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Habilitationsschrift

Untersuchungen zur Bedeutung und Pathogenese seltener Autoantikörper-assoziierter ZNS-Erkrankungen im Kindes- und Jugendalter

zur Erlangung der Lehrbefähigung
für das Fach Kinder- und Jugendmedizin

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von

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INHALTSVERZEICHNIS

ABKÜRZUNGEN

CA1/2	Cornu Ammonis 1 bzw. 2 (Bereiche des Hippocampus)
CASPR2	Contactin-associated protein-like 2
CBA	Cell-based assay
DNA	Desoxyribonukleinsäure
FACS	Fluorescence-activated cell sorting
FIRES	Febrile infection-related epilepsy syndrome
GABA	Gamma-Aminobuttersäure
GAD65	Glutamatdecarboxylase 65
HSV	Herpes-simplex-Virus
HSE	Herpes-simplex-Enzephalitis
IgG	Immunglobulin G
IVIG	Intravenöses Immunoglobulin
KLHL11	Kelch-like protein 11
LGI1	Leucine-rich glioma inactivated 1
mGluR5	Metabotroper Glutamatrezeptor 5
MOG	Myelin-Oligodendrozyten-Glykoprotein
MRT	Magnetresonanztomographie
mRS	modified Rankin Scale
NEOS	anti-NMDAR Encephalitis One-Year Functional Status
NGS	Next Generation Sequencing
NMDA	N-Methyl-D-Aspartat
NMDARE	N-Methyl-D-Aspartat-Rezeptor-Enzephalitis
NR1	NMDA receptor subunit 1
RNA	Ribonukleinsäure
RT-PCR	Reverse Transkriptase-Polymerase-Kettenreaktion)
TBA	Tissue-based assay
VGKC	Voltage-gated potassium channel
ZNS	Zentralnervensystem

1. EINLEITUNG

1.1 Autoimmunenzephalitis – Charakterisierung einer neuen Krankheitsgruppe

Der Begriff Autoimmunenzephalitis umfasst eine Gruppe akuter entzündlicher Erkrankungen des zentralen Nervensystems (ZNS), die vorwiegend junge Menschen betrifft. Sie wurde erstmals im Jahr 2007 von Dalmau et al. beschrieben (Dalmau et al., 2007). Allen Vertretern dieser Krankheitsgruppe ist gemeinsam, dass Autoantikörper gegen neuronale und gliale Zelloberflächenproteine, Rezeptoren und Ionenkanäle eine entscheidende Rolle spielen. Diese Antikörper lösen eine komplexe Kombination aus enzephalopathischen und psychiatrischen Symptomen, epileptischen Anfällen, Bewegungsstörungen sowie lebensbedrohlichen vegetativen Regulationsstörungen aus (Armangue et al., 2012; Graus et al., 2016).

Die Gruppe der Autoimmunenzephalitiden umfasst eine Vielzahl an verschiedenen Unterformen, von denen die anti-NMDA-Rezeptor Enzephalitis (NMDARE) eine der bekanntesten und die mit Abstand häufigste ist. Darüber hinaus hat diese wesentlich zum Verständnis der zugrunde liegenden Pathomechanismen beigetragen (Hughes et al., 2010; Mikasova et al., 2012). Der Weg zur Diagnose einer Autoimmunenzephalitis stellt häufig eine Herausforderung dar, da sich Symptome sehr vielfältig präsentieren und somit andere z.B. psychiatrische Erkrankungen vortäuschen können (Graus et al., 2016). Die frühzeitige und präzise Diagnose ist jedoch von großer Bedeutung, da eine zügig eingeleitete Behandlung das langfristige Outcome erheblich verbessert (Titulaer et al., 2013).

1.2 Das Spektrum an klinischen Präsentationen – Besonderheiten bei Kindern

Die Klinik einer Autoimmunenzephalitis kann vielfältig sein und von Patient zu Patient stark variieren. Am umfangreichsten ist dies für die NMDARE, die Modellerkrankung innerhalb der Autoimmunenzephalitiden, beschrieben. Diese zeigt ein breites Spektrum an Symptomen und bei erwachsenen Patienten typischerweise einen stadienartigen Verlauf (Tüzün et al., 2009).

Zu einem frühen Zeitpunkt treten in der Regel unspezifische grippeähnliche Symptome wie Fieber, Kopfschmerzen und Müdigkeit auf. Diese werden in einer zweiten Phase zunehmend von psychiatrischen Symptomen begleitet, wie Verwirrtheit, Halluzinationen, Stimmungsstörungen und Verhaltensänderungen. Im fortgeschrittenen Stadium können neurologische Symptome, wie epileptische Anfälle, Bewegungsstörungen und Sprachstörungen auftreten. Nicht selten aber

variiert dieser Verlauf und nicht alle Patienten müssen jedes Stadium durchlaufen (Dalmau et al., 2011).

Im Gegensatz zu den oft sehr ausgeprägten psychiatrischen Symptomen bei Erwachsenen präsentieren Kinder in der Regel subtilere Zeichen, wie Verhaltensänderungen, Schlaflosigkeit, Ängstlichkeit, aber auch mutistisches oder aggressives Verhalten. Oft stehen klar fassbare neurologische Symptome, wie Bewegungsstörungen, Koordinationsprobleme und epileptische Anfälle bei pädiatrischer Autoimmunenzephalitis im Vordergrund (Ariño et al., 2020; Florance et al., 2009; Hacohen et al., 2016; Wright and Vincent, 2016). Diese breite Palette an klinischen Präsentationen kann gerade bei jüngeren Kindern die korrekte Diagnosestellung einer Autoimmunenzephalitis erheblich verzögern.

1.3 Die Häufigkeit im Kindesalter – Eine unterschätze Differentialdiagnose

Seit ihrer Entdeckung vor nunmehr 15 Jahren werden Autoimmunenzephalitiden, allen voran die NMDARE, immer häufiger und immer früher erkannt und diagnostiziert. Trotz eines zunehmenden Bewusstseins für diese Differentialdiagnose wird die Häufigkeit dieser Erkrankungsgruppe gerade im Kindes- und Jugendalter oft unterschätzt.

Im Laufe der Jahre stiegen die Inzidenzen von ca. 0,4/100.000 Personenjahre (2005) auf ca. 1,2/100.000 Personenjahre (2015) und Prävalenzen bis zu 40/100.000, so dass epidemiologische Daten darauf hindeuten, dass Autoimmunenzephalitis als Krankheitsgruppe keine seltene Erkrankung im engeren Sinne ist. Betrachtet man nur die Gruppe der Enzephalitiden, wird deutlich, dass Autoimmunenzephalitis bei jungen Menschen mindestens so häufig auftritt, wie Virusenzephalitiden und ihre Prävalenz die einzelner viraler Ätiologien sogar übertrifft (Dubey et al., 2018; Erickson et al., 2020; Gable et al., 2012).

Die hohe Prävalenz der Autoimmunenzephalitis im Kindes- und Jugendalter verdeutlicht die Wichtigkeit, dieses Krankheitsbild bei der differentialdiagnostischen Abklärung von Enzephalitiden in Betracht zu ziehen.

1.4 V.a. Autoimmunenzephalitis – Die Diagnosekriterien nach Graus et al.

Das Erkennen einer Autoimmunenzephalitis kann wie bereits erläutert erheblich erschwert sein. Um eine einheitliche Diagnose zu erleichtern, wurden von Graus et al. 2016 Diagnosekriterien vorgeschlagen. Diese Kriterien basieren auf einer Kombination von klinischen Symptomen,

neurologischen Befunden, bildgebenden Verfahren und dem Nachweis von Antikörpern im Liquor oder Serum (Graus et al., 2016).

Als klinische Voraussetzung muss eine akute oder subakute Enzephalopathie vorliegen, die Symptome wie Bewusstseinsstörungen, Gedächtnisverlust, Verhaltensänderungen, Psychose oder Bewegungsstörungen umfasst. Zudem können assoziierte neurologische Befunde wie epileptische Anfälle oder fokale neurologische Defizite den Verdacht erhärten. In der zerebralen Bildgebung mittels MRT und einer Liquoranalyse zeigen sich Veränderungen, die auf eine Entzündung hinweisen. Es müssen andere Erkrankungen, die ähnliche Symptome verursachen können, ausgeschlossen werden. Hierzu gehören in erster Linie Infektionen des Zentralnervensystems, wie virale oder bakterielle Enzephalitiden, neurodegenerative Erkrankungen sowie Stoffwechselstörungen und strukturelle wie genetische Epilepsien. Eine sorgfältige Anamnese, körperliche Untersuchung, Bildgebung und Laboruntersuchungen sind entscheidend, um eine differenzialdiagnostische Abgrenzung vorzunehmen. Das wesentliche Kriterium für die Diagnose einer Autoimmunenzephalitis ist der Nachweis von spezifischen Autoantikörpern gegen neuronale und gliale Zelloberflächenproteine, Rezeptoren oder Ionenkanäle im Liquor oder Serum.

Entsprechend der Befunde kann die Diagnose einer Autoimmunenzephalitis in verschiedenen Abstufungen erfolgen. Eine gesicherte Autoimmunenzephalitis (Antikörper-positiv) liegt bei Nachweis eines spezifischen Autoantikörpers in Blut oder Liquor vor, der mit einer Autoimmunenzephalitis assoziiert wird. Diese Autoantikörper können beispielsweise gegen den NMDA-Rezeptor, LGI1, CASPR2, den $GABA_A$- oder $GABA_B$-Rezeptor gerichtet sein. Das Vorhandensein dieser Antikörper zusammen mit typischen klinischen Merkmalen bestätigt die Diagnose. V.a. Autoimmunenzephalitis besteht, wenn klinische Merkmale für diese vorliegen, aber weitere Untersuchungen erforderlich sind, um die Diagnose zu bestätigen oder auszuschließen. Eine wahrscheinliche Autoimmunenzephalitis wird diagnostiziert, wenn keine spezifischen Autoantikörper nachgewiesen wurden, aber typische klinische Merkmale der Autoimmunenzephalitis vorliegen und andere mögliche Ursachen für die Symptome ausgeschlossen wurden. In solchen Fällen kann eine immuntherapeutische Behandlung als diagnostischer Test durchgeführt werden. Wenn eine deutliche Besserung der Symptome nach der Immuntherapie auftritt, stützt dies die Diagnose einer Autoimmunenzephalitis. In einigen Fällen kann keine nachweisbare Präsenz von spezifischen Autoantikörpern festgestellt werden, obwohl klinische Merkmale einer Autoimmunenzephalitis vorliegen. In diesem Fall spricht man von einer Antikörper-negativen Autoimmunenzephalitis, sofern andere Ursachen für die Symptome ausgeschlossen wurden und eine Immuntherapie zu deutlicher Besserung der

Symptome geführt hat. In solchen Fällen wird vermutet, dass die Autoimmunenzephalitis durch Autoantikörper vermittelt wird, die derzeit noch nicht entdeckt wurden.

Die Diagnosekriterien nach Graus et al. wurden ursprünglich für erwachsene Patienten entwickelt. Inzwischen liegen jedoch modifizierte Kriterien für die Diagnose einer Autoimmunenzephalitis bei Kindern vor, welche spezifische altersabhängige Merkmale und Symptome von pädiatrischen Patienten berücksichtigen (Cellucci et al., 2020a). Die Anwendung dieser modifizierten Kriterien kann die Diagnosestellung bei Kindern beschleunigen und hierdurch ihre Prognose verbessern.

1.5 Therapie, Krankheitsverlauf und Prognose – Das Outcome bei Kindern

Die Behandlung einer Autoimmunenzephalitis umfasst unterschiedliche Ansätze, die in eine Erstlinien- und Zweitlinien-Therapie eingeteilt werden. Als Erstlinientherapie werden in der Regel immunmodulatorische Regime mittels Kortikosteroiden, intravenöser Immunglobulintherapie und Plasmapherese oder Immunadsorption eingesetzt. Diese Therapien zielen darauf ab, die im ZNS ablaufenden autoimmunologischen Prozesse zu unterdrücken und die Entzündungsreaktion zu reduzieren. Dies führt bei einem Teil der Patienten bereits zu einer deutlichen Besserung und schließlich Rückbildung der Symptome (Armangue et al., 2012; Titulaer et al., 2013). Bei mangelndem Ansprechen auf die Erstlinientherapie oder initial schwerer Symptomatik, kann eine Zweitlinientherapie in Betracht gezogen werden, bei der eine deutlichere Immunsuppression und insbesondere B-Zell-Depletion durch z.B. Rituximab angestrebt wird (Titulaer et al., 2013). Seltener kommen Cyclophosphamid oder Bortezomib zum Einsatz (Scheibe et al., 2017). Die Entscheidung für eine Zweitlinientherapie wurde in der Vergangenheit in der Regel individuell getroffen. Inzwischen gibt es jedoch zumindest für die Behandlung der NMDARE erste Metaanalysen und Consensus-Empfehlungen (Nosadini et al., 2021a, 2021b).

