBD” 9th International Weigl Conference 2024, referat wygłoszony
12. Węgrzyn G, Rintz E, Podlacha M, Gaffke L, Wiśniewska K, **Cyske Z**, Pierzynowska K ”Therapeutic potential of foxo3-dependent stimulation of autophagy by 3,5,4′-trihydroxystilbene (resveratrol) or 4′,5,7-trihydroxyisoflavone (genistein) in mucopolysaccharidosis type iiib and huntington disease ” 17th International Symposium on Mucopolysaccharidoses and Related Diseases 2024, referat wygłoszony
13. Węgrzyn G, Szulc A, Gaffke L, Rintz E, **Cyske Z,** Wiśniewska K, Żabińska M, Pierzynowska K „Transcriptomic analysis of Huntington disease cellular models with different numbers of the CAG repeats and effects of genistein treatments” EHDN and Enroll-HD Congress 2024, referat wygłoszony
14. Mazur-Marzec H, Konkel R, Gaffke L, Pierzynowska K, **Cyske Z**, Grabowski Ł, Grabski M, Węgrzyn G „Genotypic and chemotypic characteristics of Prymnesium parvum from the Odra River”, 40th International Conference of the Polish Phycological Society "Time of change: taxonomy and ecology of algae in theory and practice" 2023, referat wygłoszony
15. Pierzynowska K, Podlacha M, Gaffke L, Rintz E, Wiśniewska K, **Cyske Z**, Węgrzyn G „Correction of symptoms of Huntington disease in the mouse R6/1 model by genistein through the FOXO3-mediated autophagy stimulation” 5th Congress of Polish Biosciences "Different faces of biosciences" 2023, referat wygłoszony
16. Pierzynowska K, Podlacha M, Gaffke L, Rintz E, Wiśniewska K, **Cyske Z**, Węgrzyn G „Stymulacja autofagii koryguje odkładanie się agregatów białkowych oraz objawy choroby Huntingtona w modelach komórkowych i zwierzęcych” XI Zjazd Polskiego Towarzystwa Genetyki Człowieka 2023, referat wygłoszony
17. Żabińska M, Gaffke L, **Cyske Z,** Węgrzyn G, Pierzynowska K „Disturbances in levels of chaperone proteins as one of aspects of the pathogenesis of mucopolysaccharidoses” International Sopot Youth Conference "Where the world is heading?" 2023, poster
18. Sibirny A, Semkiv M, Dmytruk O, Vasylyshyn R, Fayura L, Gaffke L, Podlacha M, **Cyske Z**, Pierzynowska K, Węgrzyn G „Construction of the humanized strains of Komagataella phaffii producing intracellular, secreted and surface displayed Sars-CoV-2 antigens as potential vaccines against COVID-19, 1st Polish Yeast Conference 2022, referat wygłoszony