Zwar ist das initiale Therapieansprechen in der überwiegenden Mehrzahl der Fälle positiv. Dennoch hängt die Prognose einer Autoimmunenzephalitis vom Schweregrad der Erkrankung zum Zeitpunkt der Diagnose, der Dauer bis zum Beginn einer Therapie sowie von der exakten Entität und dem zugrunde liegenden Autoantikörper ab. Eine frühzeitige Diagnose und Behandlung sind jedoch generell entscheidend, um das Fortschreiten der Erkrankung zu stoppen und langfristige Schäden zu minimieren (Graus et al., 2016).

Das Outcome bei Kindern mit Autoimmunenzephalitis ist in über 80% der Fälle positiv und generell besser als das von Erwachsenen, kann jedoch ebenfalls variieren (Nosadini et al., 2015; Titulaer et al., 2013). In vielen Fällen führt eine frühzeitige und angemessene Behandlung zu

einer vollständigen Remission der Symptome und einer Wiederherstellung aller psychomotorischen und mnestischen Funktionen. Bei einigen Kindern kommt es aber zu einer partiellen Besserung und es verbleiben neurologische Defizite, Epilepsie oder lange andauernde psychische Beschwerden. Rezidive treten in ca. 10-20% aller Fälle auf (Nosadini et al., 2019a, 2021b; Wright et al., 2015). Insbesondere Langzeitstudien haben gezeigt, dass bei zahlreichen Kindern trotz scheinbarer *restitutio ad integrum* langfristige kognitive Defizite persistieren. Diese betreffen vor allem die Bereiche Aufmerksamkeit, Gedächtnis, Lernfähigkeit und exekutive Funktionen und können den Alltag, die schulische Leistung und die Lebensqualität insgesamt erheblich beeinträchtigen (Bruijn et al., 2018; Heine et al., 2021).

Eine umfassende Nachsorge und regelmäßige Verlaufskontrollen sind daher enorm wichtig, um das langfristige Outcome bei Kindern mit Autoimmunenzephalitis zu überwachen und bei Bedarf zusätzliche Unterstützung anzubieten. Eine multidisziplinäre Betreuung kann hier dazu beitragen, die Lebensqualität der betroffenen Kinder zu verbessern.

1.6 NMDAR Enzephalitis – Pathomechanismen einer Modellerkrankung

Die NMDARE ist die am besten untersuchte Entität in der Gruppe der Autoimmunenzephalitiden und gilt als Modellerkrankung. Ein Großteil des heutigen Wissens über Autoimmunenzephalitiden, insbesondere zu Pathomechanismen, entstammt Erkenntnissen über die NMDARE.

Bei dieser Form der Autoimmunenzephalitis werden Autoantikörper gegen die NR1-Untereinheit des NMDAR gebildet, welcher in den Neuronen unseres Gehirns ubiquitär exprimiert wird (Hughes et al., 2010). Diese Autoantikörper binden reversibel an NR1 und führen zu einer internalisierenden Endozytose der NMDAR, was eine gestörte Neurotransmission und Entzündungsreaktion in unserem Gehirn zur Folge hat (Mikasova et al., 2012; Moscato et al., 2014). Diese Reduktion der NMDA-Rezeptordichte an der Zelloberfläche der Neuronen hat eine Störung der Glutamat-vermittelten Neurotransmission zur Folge, welche sich unmittelbar auf die Regulation neuronaler Netzwerke und die synaptische Plastizität auswirkt, die für Lern- und Gedächtnisprozesse essenziell ist (Jézéquel et al., 2017; Ladépêche et al., 2018). Ein weiterer Effekt ist die Dysregulation im dopaminergen System, welche zu den bereits erwähnten psychiatrischen Symptomen wie Wesensveränderung, Halluzinationen oder Wahnvorstellungen führen können (Carceles-Cordon et al., 2020). Darüber hinaus löst die Bindung der Autoantikörper an die NMDAR eine Aktivierung von Mikroglia und Astrozyten im Gehirn und hierüber die Freisetzung von Entzündungsmediatoren aus, was die Gehirnfunktionsstörung

weiter verstärkt. Sowohl die veränderte Neurotransmission als auch die allgemeine Entzündungsreaktion erklären das komplexe klinische Bild der NMDARE, einer Kombination aus Bewusstseinsstörungen, psychiatrischen Symptomen, Bewegungsstörungen und epileptischen Anfälle (Armangue et al., 2013; Dalmau et al., 2017; Hacohen et al., 2016).

Über diese grundlagenwissenschaftlichen Erkenntnisse wurden in den letzten Jahren schrittweise auch Pathomechanismen anderer Autoimmunenzephalitiden entschlüsselt. Dabei konnten bei anderen Autoantikörpern zum Teil ähnliche (Petit-Pedrol et al., 2014), zum Teil aber ganz neue Mechanismen aufgedeckt werden (Ohkawa et al., 2013), was zu einem besseren Verständnis des großen klinischen Spektrums innerhalb der gesamten Gruppe der Autoimmunenzephalitiden führte (Dalmau et al., 2017).

Die genauen Ursachen für die Bildung dieser Autoantikörper sind bislang jedoch noch nicht gut verstanden. Es wird angenommen, dass auslösende Faktoren wie Infektionen, Tumore oder andere immunologische Prozesse die Bildung der Autoantikörper bei bestimmten genetisch prädisponierten Personen auslösen können. Hierzu passen insbesondere die Assoziationen zwischen NMDARE und Ovarialteratomen (Dalmau et al., 2019; Day et al., 2014; Wandinger et al., 2011) oder der Zusammenhang zu bestimmten Infektionen, wie Herpes-simplex-Virus Enzephalitis (Armangue et al., 2014; Prüss et al., 2012).

1.7 Seltene und Antikörper-negative Autoimmunenzephalitiden – Die unklare Relevanz

Die Autoimmunenzephalitis umfasst nicht nur gut charakterisierte Formen wie die NMDARE, sondern auch zahlreiche seltene Entitäten. Zu den häufigeren der hierbei auftretenden Autoantikörper gehören bei erwachsenen Enzephalitis-Patienten Anti-VGKC-Komplex-, Anti-$GABA_B$-Rezeptor- und Anti-LGI1-Antikörper (Dalmau et al., 2017; Sonderen et al., 2017; Titulaer et al., 2013). Bei Kindern hingegen sind Anti-MOG-, Anti-GAD65- und $GABA_A$-Rezeptor-Antikörper zu finden (Hardy, 2022; Ohkawa et al., 2014). Die genaue Diagnosestellung und die Identifizierung spezifischer Autoantikörper sind von großer Bedeutung, da unterschiedliche Autoantikörper mit verschiedenen klinischen Manifestationen assoziiert sein können (Armangue et al., 2013; Wright et al., 2015). Darüber hinaus divergieren Krankheitsverlauf und Therapieansprechen zwischen den unterschiedlichen Entitäten, und so ist – obwohl über die systemische Immuntherapie hinaus bislang keine Antikörper-individuellen Therapieoptionen existieren – zumindest eine Prognoseabschätzung möglich. Insgesamt hat diese immer größer werdende Gruppe an Erkrankungen somit in den letzten Jahren ganze Bereiche insbesondere in den Neurowissenschaften geprägt, aber auch diagnostischen Abläufe und Therapieansätze im

klinischen Alltag innerhalb der Neurologie, Psychiatrie und Kinderheilkunde entscheidend verändert. Und, während die Liste an neuen antineuronalen Autoantikörpern kontinuierlich wächst, bleibt unklar, wie viele noch nicht identifizierte Antikörper gegen neuronale Oberflächenstrukturen unentdeckt sind, welche pathogenetische Relevanz neu identifizierte Autoantikörper haben und wie groß die Bedeutung der sog. Antikörper-negativen Autoimmunenzephalitiden ist. Letztere stellen eine diagnostische Herausforderung dar, weil spezifische biomarkerbasierte Tests noch fehlen (Dalmau & Graus, 2023) und somit ihre Häufigkeit auch weiterhin unbekannt bleibt. Die Tatsache, dass bei einer beträchtlichen Anzahl von Patienten mit klinischem Verdacht auf Autoimmunenzephalitis trotz umfangreicher Untersuchungen keine Autoantikörper nachgewiesen werden können, macht eine Gruppe von nicht unerheblicher Größe jedoch sehr wahrscheinlich (Dalmau & Graus, 2023).

Zusätzlich ist seit längerem bekannt, dass anti-neuronale Autoantikörper auch bei verschiedenen ZNS-Erkrankungen auftreten, die sich nicht primär mit einer Enzephalitis manifestieren. Bei Erwachsenen sind dies beispielsweise Epilepsien unklarer Genese, atypische Psychosen und postinfektiöse Bewegungsstörungen (Atmaca et al., 2017; Dubey et al., 2017; Schou et al., 2016), bei Kindern Erkrankungen wie Rasmussen-Enzephalitis, FIRES (*Febrile infection related epilepsy syndrome*), Sydenham-Chorea, postinfektiöse zerebelläre Ataxie oder das Opsoklonus-Myoklonus-Syndrom (Armangue et al., 2012). Da zahlreiche in diesem Rahmen erhobene Antikörperbefunde jedoch auf Serum-Untersuchungen statt Liquoranalysen basieren, bleibt die Einschätzung der Relevanz und Pathogenität dieser Autoantikörper ungeklärt (Dahm et al., 2014; Kreye et al., 2016).

Aus diesen Gründen bleibt die Identifizierung und funktionelle Charakterisierung von antineuronalen Autoantikörpern sowie die Klärung ihrer pathogenetischen Relevanz, auch 15 Jahre nach den Entdeckungen von Dalmau et al., weiterhin von großer Bedeutung. Die so gewonnenen Erkenntnisse werden auch künftig bei der Erforschung von Pathomechanismen und klinischen Manifestationen bislang unverstandener Formen der Autoimmunenzephalitis, wie seltenen Entitäten und antikörper-negativer Autoimmunenzephalitis helfen. Zudem können sie die Grundlage für neue diagnostische Tests oder gezieltere Therapieansätze liefern. Fortschritte in der Hochdurchsatztechnologie könnten hierbei die Identifizierung neuer Autoantikörper beschleunigen (Cellucci et al., 2020b; Dalmau and Graus, 2023; Nosadini et al., 2021a).

Meine Forschung über Autoimmunenzephalitiden und die in dieser Habilitationsschrift vorgelegten Untersuchungen zur Bedeutung und Pathogenese seltener Autoantikörper-assoziierter ZNS-Erkrankungen im Kindes- und Jugendalter sollen eben jenes Verständnis dieser komplexen Erkrankungsgruppe vertiefen und darüber hinaus in einem translationalen Ansatz dazu beitragen, effektivere Diagnose- und Behandlungsstrategien zu entwickeln.

2. EIGENE ARBEITEN

2.1 Publikation 1

Wie eingangs beschrieben, dient die NMDARE als Modellerkrankung. Ihre Erforschung konnte in den letzten Jahren wertvolle Erkenntnisse über die Pathomechanismen der gesamten Erkrankungsgruppe der Autoimmunenzephalitis liefern. So haben die inzwischen detaillierten Erkenntnisse zu Antikörper-vermittelter Internalisierung des NMDAR dazu beigetragen, die pathologischen Effekte z.B. von Anti-LGI1- und anderer antineuronaler Autoantikörper zu entschlüsseln (Ladépêche et al., 2018; Nosadini et al., 2019b; Ohkawa et al., 2013; Sonderen et al., 2017).

Weit weniger ist jedoch über die immunologischen Ursachen bekannt, welche zur Bildung solcher Autoantikörper führen und inwieweit diese mit der Assoziation zwischen Autoimmunenzephalitiden und verschiedenen Tumorerkrankungen zu tun haben. Zur Klärung dieser Fragen konnten wir in zwei Arbeiten beitragen.

Schnell S, Knierim E, Bittigau P, Kreye J, Hauptmann K, Hundsdoerfer P, Morales-Gonzalez S, Schuelke M, Nikolaus M. Hodgkin Lymphoma Cell Lines and Tissues Express mGluR5: A Potential Link to Ophelia Syndrome and Paraneoplastic Neurological Disease. *Cells*. 2023. https://doi.org/10.3390/cells12040606 (IF=7,7)

In der ersten Studie untersuchten wir die Expression des metabotropen Glutamatrezeptors 5 ($mGluR_5$) im klassischen Hodgkin-Lymphom und seine mögliche Rolle bei der Entstehung des sogenannten Ophelia-Syndroms.

$mGluR_5$ wird in der Regel in den Nervenzellen des Gehirns exprimiert. Das Ophelia-Syndrom wiederum ist eine seltene neuroimmunologische Erkrankung aus der Gruppe der Autoimmunenzephalitiden. Es zeichnet sich durch die Kombination aus einem akut auftretenden komplexen neuropsychiatrischen Syndrom, dem Nachweis von Antikörpern gegen $mGluR_5$ im Liquor und der Assoziation mit einem im Verlauf auftretenden Hodgkin-Lymphom aus. Die genaue Pathophysiologie des Ophelia-Syndroms und vor allem der Grund für die Entstehung des Tumors sind bisher nicht vollständig verstanden.

Durch eine Reihe von Methoden, u.a. Immunhistochemie, Western Blot, RT-PCR und FACS konnten wir nachweisen, dass $mGluR_5$ sowohl in Tumorgewebe von Patienten mit Ophelia

Syndrom als auch in verschiedenen Hodgkin-Lymphom-Zelllinien exprimiert wird. Diese Expression zeigte sich über verschiedene Zelllinien hinweg sehr heterogen ausgeprägt und war in den Lymphomzellen im Vergleich zu normalen Geweben signifikant erhöht.

Mittels RNA-Sequenzierungsdaten aus verschiedenen Hodgkin-Zelllinien konnten wir darüber hinaus zeigen, dass in Abhängigkeit der $mGluR_5$ Expression zahlreiche Gene überexprimiert werden, welche mit Zellproliferation und Apoptose assoziiert sind. Somit führt eine Aktivierung von $mGluR_5$ durch Bindung des natürlichen Liganden Glutamat oder – wie im Falle des Ophelia Syndroms – des anti-$mGLuR_5$ Autoantikörpers zu einer Aktivierung von intrazellulären Signalwegen in Richtung Zellproliferation und Tumorprogression.

Insgesamt zeigt diese Arbeit erstmals die Expression von $mGluR_5$ im Hodgkin-Lymphom. Mit unseren Ergebnissen stellen wir eine pathomechanistische Verbindung zwischen der anti-$mGluR_5$ Enzephalitis und der Entstehung des Hodgkin-Lymphoms beim Ophelia-Syndrom her. Diese Erkenntnisse könnten sowohl dazu beitragen, neue potenzielle Angriffspunkte bei der Behandlung des Hodgkin-Lymphoms zu untersuchen, als auch spezifischere Therapiekonzepte für das Ophelia Syndrom und Autoimmunenzephalitiden im Allgemeinen zu finden. Hierzu sind jedoch Untersuchungen erforderlich, um die klinische Relevanz dieser Befunde zu bestätigen.

cells

MDPI

Article

Hodgkin Lymphoma Cell Lines and Tissues Express mGluR5: A Potential Link to Ophelia Syndrome and Paraneoplastic Neurological Disease

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Abstract: Ophelia syndrome is characterized by the coincidence of severe neuropsychiatric symptoms, classical Hodgkin lymphoma, and the presence of antibodies to the metabotropic glutamate 5 receptor (mGluR5). Little is known about the pathogenetic link between these symptoms and the role that anti-mGluR5-antibodies play. We investigated lymphoma tissue from patients with Ophelia syndrome and with isolated classical Hodgkin lymphoma by quantitative immunocytochemistry for mGluR5-expression. Further, we studied the L-1236, L-428, L-540, SUP-HD1, KM-H2, and HDLM-2 classical Hodgkin lymphoma cell lines by FACS and Western blot for mGluR5-expression, and by transcriptome analysis. mGluR5 surface expression differed significantly in terms of receptor density, distribution pattern, and percentage of positive cells. The highest expression levels were found in the L-1236 line. RNA-sequencing revealed more than 800 genes that were higher expressed in the L-1236 line in comparison to the other classical Hodgkin lymphoma cell lines. High mGluR5-expression was associated with upregulation of PI3K/AKT and MAPK pathways and of downstream targets (e.g., *EGR1*) known to be involved in classical Hodgkin lymphoma progression. Finally, mGluR5 expression was increased in the classical Hodgkin lymphoma-tissue of our Ophelia syndrome patient in contrast to five classical Hodgkin lymphoma-patients without autoimmune encephalitis. Given the association of encephalitis and classical Hodgkin lymphoma in Ophelia syndrome, it is possible that mGluR5-expression in classical Hodgkin lymphoma cells not only drives tumor progression but also triggers anti-mGluR5 encephalitis even before classical Hodgkin lymphoma becomes manifest.

Keywords: metabotropic glutamate 5 receptor; anti-mGluR5 encephalitis; neuroimmunology; pediatric neurology; pediatric oncology; transcriptome analysis; Hodgkin lymphoma; Ophelia syndrome

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1. Introduction

Classical Hodgkin lymphoma is a B-cell lymphoma characterized by the presence of a few giant multinucleated Hodgkin and Reed–Sternberg cells [1]. These cells make up less than 1% of the infiltrated lymphoid tissue and are surrounded by inflammatory cells that form the tumor microenvironment [1,2]. Hodgkin and Reed–Sternberg cells express the CD30 surface marker and thereby define classical Hodgkin lymphoma. The disease is divided into the subtypes (i) nodular sclerosis, (ii) mixed cellularity, (iii) lymphocyte-depleted, and (iv) lymphocyte-rich, which represent the majority of Hodgkin lymphoma [2].

Although Hodgkin lymphoma accounts for only 5–6% of all childhood cancers, it is the most frequent neoplasia in adolescents and young adults between 15 and 19 years of age with a second peak in elderly individuals [3–5].

Classical Hodgkin lymphoma is not only known to be associated with viral infections and autoimmune diseases [6–8], but also with atypical immune-mediated phenomena such as paraneoplastic neurological syndromes [9–11]. These are often caused by an antibody-mediated response against so-called "onconeural" antigens, e.g., antigens that are expressed by both the nervous system and tumor [12]. Thus, ectopic expression of these antigens by classical Hodgkin lymphoma may trigger a misdirected autoimmune response against neuronal structures due to molecular mimicry [13,14]. Paraneoplastic neurological symptoms can precede, accompany, or occur in the wake of classical Hodgkin lymphoma [6,15,16]. Examples are the paraneoplastic cerebellar degeneration (PCD) syndrome, subacute cortical cerebellar degeneration (SCCD), and limbic encephalitis (LE) [9,14,17]. A form of the latter was first described as "Ophelia syndrome" by Ian Carr (1982) [14,18–20]. It primarily and exceedingly affects children and young adults, who develop severe psychosis with extensive hallucinations, behavioral changes, cognitive dysfunction, seizures, movement disorders, and sleep disturbance [10,18,21,22]. Lancaster et al. (2011) described pathogenic autoantibodies in Ophelia syndrome that target the metabotropic glutamate receptor 5 (mGluR5) and cause a decrease in mGluR5 density on neurons [19]. Early recognition of this anti-mGluR5 encephalitis by cerebrospinal fluid antibody screening is crucial, as patients respond favorably to antibody removal. Most patients recover and benefit from thorough follow-up with early detection of classical Hodgkin lymphoma [18,19]. Although lymphoma occurs in half of all Ophelia cases, there are no reports that have investigated the pathophysiological or functional association between anti-mGluR5 encephalitis and classical Hodgkin lymphoma [18]. Therefore, we set out (i) to investigate the expression of mGluR5 in patients with classical Hodgkin lymphoma (some with and some without a prior encephalitis), (ii) to investigate mGluR5 positivity on $CD30^+$ Hodgkin and Reed–Sternberg cells from biopsy material of these patients, and (iii) to analyze the transcriptome of various established classical Hodgkin lymphoma cell lines for a correlation between mGluR5 mRNA expression and downstream target activation that might promote tumor growth.

2. Materials and Methods

2.1. Patient

We diagnosed anti-mGluR5 encephalitis (Ophelia syndrome) in a 15-year-old boy who presented with acute psychosis, severe encephalopathy, and autonomic dysregulation. Antibody screening revealed the presence of anti-mGluR5 IgG-antibodies in cerebrospinal fluid and serum (Figure 1A,B). Full recovery was only achieved after 22 weeks of ICU treatment and immunotherapy including methylprednisolone, IV immunoglobulins, immunoadsorption, IV and intrathecal rituximab as well as IV bortezomib application. At the time of Ophelia syndrome manifestation, we extensively investigated our patient for the presence of Hodgkin lymphoma including serial abdominal ultrasounds, chest X-ray, whole body MRI, and PET-MRI, as well as laboratory investigations (βHCG, NSE, LDH, uric acid), but did not detect any abnormalities. However, sixteen months later, the patient developed advanced classical Hodgkin lymphoma of the nodular sclerosing subtype, which responded to EuroNet-PHL protocol treatment [23], obtaining complete remission.

2.2. Immunological Studies on Tumor Tissue from Patients and Controls

We examined samples obtained from the lymph nodes of the index patient and tissue from n = 5 age-matched classical Hodgkin lymphoma-cases without autoimmune encephalitis. Staining was performed on 3-µm tissue sections from formalin-fixed, paraffin-embedded (FFPE) lymph node biopsy specimens using an automated slide staining system (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA). We performed heat-induced epitope retrieval (Cell Conditioning 1, Ventana), primary staining with anti-mGluR5 (1:250, Cat# PA5-33823, ThermoFisher, Waltham, MA, USA, or Cat# ab27190, Abcam, Cambridge,

UK) and automated secondary antibody staining with the DAB detection kit (iVIEW, Ventana Medical Systems).

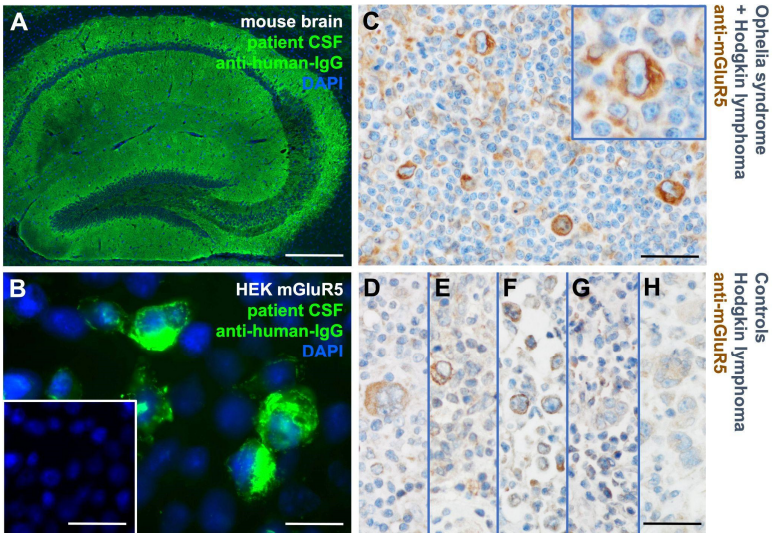

Figure 1. Detection of anti-mGluR5 antibodies in the cerebrospinal fluid and of mGluR5 protein expression in the tumor cells of a patient with Ophelia syndrome. (**A**) Immunostaining of fresh frozen mouse hippocampus with the patient's cerebrospinal fluid depicts reactivity typical for anti-mGluR5 antibodies. (**B**) Patient's cerebrospinal fluid immunostaining on mGluR5-expressing HEK293T cells shows clear reactivity in contrast to control (inset). (**C**) ICC with commercial anti-mGluR5 antibodies on a tumor biopsy specimen of the patient after developing classical Hodgkin lymphoma reveals mGluR5^{+} Hodgkin and Reed–Sternberg cells. The inset depicts a magnified multinuclear Reed–Sternberg cell. (**D**–**H**) Biopsy specimens from patients with classical Hodgkin lymphoma but without encephalitis (controls) demonstrate only weak or absent anti-mGluR5 reactivity. Size bars: 100 µm (**A**, inset of **B**, **C**–**H**), 20 µm (**B** and inset of **C**).

2.3. Cell Lines and Culture Conditions

We used standard cell lines from classical Hodgkin lymphoma patients of the nodular sclerosing (L-428, HDLM-2, L-540, SUP-HD1) or mixed cellularity (KM-H2, L-1236) subtypes, established from pleural effusions (HDLM-2, KM-H2, L-428, SUP-HD1), bone marrow (L-540), or peripheral blood (L-1236) [24–29]. Jurkat cells served as negative control. The cells were cultured in RPMI-1640 GlutaMAX (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained in a 5% CO_2 humidified atmosphere at 37 °C. The source of the classical Hodgkin lymphoma cell lines and the links to the accompanying data sheets are provided in the Supplementary Material section.

2.4. Immunocytochemistry—Surface Antigen Labeling and Quantification

We performed immunocytochemistry (ICC) with patient cerebrospinal fluid on fresh-frozen murine brain sections and on mGluR5-expressing HEK293T cells as described previously [30,31]. Further, we seeded 5×10^5 classical Hodgkin lymphoma cells on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated coverslips in 12-well plates. The cells were fixed with 4% paraformaldehyde, blocked with 5% normal goat serum and 2% bovine serum albumin for 1 h at RT and incubated with the primary antibodies against mGluR5 (1:250, Cat# ab76316, Abcam, Cambridge, UK) and CD30 (1:80, Ber-

H2, Cat# M0751, Dako, Jena, Germany) O/N at 4°C. Secondary staining was performed with a fluorophore-conjugated anti-rabbit-IgG antibody (Alexa Fluor 488, 1:1000, Cat# A-11008, Invitrogen, Waltham, MA, USA) and anti-mouse antibody (Alexa Fluor 568, 1:250, Cat# A-11004, Invitrogen) for 1 h followed by incubation with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000; Cat# D1306, Invitrogen). Fluorescence was recorded using a THUNDER Imager DMi8 with a Leica DFC9000 GT camera and the LAS(X) software (Leica Microsystems, Wetzlar, Germany). The imaging parameters (illumination light intensity, aperture, exposure time, and camera sensitivity) were kept strictly constant for all recordings. For comparison of anti-mGluR5 and anti-CD30 staining intensities on the classical Hodgkin lymphoma cell lines, we generated four visual fields with a 20× microscope lens on the blue channel (DAPI), the green channel (mGluR5), and the red channel (CD30). We analyzed the layered images using the Fiji/ImageJ v.2.3.0/1.53 software. We used the ROI of the nuclear DAPI signal to find the single cells and radially extended the respective ROIs by 1 µm to also cover the cytosol and cell membrane. We then recorded the integrated densities over all ROIs for each fluorophore from a total of 1000 cells per sample. For this analysis, we started with the cells on the top left and worked our way down the image until we had gathered 1000 cells. The absolute number of the analyzed cells had to be kept constant for statistical reasons. The distributions of the integrated density values were visualized on a dotplot using the R version 4.2.2 from within the RStudio v.1.4.1106 software (Figure 2C).