19. Węgrzyn G, Pierzynowska K, Gaffke L, **Cyske Z** „Stymulacja autofagii jako potencjalna metoda leczenia choroby Huntingtona, V Zjazd Naukowy Polskiego Towarzystwa Biologii Medycznej "Biologa - medycyna - terapia" 2022, referat wygłoszony
20. **Cyske Z**, Gaffke L, Pierzynowska K, Węgrzyn G „Mechanisms of changes in the efficiency of the expression of many genes in mucopolysaccharidoses, VI Polski Kongres Genetyki 2022, poster
21. **Cyske Z**, Pierzynowska K, Gaffke L, Węgrzyn G „Some, but not all, commonly used drinks can induce Shiga toxin-converting prophage in Escherichia coli, International Conference "The last word belongs to microbes" 2022, poster
22. Gaffke L, **Cyske Z**, Pierzynowska K, Firyn N, Węgrzyn G „Autophagy induction as a therapy for neuronopathic forms of Mucopolysaccharidosis, VI Polski Kongres Genetyki 2022, poster
23. Gaffke L, Kubiak K, **Cyske Z**, Węgrzyn G „Dysfunctions of the Escherichia coli hfq gene affect bacterial antibiotic resistance, International Conference "The last word belongs to microbes" 2022, poster
24. Grabowski Ł, Podlacha M, Rintz E, Mantej J, Myślińska D, Nowak-Zaleska A, Gaffke L, Pierzynowska K, Piotrowska E, **Cyske Z** „The effect of phage therapy on the level of immunological parameters in the chicken model, Viruses of Microbes Conference 2022, poster
25. Jaroszewicz W, Morcinek-Orłowska J, Pierzynowska K, Gaffke L, **Cyske Z**, Węgrzyn G „The first thermophilic phage display system employing bacteriophage TP-84 and Geobacillus stearothermophilus, International Conference "The last word belongs to microbes" 2022, poster
26. Kosznik-Kwaśnicka K, Ciemińska K, Nowak-Zaleska A, Podlacha M, Myślińska D, Pierzynowska K, **Cyske Z**, Gaffke L, Rintz E, Mantej J „The story of a phage cocktail: effectiveness of phage cocktail against S. Typhimurium on in vitro and in vivo models, Viruses of Microbes Conference 2022, poster
27. Pierzynowska K, Gaffke L, Podlacha M, **Cyske Z**, Węgrzyn G „Efficacy of genistein-mediated autophagy induction in treatment of the mouse model of Huntington's disease, VI Polski Kongres Genetyki 2022, poster
28. **Cyske Z** „Changes in the cytoskeleton in mucopolysaccharidoses, 17th International Conference on Rare Diseases "Don't miss a rare disease" 2019, referat wygłoszony
29. Gaffke L, Pierzynowska K, Brokowska J, **Cyske Z,** Węgrzyn G „Changes in the vacuolar transport as a unknown aspect of mucopolysaccharidosis patogenesis, 17th International Conference on Rare Diseases "Don't miss a rare disease" 2019, referat wygłoszony
30. Pierzynowska K, Gaffke L, Brokowska J, **Cyske Z**, Węgrzyn G „Disturbances in the effectiveness of the autophagy process as a new aspect of mucopolisaccharidosis pathogenesis: transcriptomic and cellular studies, 17th International Conference on Rare Diseases "Don't miss a rare disease" 2019, referat wygłoszony

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35. Węgrzyn G, Pierzynowska K, Gaffke L, Lopez-Lugo S, **Cyske Z**, Rintz E, Pankanin D „Genistein-mediated correction of differential defects in the cytoskeleton in cellular models of various neurodegenerative diseases,18th European Congress On Biotechnology 2018, referat wygłoszony

Udział w projektach:

Granty finansowane przez Narodowe Centrum Nauki:

1. Opus 13: „Changes in cellular processes as key defects in pathogenesis of inherited metabolic diseases from the group of mucopolysaccharidoses” kierownik: prof. dr hab. Grzegorz Węgrzyn
2. Preludium 15: „Stimulation of different pathways of autophagy induction in the light of the efficiency of glycosaminoglycan degradation in the neuronopathic types of mucopolysaccharidosis” kierownik: dr hab. Lidia Gaffke
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Kierowanie projektami:

Wydział Biologii Uniwersytetu Gdańskiego:

1. Granty Młodych Naukowców: „Rola czynników transkrypcyjnych AP-1, PPAR-gamma1, PPAR-gamma2 w zaburzeniach regulacji ekspresji genu CLU w lizosomalnych chorobach spichrzeniowych z grupy mukopolisacharydoz" **kierownik: mgr Zuzanna Cyske**
2. Granty Młodych Naukowców: „Efektywność działania ambroksolu i genisteiny w zespole MPS-plus" **kierownik: mgr Zuzanna Cyske**"
3. Granty Młodych Naukowców: „Terapia łączona genisteiną i ambroksolem w leczeniu choroby Gaucher'a" **kierownik: mgr Zuzanna Cyske**

Uniwersytet Gdański:

1. Program Małych Grantów UGrants-Start: „Rola wybranych niekodujących cząsteczek RNA w patogenezie mukopolisacharydozy typy IIIC" **kierownik: mgr Zuzanna Cyske**
2. Program Małych Grantów UGrants-First: „Rola interakcji wybranych lncRNA-miRNA-mRNA w patogenezie mukopolisacharydozy typu I" **kierownik: mgr Zuzanna Cyske**
3. Program Małych Grantów UGrants-Start: „Analiza zaburzeń morfologii i funkcji organelli komórkowych w Zespole Mukopolisacharydozy Plus" **kierownik: mgr Zuzanna Cyske**
4. Program Małych Grantów UGrants-Start: „Terapia kombinowana genisteiną i ambroksolem w zapobieganiu i leczeniu choroby Huntingtona" **kierownik: mgr Zuzanna Cyske**