2.5. Western Blot

We extracted protein with cell lysis RIPA Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete® protease inhibitors). Samples were treated with 4× LDS loading buffer (NuPAGE®) and DTT (NuPAGE® sample reducing agent). A 150-µg sample of each classical Hodgkin lymphoma cell line and Jurkat cells (negative control) were loaded onto a gradient 4–12% NuPAGE® Bis-Tris gel (MES Running buffer; 150 V; XCell Surelock Mini-cell) without boiling. The blots were incubated with primary anti-mGluR5 (monoclonal rabbit antibody, 1:1000, Cat# ab76316, Abcam) and anti-α-Tubulin (1:1000, Cat# MCA77G, Bio Rad, Hercules, CA, USA) antibodies O/N at 4 °C and stained with the corresponding HRP-labeled secondary antibodies: anti-rabbit-IgG (1:2000, Cat# DC03L, Calbiochem, San Diego, CA, USA) and anti-rat-IgG (1:2000, Cat# DC01L, Calbiochem). For detection, we used ECL (Amersham plc, Amersham, UK) and a gel imager (VWR Image Capture Software, Radnor, PA, USA). For verification of the characteristic mGluR5 banding pattern, we loaded protein extracts from the L-1236 Hodgkin lymphoma cell line and 1 µg of mouse brain (positive control) side-by-side and performed a Western blot as described above (Supplementary Figure S1).

2.6. Quantitative Reverse-Transcription Quantitative Polymerase-Chain-Reaction

We performed reverse-transcription quantitative polymerase-chain-reactions (RT-qPCR) for relative quantification of *GRM5* gene expression on an ABI 7000 Prism sequence detection system (Applied Biosystems, Waltham, MA, USA) with triplicates in three independent reactions for each sample. Each RT-qPCR reaction contained transcript-specific oligonucleotide primers (for primer sequences see Supplementary Table S1), cDNA of the cell lines of interest, and SYBR Green Master Mix (#4309155; Life Technologies, Carlsbad, CA, USA) in a 20 µL volume. The following cycling conditions were used: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and a final ramp from 60–90 °C for melting curve recording. For generation of a standard curve we prepared a 1:10 serial dilution of 16 steps from the respective PCR-products and ran them under the above conditions. For calculation of the PCR-efficiency we used the n = 7 dilutions that provided the best linear regression line. Relative target gene expression was calculated by the efficiency corrected $\Delta\Delta C_t$ method by Pfaffl et al. (2001) using *HPRT* as reference gene [32].

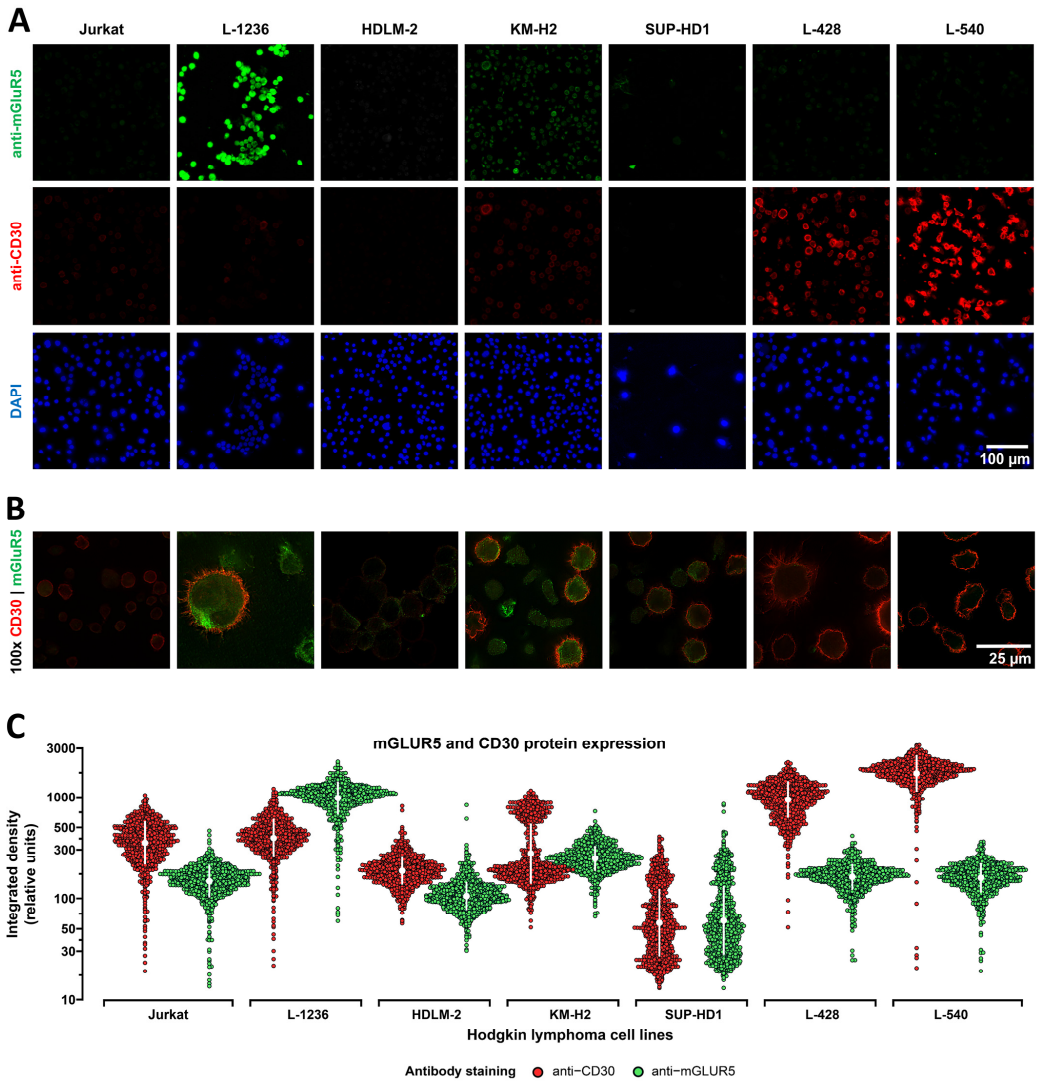

Figure 2. Comparative analysis and quantification of mGluR5 immunostaining in classical Hodgkin lymphoma cell lines. (**A**) Immunostaining of classical Hodgkin lymphoma cell lines using exactly the same imaging parameters to be able to distinguish between the various expression levels for CD30 (red) and mGluR5 (green). Nuclei were stained by DAPI (blue). Jurkat cells, as negative controls, expressed very low levels of CD30 and mGluR5. (**B**) Co-immunostaining of anti-CD30 (red) and anti-mGluR5 (green). (**C**) Dotplots generated with the ggbeeswarm::geom_beeswarm() feature of R!. Staining intensities were given as relative integrated density units of the 8 bit b/w images. A total of 1000 cells from each sample were plotted; the white dot depicts the mean and the white whiskers the SD. The *Y*-axis is adjusted to a log10 scale.

2.7. Fluorescence-Activated Cell Sorting (FACS)

For FACS analysis, a standard staining procedure was applied. Each classical Hodgkin lymphoma cell line was harvested, washed, and resuspended in FACS buffer (PBS, 10% FBS, 0.1% NaN_3) to a concentration of 5×10^6 cells/mL. Cells The cells were incubated with primary antibodies against mGluR5 (1:250, Cat# ab27190, Abcam, Cambridge, UK) and CD30 (1:80, Ber-H2, Cat# M0751, Dako, Jena, Germany) for 2 h on ice. The cells were washed thrice with 1× PBS. The secondary antibodies (anti-rabbit-IgG antibody, Alexa Fluor 488, 1:1000, Cat# A-11008, Invitrogen, Waltham, MA, USA and anti-mouse antibody, Alexa Fluor 568, 1:250, Cat# A-11004, Invitrogen) were added and incubated for 2 h on ice. The labeled cells were washed thrice with 1× PBS, centrifuged each time at 500× *g* for 5 min at 4 °C, and resuspended in 500 µL of ice-cold FACS buffer for flow cytometry. The cells were double-labeled for Hodgkin lymphoma tumor marker CD30 and for cell surface receptor mGluR5. The gating strategy comprised the exclusion of dead cells and debris (forward and side scatter) as well as doublets (plotting height and width against area), and aimed for the double-positive ($mGluR5^+ | CD30^+$) cells, using a fluorescence activated cell sorter (FACS) Aria II (Beckton Dickinson, Heidelberg, Germany).

2.8. Gene Expression Profiling

We extracted total RNA from all six classical Hodgkin lymphoma cell lines (5×10^5 cells each) using the NucleoSpin® NA Plus Kit (Macherey and Nagel, Düren, Germany). The quality of the RNA was controlled via the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA-sequencing was performed on a $polyA^+$ enriched cDNA library on the BGISEQ-500 RNA-Sequencing pipeline (Beijing Genomics Institute, Hong Kong) yielding >40 Mio FASTQ 100 paired-end stranded reads that were quality controlled using FastQC v0.11.8 and then aligned to the human reference sequence (GRCh37.75) with the STAR 2.4.0.1 aligner [33]. The resulting BAM files were further investigated and normalized with the StringTie v2.2.1 pipeline [34,35], which yielded the relative mRNA quantities for each transcript present in the transcriptome dataset. As a quantitative measure for gene abundance, we used the FPKM value (fragments per kilobase of transcript per million mapped reads), which is calculated by the StringTie software. To control for multiple testing in the gene expression studies, we calculated the false discovery rate (FDR) [36]. Gene expression differences between the n = 1 "*GRM5* high-expressing" (L-1236) and the n = 5 "*GRM5* low-expressing" (L-428, L-540, SUP-HD1, KM-H2, HDLM-2) cell lines were considered significant if the up- or down-regulation was by a factor of five or larger and the FDR was <0.05. Differential gene expression yielded over 800 genes that were upregulated by >10-fold in L-1236. The genes of interest were annotated using the database for Annotation, Visualization, and Integrated Discovery (DAVID) tool [37].

2.9. Statistics

Quantitative data are presented as the mean ± SD of results obtained from at least three independent experiments. Significance levels of ICC signal variation, RT-qPCR relative expression levels, and Western blot densities were determined by one-way analyses of variance (ANOVA) and Tukey's Multiple Comparison Test. A probability of < 0.05 was considered statistically significant, indicated in the graphs as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); n.s., not significant. Statistical analyses and graphing were performed with RStudio version 1.4.1106, and R version 4.2.2 software packages. Image J, and GraphPad Prism v9.2 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. mGluR5 Is Expressed on Hodgkin Lymphoma Tissue at Varying Levels

To investigate a possible link between encephalitis and classical Hodgkin lymphoma, we performed immunostaining with anti-mGluR5 antibodies on tumor biopsy specimens from our patient with Ophelia syndrome and in n = 5 classical Hodgkin lymphoma patients without autoimmune encephalitis.

The index patient's tumor revealed an intensive mGluR5 signal on classical Hodgkin lymphoma cells (Figure 1C). The controls showed a heterogeneous immunoreactivity pattern with far fewer positive cells and weaker intensities (Figure 1D–H). These initial findings prompted our further examination of mGluR5 expression in classical Hodgkin lymphoma cell lines.

3.2. Heterogeneous mGluR5 Expression Patterns

To characterize anti-mGluR5 staining intensities and distributions, as well as mGluR5/*GRM5* expression levels, we investigated six classical Hodgkin lymphoma cell lines. Jurkat cells served as controls. We performed immunofluorescence co-staining of mGluR5 and CD30 and were able to detect mGluR5 predominantly in the L-1236 and, to a lesser extent, in the KM-H2 cell lines. In the other cell lines, we mainly recorded low background fluorescence (Figure 2). Immunostaining of the L-1236 line revealed a specific distribution of the mGluR5 signals not seen in the other cell lines, with mGluR5 appearing to form a cluster on the cell surface, whereas a more even cell membrane distribution was seen in the KM-H2 line (100× magnification on Figure 2B; for 3D-reconstruction see Supplementary Figure S2). In the L-1236 and KM-H2 lines, anti-mGluR5 signals varied considerably, and cells that were anti-CD30 positive showed a tendency for higher anti-mGluR5 staining intensities (Figure 2A,B). The anti-CD30 staining also varied across the cell lines. The highest percentages of anti-CD30 positive cells were seen in the L-428 and L-540 lines, which had the lowest anti-mGluR5 signals (Figure 2).

In summary, immunostaining detected strong, moderate, and weak/absent staining intensities, depending on the cell line. Clearly detectable immune signals in the majority of cells were only seen in the L-1236 and KM-H2 cell lines. The overall staining intensities of the HDLM-2, SUP-HD1, L428, and L-540 lines were very low (in the range of background fluorescence). The distribution pattern of mGluR5 on the cell surface varied considerably between the different cell lines but also between single cells of the same line. A similar tendency of non-uniform staining, with the exception of the L-540 line, was seen for the CD30 marker. This staining pattern is typical for classical Hodgkin lymphoma and shows that not every tumor cell expresses this characteristic marker permanently [38,39].

To further quantify mGluR5/*GRM5* expression in classical Hodgkin lymphoma cells, we extended our investigations using immunoblotting and RT-qPCR (Figure 3A–E). On the protein level, we detected two major bands: one at ~150 kDa corresponding to the known monomeric form of mGluR5 and another at ~280 kDa corresponding to the disulfide-bond linked dimeric form of mGluR5 [40,41]. Considerably weaker bands at 140 kDa and 270 kDa were seen in all cell lines, including the Jurkat controls, and were thus regarded as non-specific (Figure 3A). Overall, mGluR5 protein abundance was by far highest in the L-1236 line ($p < 0.0001$) compared with all other classical Hodgkin lymphoma cell lines.

In RT-qPCR, the mGluR5 encoding *GRM5* gene could be consistently amplified only in the two L-1236 and KM-H2 lines. In the RNA-sequencing experiment, this high variance in GRM5 mRNA copy numbers was confirmed (Figure 4A).

These findings encouraged us to proceed with flow cytometry to quantify mGluR5-positive cells in all six classical Hodgkin lymphoma cell lines in an unbiased manner. Looking at the FACS data from the L-1236 line, a subpopulation of double-positive ($mGluR5^+ | CD30^+$) cells was easily distinguishable (Figure 3F), amounting to 10% of all viable cells. In the other classical Hodgkin lymphoma cell lines, either no distinct subpopulation of double-positive cells was found (L-428, HDLM-2), or the subpopulation was barely visible and the percentage of $mGluR5^+ | CD30^+$ cells was significantly lower (below 6% for L-540, KM-H2, and SUP-HD1) (Figure 3F, Supplementary Figure S3).

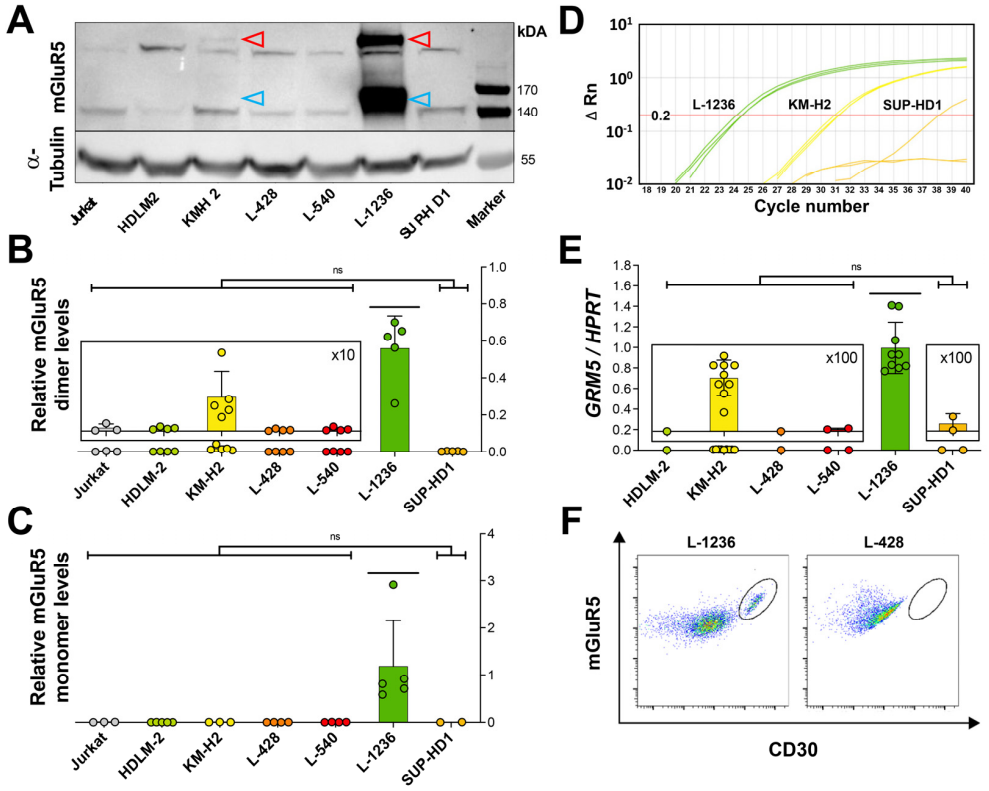

Figure 3. Relative mGluR5 protein levels and *GRM5* mRNA expression. (**A**) Representative Western blot with different mGluR5 expression levels on six classical Hodgkin lymphoma cell lines. The anti-mGluR5 antibody detects two specific bands: one band of higher molecular weight at ~280 kDa representing the mGluR5 dimer and its monomeric form at ~150 kDa. Signal intensities of dimeric (**B**) and monomeric (**C**) mGluR5 from three immunoblots were normalized to α-Tubulin expression. The L-1236 cell line (green) shows by far the highest expression of mGluR5. Jurkat cells serve as a negative control. (**D**) Amplification plot of RT-qPCR from classical Hodgkin lymphoma cell lines, depicting consistent amplification of the *GRM5* target gene only in the L-1236 and KM-H2 lines in all three RT-qPCR reactions and in SUP-HD1 in only one out of three RT-qPCR reactions with a high C*t* value. Fluorescence is plotted on a log-scale with ΔRn (horizontal lines) against cycle numbers (C*t* value) (vertical lines). The threshold for detection was set at ΔRn = 0.2. (**E**) Large variations in *GRM5* expression determined by RT-qPCR. L-1236 shows by far the highest amount of *GRM5* per *HPRT* mRNA copy number. (**F**) Representative dot plots from flow cytometry analysis with the six classical Hodgkin lymphoma cell lines. L-1236, but not L-428, shows a distinct mGluR5$^+$ | CD30$^+$ subpopulation (gate) (after exclusion of dead cells and doublets). All bars represent the mean and SD of three independent experiments. ns = not significant. Significance values were calculated with one-way ANOVA followed by Tukey's multiple comparison test.

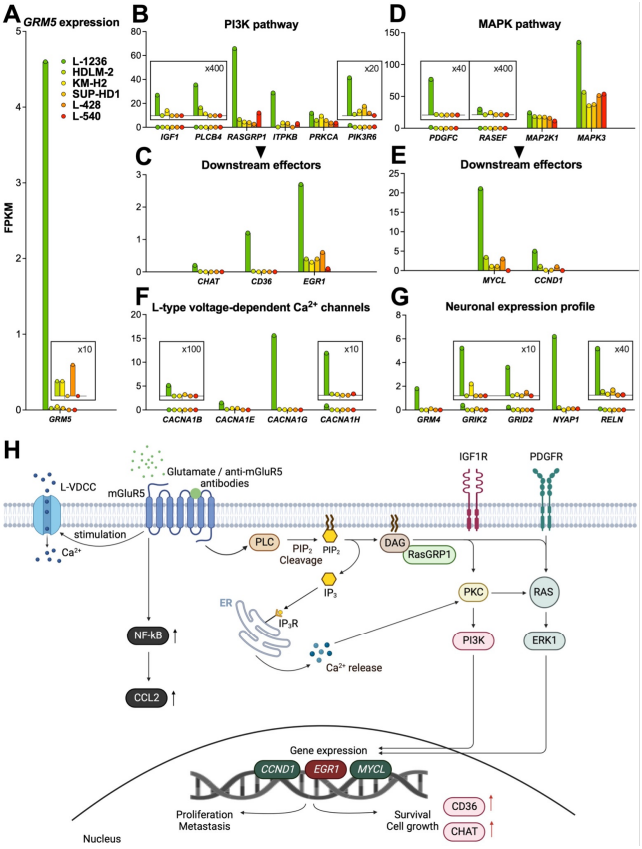

Figure 4. *GRM5*-related gene expression profile of classical Hodgkin lymphoma cell lines. RNA-sequencing data of classical Hodgkin lymphoma cell lines on *GRM5* expression and activation of mGluR5-related pathways. (**A**) L-1236 (green) shows by far the highest *GRM5* expression. Representative genes from PI3K (**B**) and MAPK (**D**) pathways with respective downstream effectors (**C**,**E**), calcium signaling (**F**), and neuronal expression profiles (**G**) are most activated in L-1236 (green). *Y*-axes are adjusted to considerably differing FPKM values. For details see magnified insets (scaling factors are indicated). (**H**) A schematic overview displays the known signal transduction pathways that are activated by mGluR5 in L-1236 according to our RNA-sequencing data. Expression of mGluR5 and activation by its corresponding ligands (e.g., L-glutamate or anti-mGluR5 antibodies) results in coupling with G-protein (not shown) and activation of protein lipase C (PLC). This is followed by the (over-)activation of signaling pathways promoting cell survival (PI3K), upregulating cell proliferation (MAPK), and increasing Ca^{2+}-influx via L-type voltage-dependent Ca^{2+} channels (LVDCCs) leading to downstream modifications, all of which are implicated in classical Hodgkin lymphoma progression. CCND1, Cyclin D1; ChAT, Choline acetyltransferase; DAG, Diacylglycerol; EGR1, Early growth response 1; ERK1, Extracellular signal-regulated kinase 1; IP3, Inositol 1,4,5-triphosphate; IP3R, Inositol 1,4,5-triphosphate receptor; PIP2, Phosphatidylinositol 4,5-bisphosphate; PKC, Protein kinase C; PI3K, Phosphoinositide 3-kinase; RasGRP1, Ras guanyl-releasing protein 1. Created with BioRender.com.

Overall, immunohistochemistry, Western blot, RT-qPCR, and FACS consistently indicate heterogeneous mGluR5 expression, which was exceptionally high in the L-1236 line compared with the other classical Hodgkin lymphoma cell lines.

To verify the very heterogeneous *GRM5* expression in a larger number of classical Hodgkin lymphoma patients at the population level, we reanalyzed published gene expression data (Affymetrix microarray data) from a n = 130 cohort of patients with classical Hodgkin lymphoma [42]. In a subset of n = 6 patients (~5%), we detected a high expression of *GRM5* (Supplementary Figures S2 and S4). These results confirm the findings from our analysis of classical Hodgkin's lymphoma cell lines, but do not allow us to draw conclusions about the exact pathophysiology of Ophelia syndrome because we lack the anamnestic and clinical data from this large cohort, particularly with regard to the presence of autoimmune encephalitis.

3.3. mGluR5 Expression Is Correlated with Sustained Activation of Glutamatergic Signaling Pathways

To investigate whether the highly variant mGluR5 expression would have an effect on cell physiology, we performed RNA-sequencing of all six classical Hodgkin lymphoma cell lines. The gene expression profile of L-1236 was markedly different from that of the other cell lines, and data analysis revealed that together with *GRM5*, more than 800 genes were upregulated (≥10-fold). However, comparing the expression pattern of a single cell line (L-1236 in this case) with five other cell lines could lead to random results that would not have occurred if more cell lines of each type had been examined. Therefore, the results below should be interpreted with caution.

For further analysis, we grouped the genes by G-protein coupled receptor (GPCR)-related signaling pathways (Supplementary Table S3). Most strikingly, several genes involved in mGluR5-linked signal transduction were overexpressed, indicating hyperactivity of two fundamental pathways (PI3K and MAPK) that control many processes essential for tumor growth and survival (Figure 4A–H). In the following paragraphs, we depict the fold increase in mRNA transcript numbers (given as FPKM values) of L-1236 over the average expression in the remaining n = 5 classical Hodgkin lymphoma cell lines. In summary, we identified upregulation of several genes, particularly of the PI3K and MAPK pathways, as a characteristic signature of the L-1236 cell line.

3.3.1. PI3K Pathway

We detected upregulation of *PLCB4* (Phospholipase Cβ4, 15.6-fold upregulation), which is directly involved in mGluR5-related signal transduction by cleaving phosphatidylinositol-4,5-diphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [43,44]. The DAG-regulated nucleotide exchange factor RASGRP1 (Ras guanyl releasing protein 1) specifically activates Ras [45]. IP3 binds to its corresponding receptor (IP3R) at the endoplasmic reticulum (ER) resulting in Ca^{2+} release into the cytoplasm [43]. Both the elevated intracellular Ca^{2+} and DAG activate protein kinase C (PKC), which in turn activates Ras, followed by ERK1 phosphorylation. We found upregulation of *RASGRP1* (11.1-fold upregulation), *ITPKB* (Inositol 1,4,5-triphosphate receptor, IP3R; 13.9-fold upregulation) and *PIK3R6* (Phosphoinositide 3-kinase, PI3K; 10.1-fold upregulation). Elevated gene expression of the downstream targets *EGR1* (Early growth response 1, EGR1; 7.5-fold upregulation), *CHAT* (Choline acetyltransferase, ChAT; 112.4-fold upregulation) and *CD36* (208.5-fold upregulation) confirmed activation of the PI3K pathway.

Growth factors are key constituents that help sustain G-protein coupled receptor (GPCR) signaling. *IGF1* (Insulin-like growth factor, IGF1; 15.4-fold upregulation) is an upstream regulator of PI3K and was upregulated as well, suggesting receptor binding and activation upon enhanced mGluR5 expression (Figure 4B,C).

3.3.2. MAPK Pathway

The MAPK cascade is overactive in about one-third of all human cancers [46]. Inhibition of components of this cascade by targeted inhibitors represents an important

antitumor strategy [46]. mGluR5-dependent overexpression was found in *PDGFC* (platelet-derived growth factor C, 332.7-fold upregulation), *RASEF* (RAS and EF-hand domain containing protein, 13.4-fold upregulation), as well as downstream effectors *MAP2K1* (mitogen-activated protein kinase kinase 1, MEK1, 1.5-fold upregulation), *MAPK3* (extracellular signal-regulated kinase 1, ERK1, 2.9-fold upregulation), nuclear transcription targets *MYCL* (L-Myc proto-oncogene BHLH transcription factor, 12.4-fold upregulation), and *CCND1* (cyclin D1, 10.7-fold upregulation) that are all involved in the MAPK pathway (Figure 4D,E).

3.3.3. Calcium Signaling

Activation of mGluR5 followed by membrane depolarization through Ca^{2+}-release opens L-type voltage-dependent Ca^{2+}-channels (L-VDCCs) [47]. This implies a crosstalk between mGluRs, intracellular Ca^{2+} and membrane Ca^{2+}-channels.

We found L-VDCC genes, including *CACNA1B* (29.7-fold upregulation), *CACNA1E* (10.5-fold upregulation), *CACNA1G* (137.5-fold upregulation), and *CACNA1H* (54.4-fold upregulation) (Calcium voltage-gated channel subunit α-1B, E, G, and H, respectively) were upregulated in L-1236 along with high *GRM5* expression (Figure 4F).

3.3.4. NF-kB Pathway

Classical Hodgkin lymphoma is also characterized by a high constitutive activity of the NF-kB pathway. Examining genes involved in this pathway, we found a significant overexpression of *CCL2* (C-C chemokine ligand 2, 14.4-fold upregulation, Supplementary Table S3) in L-1236.

4. Neuronal Expression Profile

Associated with a high expression of *GRM5*, we detected upregulation of other genes involved in glutamatergic signaling, including *GRM4*, which is a member of group III mGluRs. In contrast to the other cell lines, L-1236 expressed ionotropic glutamate receptors (iGluRs), including *GRIK2* (glutamate ionotropic receptor kainate type subunit 2) and *GRID2* (glutamate ionotropic receptor delta type subunit 2). Interestingly, genes normally exclusively turned on in neurons, were also upregulated in L-1236. These included *NYAP1* (neuronal tyrosine-phosphorylated phosphoinositide 3-kinase adaptor 1) that regulates neuronal morphogenesis and *RELN* (Reelin, extracellular matrix glycoprotein), a regulator of neuronal migration, which also activates N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) (Figure 4G) [48,49].

5. Discussion

Previous work has focused on the identification of mGluR5 as a neuronal antigen target of autoantibodies in Ophelia syndrome, a disorder characterized by classical Hodgkin lymphoma in association with anti-mGluR5 mediated encephalitis [18,19]. However, little attention has been paid to the question, whether mGluR5 can be found in classical Hodgkin lymphoma tumor tissue. Thus, the aim of this study was to search for mGluR5 expression in classical Hodgkin lymphoma cells and gain a more in-depth understanding of the link between tumor growth and autoimmunity.

In neurons, stimulation of mGluR5 activates the PI3K and MAPK pathways and causes increased Ca^{2+}-influx into the cytosol [50,51]. In classical Hodgkin lymphoma, these glutamatergic signaling cascades are the most frequently dysregulated ones [52–54], with glutamate stimulating proliferation and migration of tumor cells via GPCR activation [44].

Our study shows that the two fundamental pathways PI3K and MAPK with PDGFC (platelet-derived growth factor C), in conjunction with the increased recruitment of its receptor PDGFR (platelet-derived growth factor receptor), which in turn also enhances MAPK signaling [55,56], were upregulated along with high mGluR5 expression in classical Hodgkin lymphoma cell lines. In addition, we detected upregulation of downstream

targets of the PI3K and MAPK pathways that are known to be generally involved in tumor progression (Figure 4H). High expression of the surface protein CD36 [57], the transcription factors Early Growth Response Protein 1 [58], Cyclin D1 [59], and L-Myc [60,61] and of the enzyme ChAT [62,63] have been linked to tumor progression, poor survival and increased metastasis in several cancers, including melanoma [59] and recently also in lymphoma [60,61].

ChAT is a critical enzyme for acetylcholine synthesis. Acetylcholine can be produced in lung and colon cancer cells and acts as an autocrine and paracrine growth factor [62,63]. High *CHAT* overexpression ($\geq$100-fold), as shown in L-1236, may result in non-neuronal production and release of acetylcholine by Hodgkin and Reed–Sternberg cells, thereby self-promoting lymphoma growth.

Transcription factor L-Myc, a member of the Myc-proto-oncogene family, is amplified and overexpressed in 70% of all human malignancies including classical Hodgkin lymphoma. Its upregulation contributes to uncontrolled cell proliferation, survival, and escape from immune surveillance [60,61]. As classical Hodgkin lymphoma shares many characteristics with acute inflammatory processes, NF-kB signaling plays a major role by mediating the immediate-early expression of various cytokines including the chemokine CCL2 [56]. Upregulation of *CCL2*, as shown in our data (Supplementary Table S3), has been associated with cancer advancement, metastasis, and relapse [64,65].

In addition to this, other glutamate receptors are differentially expressed in tumors [66–68], e.g., mGluR1 in melanoma [55] or mGluR3 in malignant gliomas and mGluR4 in medulloblastomas [66]. Activation of these receptors stimulates tumor growth and development of metastasis in a MAPK-dependent manner [66]. Moreover, investigations showed that glutamate receptor antagonists, including the potent mGluR3 antagonist LY341495, limit tumor growth [66,69]. Although mGluR5 was not considered an oncogene previously, it has recently been found to play an important role in promoting tumor growth, e.g., in melanoma and oral squamous cell carcinoma [70–72], and to have an influence on astrocyte proliferation upon chemical and mechanical injury [73].

Besides these data on various tumors, non-malignant immune cells, e.g., B- and T-lymphocytes also show baseline expression of mGluR5 supporting our findings [74]. This indicates that the basic machinery required for mGluR5 signaling is already present prior to the development of Hodgkin and Reed–Sternberg cells and the transformation to classical Hodgkin lymphoma. Interestingly, our analysis also showed several neuronal genes activated in L-1236, suggesting that some classical Hodgkin lymphoma cells might exploit neuronal and neurodevelopmental pathways for tumorigenesis. Consistent with this, studies have reported that tumors behave more aggressively when expressing genes related to the nervous system [75,76].

The six classical Hodgkin lymphoma cell lines L-1236, HDLM-2, KM-H2, SUP-HD1, L-428, and L-540 investigated in this study were established between the 1970s and 1990s. They have been used extensively as model systems in research for many years and are considered as "the classical Hodgkin lymphoma cell lines" [77]. The L-1236 line was obtained from the peripheral blood of a 31-year-old patient diagnosed with classical Hodgkin lymphoma of mixed cellularity subtype, who developed an advanced disease with rapid progression and multiple relapses [28]. Unlike our index patient with Ophelia syndrome, no neurologic or psychiatric symptoms suggestive of encephalitis were reported from the L-1236 donor [28]. However, data on the clinical course and medical history of that patient are incomplete and signs of previous paraneoplastic neurological symptoms may have gone unnoticed or unreported. In any case, both our patient and the L-1236 donor suffered from advanced stage disease, an aggressive clinical course. The strong expression of mGluR5 and its downstream effectors (e.g., *MYCL* and *EGR1*) may have contributed to the aggressive clinical course.

Given the observed occurrence of classical Hodgkin lymphoma in the wake of half of all reported Ophelia syndromes and reports of additional cases of classical Hodgkin lymphoma with preceding paraneoplastic symptoms before [78–81], we believe it is possible

that mGluR5 expression on classical Hodgkin lymphoma cells not only drives tumor progression but also plays a role in triggering anti-mGluR5 encephalitis early during tumor development, long before classical Hodgkin lymphoma is clinically detected.

However, classical Hodgkin lymphoma does not follow in all cases of anti-mGluR5 encephalitis, and conversely, Ophelia syndrome occurs in only a minority of patients with classical Hodgkin lymphoma. According to our reanalysis of a large mRNA expression dataset of Hodgkin tumor tissues, high mGluR5 expression does not seem to be all that rare in classical Hodgkin lymphoma. We hypothesize that other factors must play a role in the manifestation of Ophelia syndrome. Such factors may include the histological subtype, mechanisms of immune tolerance or preceding viral infections, which may trigger both encephalitis and subsequent tumor progression as a "second hit". Further prospective studies are needed in cases of paraneoplastic neurological disease and/or classical Hodgkin lymphoma with respect to mGluR5 expression on tumor cells.

6. Conclusions

In recent years, significant progress has been made in our understanding of Ophelia syndrome. However, the underlying molecular mechanisms leading to both anti-mGluR5 encephalitis and classical Hodgkin lymphoma were poorly understood. This study contributes to their understanding and may have clinical implications.

Detection of paraneoplastic neurological disease preceding classical Hodgkin's lymphoma can be challenging [21]. However, early diagnosis of an Ophelia syndrome is paramount both for the treatment of anti-mGluR5 encephalitis and the earliest possible recognition of a subsequent classical Hodgkin lymphoma. Based on our findings, we recommend (i) thorough follow-up and close screening for classical Hodgkin lymphoma after detection of anti-mGluR5 encephalitis, (ii) searching for mGluR5 expression in classical Hodgkin lymphoma biopsy material of patients with autoimmune encephalitis, and (iii) inclusion of mGluR5 screening alongside the standard diagnostic procedure for classical Hodgkin lymphoma as this could provide further information on tumor progression and may serve as a prognostic marker.

Other factors may also be implicated in the pathogenesis of the Ophelia syndrome in the setting of classic Hodgkin lymphoma, because mGluR5 expression is not so rare in classic Hodgkin lymphoma, in contrast to the rarity of the Ophelia syndrome. Therefore, further studies on the clinical relevance of mGluR5 expression in classical Hodgkin lymphoma, as well as its functional impact on tumor development are needed to gain a more in-depth understanding of the pathogenesis of anti-mGluR5 encephalitis and its link to lymphoma development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12040606/s1. Figure S1: mGluR5 banding pattern of human Hodgkin cells and mouse brain. Figure S2: Distribution of mGluR5 on the surface of Hodgkin lymphoma cells—3D-reconstruction. Figure S3: FACS analysis of classical Hodgkin lymphoma cells. Figure S4: Calculation of *GRM5* gene expression intensities using the public GEO GSE17920 dataset. Figure S5: mRNA expression levels of housekeeping genes in all six classic Hodgkin lymphoma cell lines. Table S1: Primers used in RT-qPCR. Table S2: Clinical details of classical Hodgkin lymphoma patients from GEO GSE17920 with high *GRM5* expression. Table S3: Main upregulated signaling pathways in the L-1236 cell line. Information about the source and character of the investigated classic Hodgkin lymphoma cell lines here.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. It involved the use of material of human origin. The study was approved by the Ethics Committee of Charité-Universitätsmedizin Berlin (EA2/121/17). The control classical Hodgkin lymphoma samples were obtained in anonymous form from the author K.H., for which no ethics approval is required. The classical Hodgkin lymphoma cell lines can be freely used for research and can be obtained from standard repositories such as the Leibniz-Institute (DSZM-German Collection of Microorganisms and Cell Cultures GmbH).

Informed Consent Statement: The parents of the index patient provided written informed consent for research use and publication of clinical details and material. No patient-identifying information nor face image is published with this article.

Data Availability Statement: The raw FASTQ sequence files of all the RNA-sequencing runs as well as the StringTie result files have been deposited in NCBI's Gene Expression Omnibus [82] and are accessible through GEO Series accession number GSE212326 "https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE212326, accessed on 12 February 2023".

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2.2 Publikation 2

Bekannter als der Zusammenhang zwischen Ophelia Syndrom und Hodgkin Lymphom ist die Assoziation zwischen NMDARE und Ovarialteratomen (Dalmau et al., 2019; Day et al., 2014; Wandinger et al., 2011). Doch auch hier ist ein möglicher Pathomechanismus, der dieser Assoziation zugrunde liegt, noch nicht gefunden. In einer weiteren Studie über den Zusammenhang zwischen Autoimmunenzephalitis und Tumorerkrankungen konnten wir Daten präsentieren, die ein neues Erklärungsmodell hierzu liefern.

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In dieser Arbeit untersuchten wir die Expression NMDAR in Hirntumoren als potenziellen Auslöser für eine Autoimmunenzephalitis.

Etablierte Trigger für eine NMDARE sind virale Infektionen und Ovarialteratome. Bekannt ist auch, dass bei NMDARE-Patienten Ovarialteratome glioneuronales Gewebe enthalten, welches den NMDAR exprimiert. Dies könnte zur Bildung von Autoantikörpern beitragen und schließlich eine Enzephalitis auslösen. Interessanterweise wurde zuvor noch nie berichtet, dass glioneuronale Hirntumore NMDARE auslösen können. Um einen möglichen Zusammenhang zwischen NMDARE und glioneuronalen Hirntumoren zu untersuchen, analysierten wir eine atypische, refraktäre NMDARE bei einem Kind mit einem niedriggradigen glioneuronalen Tumor des Cerebellums. Klinische Daten, Liquor und Tumorgewebe wurden mittels Immunhistochemie, konfokaler Mikroskopie und molekularer Pathologie untersucht. Wir verglichen die NMDAR-Expression im Tumorgewebe des Indexpatienten mit Proben weiterer Patienten mit unterschiedlichen Hirntumoren, Herpes-simplex-Enzephalitis (HSE), NMDARE ohne Hirntumor sowie gesunden Kontrollen. Der glioneuronale Tumor des Indexpatienten zeigte eine atypische, auf die Somata dysplastischer Ganglienzellen beschränkte NMDAR-Expression. Anti-NMDAR-Antikörper aus dem Liquor des Patienten richteten sich gegen ebendiese Zellen. Die atypische NMDAR-Expression war spezifisch für neuroepitheliales Gewebe. Wir fanden sie nicht in Tumorarten ohne dysplastische Ganglienzellen, wohl aber auf Somata von Neuronen in schwer entzündetem Gewebe bei HSE. Eine Tumorresektion beim Indexpatienten führte schließlich zu einem Abfall des Anti-NMDAR-Titers, deutlicher klinischer Verbesserung und schließlich der Ausheilung der Enzephalitis.

Insgesamt konnten wir mit dieser Arbeit erstmals Daten für eine atypische NMDAR-Expression in glioneuronalen Tumoren zeigen. Somit ist denkbar, dass NMDAR-positive dysmorphe Ganglienzellen die Bildung von Antikörpern provozieren, welche schließlich einer Autoimmunenzephalitis auslösen. Basierend auf den Erkenntnissen über NMDARE und Ovarialteratome schlagen wir ein ätiologisches Konzept vor, bei dem die immunogenen Eigenschaften von neuroepithelialem Tumorgewebe allgemein, innerhalb wie außerhalb des Gehirns, als Auslöser für eine NMDARE dienen.

Die Ergebnisse dieser Arbeit haben unmittelbare klinische Implikationen und können zur Verbesserung von Diagnose und Therapie von Patienten mit Autoimmunenzephalitis beitragen. Zukünftige Studien zum spezifischen Wechselspiel zwischen Antikörper-getriggerter Autoimmunreaktion und Tumorgenese könnten dabei helfen, personalisierte Therapieansätze zu entwickeln und die Prognose für Patienten mit Autoimmunenzephalitis zu verbessern.

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CORRESPONDENCE

Atypical NMDA receptor expression in a diffuse astrocytoma, MYB- or MYBL1-altered as a trigger for autoimmune encephalitis

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Triggers of anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) include *Herpes simplex* encephalitis (HSE) [11] and ovarian teratomas [14]. The latter occur in 50% of adult NMDARE patients and are distinct from ovarian teratomas not associated with encephalitis. They contain neuronal tissue more often and exhibit (ganglio)glioma-like features with dysmorphic neurons and atypical NMDAR expression [4, 7]. Ectopic NMDARs could, therefore, initiate or maintain the formation of anti-NR1 antibodies [8]—an anti-tumor response leading to autoimmune encephalitis. Interestingly, brain tumors have not been implicated as triggers of NMDARE [2], despite their prevalence and expression profile that includes NMDARs [12].

Here, we present data on atypical NMDAR expression in glioneuronal brain tumors, in which dysmorphic neurons might induce antibody formation leading to autoimmune encephalitis.

We followed an atypical refractory NMDARE in a 21-month-old girl for more than 2 years. After a 4-week history of gait disturbance, behavioral changes, and seizures, MRI showed a T_2-hyperintensity in the cerebellar white matter, which was interpreted as inflammatory lesion (Fig. 1d). CSF analysis revealed pleocytosis, type 2 oligoclonal bands, and intrathecal antibody synthesis. Screening for anti-neuronal antibodies detected anti-NR1 IgG in CSF (1:1,000) and serum (> 1:10,000) confirming NMDARE (Fig. 1a–c). With intensive immunotherapy, the child's condition initially improved but a protracted course developed with fluctuating antibody titers and relapses refractory to therapy (Fig. 1g). When subsequent imaging showed growth of the presumed inflammatory lesion, navigated needle biopsy of the now suspected cerebellar tumor was performed (Fig. 1e). Based on histomorphology and molecular profile, we detected a diffuse astrocytoma, *MYB* or *MYBL1*-altered with *MYBL1:MMP16*-fusion [15] (Fig. 1i–o). In contrast to the *MYB/MYBL1*-altered astrocytoma presented here, these tumors are usually not located in the cerebellum and do not contain dysmorphic neurons [5]. After a third recurrence, the patient underwent subtotal tumor resection (Fig. 1f). This resulted in a sharp decrease in anti-NR1 titer (1:3) and significant sustained clinical improvement (Fig. 1g, h, detailed case in Supplementary 1, online resources).

In contrast to this case and unlike adults, the clinical presentation of NMDARE in children is dominated by neurologic rather than psychiatric symptoms, associated tumors are less common, and complete recovery is more likely [14]. In most children, no triggers are found.

To investigate a possible association between NMDARE and brain tumors, we analyzed tumor samples from the index patient (methods in Supplementary 2, online resources). Immunohistochemistry revealed atypical NR1-specific NMDAR positivity in somata of dysmorphic neurons detectable in the tumor (Fig. 1p, q). Glial tumor areas were negative. In contrast to cortical, hippocampal, and cerebellar control tissue, no neuropil signal was seen (Fig. 1r). Immunostaining with patient CSF was consistent with anti-NR1 signal of commercial antibodies. This confirmed atypical NMDAR expression on the patient's dysmorphic neurons and suggested that autoantibodies binding the tumor were also responsible for NMDARE (Fig. 1s). Atypical NMDAR expression was specific for neuronal tissue. When analyzing low-grade tumors with different glial and neuronal composition from patients without reported autoimmune encephalitis, e.g., *pilocytic astrocytoma*, *dysembryoplastic neuroepithelial tumor*, or *ganglioglioma*, atypical NMDAR expression was detected only on dysmorphic neurons in

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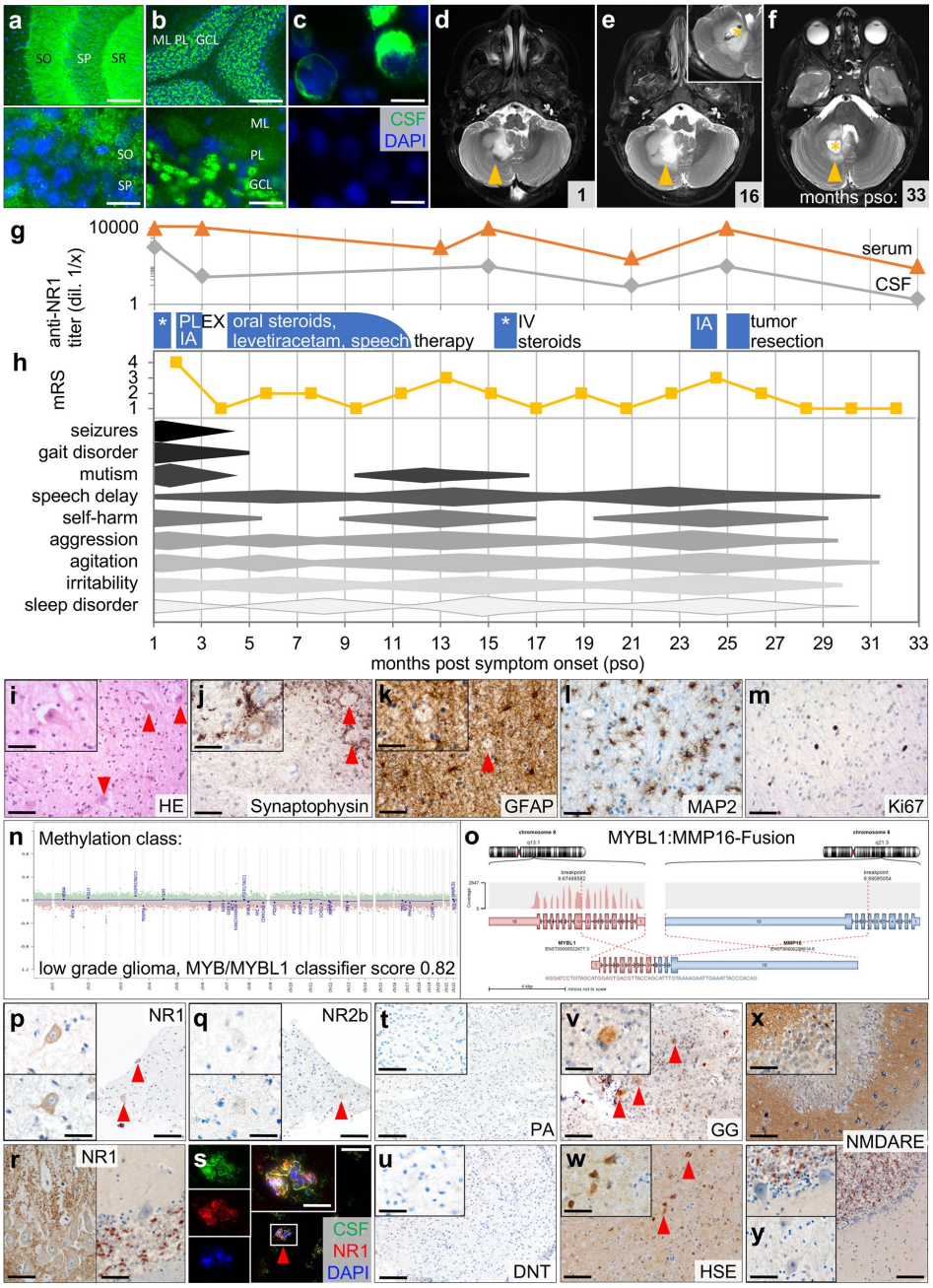
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MAP2
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Ki67
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Methylation class:
low grade glioma, MYB/MYBL1 classifier score 0.82
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◂**Fig. 1** Atypical NMDAR expression in dysmorphic neurons of diffuse astrocytoma, MYB/MYBL1-altered, detected in a patient with NMDAR encephalitis. Immunostaining with CSF shows typical anti-NMDAR neuropil signal (green, DAPI co-localization blue) in mouse hippocampus (**a**) and cerebellum (**b**), and binding to NR1-expressing HEK293T-cells (negative control below) (**c**). SO: *Stratum oriens*, SP: *Stratum pyramidale*, SR: *Stratum radiatum*, ML: molecular layer, PL: Purkinje cell layer, GCL: granular cell layer. Size bars: 100 µm (top **a**, **b**), 20 µm (bottom **a**, **b**; both **c**). MRI 1 month post-symptom onset (pso) shows white matter T_2-hyperintensity in right cerebellar hemisphere with minimal contrast uptake (**d**). Navigated biopsy (inset) of the growing mass 16 months pso allows tumor diagnosis (**e**). Follow-up 6 months after subtotal resection shows decrease in residual mass (**f**). Arrows indicate tumor; asterisks indicate biopsy and resection sites. Patient's CSF and serum anti-NR1 titers fluctuate during treatment with steroids, plasmapheresis (PLEX), and immunoadsorption (IA), and drop after surgery (**g**). Symptoms, progression and treatment response assessed by physical examination and modified Rankin Scale (mRS) are presented semiquantitatively (**h**). Histomorphologic characterization reveals moderately cell-rich isomorphic glial tumor with narrow, pale eosinophilic cytoplasm and fibrillary processes. Dysmorphic neurons are seen within glial tumor matrix (**i**). Dysmorphic neurons located within tumor tissue are labeled with antibodies against synaptophysin (**j**). Tissue shows diffuse matrix-related GFAP reaction (**k**). Glial tumor cells with fibrillary processes are highlighted in MAP2 staining (**l**). There is little proliferative activity (Ki67-labeling index 3%) (**m**). On methylation analysis (EPIC), tumor is assigned to "low grade glioma, MYB/MYBL1" (classifier score v11b4 0.82 and v12.5 0.93). There is a flat CNV profile with no significant chromosomal loss or gain (**n**). RNA sequencing demonstrates MYBL1:MMP16 fusion (**o**). Dysmorphic neurons (arrowheads) in patient's tumor show NMDAR-positivity with atypical concentration in somata rather than in neuropil (**p**). Signal is NR1-specific (**q**). Hippocampus (left) and cerebellum of healthy controls show typical neuropil signal (**r**). Immunostaining of fresh–frozen patient tumor with patient CSF (green) and commercial anti-NR1 (red) overlaps (yellow) confirming atypical NMDAR expression (**s**). Size bars: 200 µm (**p**, **q**, **s**), 100 µm (**r**), 20 µm (insets). Immunostaining with tumors of different glial and neuronal composition from individuals without autoimmune encephalitis show no NR1 signal in *pilocytic astrocytoma* (PA) (**t**), weak neuropil staining without NMDAR-positive somata in *dysembryoplastic neuroepithelial tumor* (DNT) (**u**), and neuropil staining with atypical NMDAR expression on somata of dysmorphic neurons (arrowheads) in *ganglioglioma* (GG) (**v**). Similar NR1 signal is seen in CNS tissue from patients with *Herpes simplex* encephalitis (HSE), where areas of inflammatory infiltration show reduced neuropil staining but neurons with NMDAR-positive somata (**w**). NMDARE without brain tumor or viral infection shows normal NMDAR pattern in hippocampus (**x**, top), cerebellum (bottom), and dentate nucleus (**y**). Size bar: 200 µm (insets 100 µm)

ganglioglioma—and in the aforementioned NMDARE-associated ovarian teratomas (Fig. 1v, Supplementary Fig. 3 and 4, online resources). Tumors without such neurons were negative (Fig. 1t, u). However, we found similar atypical NMDAR expression within inflamed CNS tissue of HSE patients. Areas of severe inflammatory infiltration showed reduced neuropil signal, whereas NMDAR was expressed in the somata of neurons (Fig. 1w). Tissue from NMDARE patients without brain tumor or infection showed normal neuropil staining (Fig. 1x, y), making an epiphenomenon unlikely. Rather, atypical NMDAR expression is either a response to anti-tumor immune reactions or property of the dysmorphic neuronal tissue that facilitates such an anti-NMDAR antibody-mediated response.

Based on this example, it cannot be clarified with certainty to what extent dysmorphic cells present in tumor tissue or local neurons, e.g., from deep cerebellar nuclei, altered by inflammatory processes may induce NMDARE. The latter usually do not show atypical NMDAR expression (Supplementary Fig. 5, online resources).

Most patients with diffuse MYB/MYBL1-altered astrocytoma had epileptic seizures since childhood [15] and symptoms such as movement disorders, behavioral or mnestic changes occur with both encephalopathy and glioneuronal tumors [13, 14]. This symptom overlap complicates differentiation and may lead to overlooking encephalitis in individual cases. Conversely, not every MRI abnormality should be attributed to encephalitis, but should be carefully investigated even in patients with known NMDARE, as it may mask an underlying neoplasm.

Recently, two isolated cases of NMDARE and astrocytoma have been reported [1, 9]. Both studies speculated a link between tumor and encephalitis, but did not perform further analyses. While astrocytes do indeed express NMDARs, this is rarely the case in astrocytomas. Notably, RNA-seq data here show no expression of NR1 [3], the major target of autoantibodies in NMDARE [6]. Together with the high prevalence of common astrocytomas [10], it remains unclear whether these tumors are indeed associated with NMDARE or whether this is coincidence.

This study suggests a link between certain brain tumors and NMDARE. We show atypical NMDAR expression on dysmorphic neurons that may have prompted antibody formation and subsequent autoimmune encephalitis. Tumor resection led to a decrease in these antibodies and terminated a previously refractory encephalitis, supporting the association *ex juvantibus*. Thus, following findings on NMDARE in ovarian teratomas, we propose an etiologic concept in which, in addition to HSE, the immunogenic properties of dysmorphic neurons, whether outside or inside the brain, serve as triggers for NMDARE.

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Author contributions MN contributed to the conception of the study, performed and analyzed immunologic studies with patient CSF on mouse brain tissue, analyzed histologic data, and wrote the first draft of the manuscript. AK, WS, and FS performed histologic examination and tumor characterization. AT performed the radiological evaluation. LS

and JK performed cell-based immunoassay. UT took care of the patient. AK, WS, PHD, AMK and MS critically read the manuscript and contributed to the design of the study. EK contributed to the conception of the study, took care of the patient, analyzed and interpreted the patient data as well as the immunofluorescence data, and was instrumental in writing the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials Data are available on request.

Declarations

Conflict of interest We have no competing interests to declare.

Consent for publication Consent for publication of individual images/details was obtained from patient's caregivers. Ethical approval for the study was obtained from the IRB of Charité (EA2/121/17).

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2.3 Publikation 3

Neben der detailliert charakterisierten NMDARE oder dem weniger gut verstandenen jedoch seit langem bekannten Ophelia Syndrom, wurden in jüngster Zeit zahlreiche weitere antineuronale Autoantikörper entdeckt, die neue Einblicke in die Pathophysiologie der Gruppe der Autoimmunenzephalitiden bieten. Einer dieser erst kürzlich entdeckten Vertreter ist die $GABA_A$-Rezeptor ($GABA_AR$) Enzephalitis, bei der Patienten in der Regel schwere therapierefraktäre epileptische Anfälle erleiden und im Liquor Autoantikörper gegen den $GABA_AR$ detektiert werden (Ohkawa et al., 2014; Petit-Pedrol et al., 2014). Vor der im Folgenden vorgestellten Arbeit konnten wir einen ersten pädiatrischen Fall einer $GABA_AR$ Enzephalitis beschreiben, der sich ohne epileptische Anfälle präsentierte und so das klinische Spektrum dieser Erkrankung deutlich erweiterte (Nikolaus et al., 2018b). Diese Arbeit verdeutlichte die Notwendigkeit einer detaillierteren Analyse der Spezifität von Autoantikörpern gegen $GABA_AR$, um mögliche Zusammenhänge zwischen den immunologischen Mechanismen und dem variablen klinischen Bild dieser Erkrankung zu identifizieren.

Nikolaus M, Kreye J, Turko P, Vida I, Knierim E, Prüss H. CSF reactivity in $GABA_A$ receptor antibody encephalitis – Immunocytochemical distribution in the murine brain. *Brain Research.* 2019. https://doi.org/10.1016/j.brainres.2018.10.019 (IF=3,3)

$GABA_AR$ sind für eine Vielzahl neurophysiologischer Prozesse von zentraler Bedeutung. Mutationen in diesem Rezeptor sind mit Epilepsie und psychiatrischen Erkrankungen assoziiert. Vor wenigen Jahren wurde eine schwerwiegende Form der Enzephalitis beschrieben, die mit therapierefraktären epileptischen Anfällen und Antikörpern gegen $GABA_AR$ einhergeht. Aufgrund der komplexen Verteilung der Untereinheiten des $GABA_AR$ ist es von großem Interesse, die Bindungsmuster von humanen Anti-$GABA_AR$-Antikörpern zu untersuchen, um die zugrunde liegende Pathophysiologie verschiedener klinischer Erscheinungsbilder besser zu verstehen. Das Ziel der hier vorliegenden Publikation war es daher, die Neuroreaktivität in Liquorproben von $GABA_AR$ Enzephalitis Patienten zu analysieren, um eine spezifische Lokalisierung der Antikörperbindung im Gehirn zu ermöglichen und so Rückschlüsse auf mögliche klinisch-neurologische Auswirkungen zu ziehen.

In den durchgeführten immunhistochemischen Untersuchungen verglichen wir die Neuroreaktivität der Liquorproben auf murinem Gehirngewebe und hierüber die Spezifität der Patienten-Antikörper mit kommerziellen monoklonalen Anti-$GABA_AR$-Antikörpern. Zur Validierung der neuronalen Spezifität der $GABA_AR$-Reaktivität erfolgte die Kolokalisation mit neuronalen und glialen Markern. Die Neuroreaktivität des Patienten-Liquors zeigte eine hohe

Übereinstimmung mit bekannten mRNA-Expressionsmustern und wir konnten in spezifischen Gehirnregionen eine besonders starke Antikörperbindung nachweisen – darunter in den äußeren Schichten des Bulbus olfactorius, CA1 und CA2 des Hippocampus, im Neokortex sowie Pallidum und der zerebellären Körnerzellschicht. Der Vergleich mit kommerziellen Antikörpern ergab eine erhebliche Überschneidung, aber auch spezifische Unterschiede in der Färbung – z.B. reicherte der kommerzielle Antikörper bevorzugt an Dendriten an, während Liquor ein homogenes Signal im Neuropil bildete. In einigen Regionen ergaben sich sogar komplementäre Färbemuster von GABAergen Neuronen zwischen kommerziellen Antikörpern und Liquor.

Dieses Verteilungsmuster legte nahe, dass es sich um polyklonale $GABA_AR$-Antikörper handelte, was mit der Existenz zusätzlicher antineuronaler Autoantikörper im Liquor der Patienten erklärt wäre. Dies könnte in der Pathophysiologie eine entscheidende Rolle spielen und zur variablen klinischen Präsentation von Patienten mit $GABA_AR$-Enzephalitis beitragen.

Unsere Arbeit bereitete die Grundlage für eine Folgestudie, in der diese Hypothese durch Untersuchungen mit aus Patienten-Liquor isolierten, rekombinanten monoklonalen Antikörpern bestätigt werden konnte (Kreye et al., 2021).

Publikation 3 ist keine open access Veröffentlichung. Daher wird an dieser Stelle nur der Literaturhinweis veröffentlicht:

Nikolaus, Marc, Jakob Kreye, Paul Turko, Imre Vida, Ellen Knierim, and Harald Prüss. "CSF Reactivity in GABAA Receptor Antibody Encephalitis - Immunocytochemical Distribution in the Murine Brain." *Brain Research* 1704 (October 19, 2018): 249–56. https://doi.org/10.1016/j.brainres.2018.10.019.

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Nikolaus, Marc, Jakob Kreye, Paul Turko, Imre Vida, Ellen Knierim, and Harald Prüss. "CSF Reactivity in GABAA Receptor Antibody Encephalitis - Immunocytochemical Distribution in the Murine Brain." *Brain Research* 1704 (October 19, 2018): 249–56. https://doi.org/10.1016/j.brainres.2018.10.019.

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2.4 Publikation 4

Wie bereits beschrieben, haben wir inzwischen detaillierte Kenntnisse über die Autoantikörper-vermittelten Pathomechanismen der NMDARE. Darüber hinaus ermöglichen etablierte diagnostische Kriterien eine schnelle Diagnose und Behandlung dieser Erkrankung (Graus et al., 2016). Im Gegensatz dazu ist über den klinischen Verlauf und die langfristige Prognose von Patienten – insbesondere Kindern – mit NMDARE nur wenig bekannt. Zwar sprechen die allermeisten Erkrankten gut auf eine immunsuppressive Therapie an und haben eine positive funktionell-neurologische Prognose mit hoher Genesungsrate (Titulaer et al., 2013). Andererseits durchleben einige Patienten sehr langwierige Genesungsprozesse mit andauernden kognitiven Defiziten oder erleiden ein Rezidiv. Aufgrund dessen wurde vor kurzem von Balu et al. mit dem NEOS (*anti-**N**MDAR **E**ncephalitis **O**ne-Year Functional **S**tatus*) Score ein Instrument zur Vorhersage des Verlaufs von NMDARE entwickelt (Balu et al., 2018). Wir konnten diesen Score in der im Folgenden vorgestellten Arbeit für Kinder validieren.

Nikolaus M, Rausch P, Rostásy K, Bertolini A, Wickström R, Johannsen J, Denecke J, Breu M, Schimmel M, Diepold K, Haeusler M, Quade A, Berger A, Rosewich H, Steen C, von Au K, Dreesmann M, Finke C, Bartels F, Kaindl AM, Schuelke M, Knierim E. Retrospective Pediatric Cohort Study Validates NEOS Score and Demonstrates Applicability in Children With Anti-NMDAR Encephalitis. *Neurology, Neuroimmunology and Neuroinflammation.* 2023. https://doi.org/10.1212/NXI.0000000000200102 (IF=11,4)

Der NEOS-Score ist ein klinisches Bewertungsinstrument zur Beurteilung des akuten neurologischen Zustands und der Prognose des funktionell-motorischen Outcomes von Patienten mit NMDARE ein Jahr nach Krankheitsbeginn. Er basiert auf unabhängigen Variablen (Aufnahme auf eine Intensivstation; verzögerter Therapiestart > 4 Wochen nach Krankheitsbeginn; fehlendes Therapieansprechen innerhalb von 4 Wochen; pathologisches MRT; Liquorpleozytose > 20 Zellen/µl), die den Schweregrad bei Krankheitsbeginn abbilden. Der NEOS-Score ermöglicht eine objektive Einschätzung basierend auf der modifizierten Rankin-Skala (mRS) und kann dabei helfen, den Verlauf der Krankheit vorherzusagen, Therapieentscheidungen zu unterstützen und Prognosen für das langfristige Outcome zu stellen. Das Ziel dieser retrospektiven Kohortenstudie war die Validierung des NEOS-Scores in einer pädiatrischen Population und die Überprüfung seiner Anwendbarkeit bei Kindern mit NMDARE.

Hierzu analysierten wir die klinischen Daten von 59 pädiatrischen NMDARE Patienten, welche über eine mittlere Follow-up Zeit von 20 Monaten nachbeobachtet wurden. Wir rekonstruierten

den ursprünglichen Score, passten ihn an die pädiatrische Kohorte an und versuchten ihn durch zusätzliche Variablen zu optimieren und seine Vorhersagekraft zu testen. Wir verwendeten hierzu verschiedene lineare Regressionsmodelle, um die Vorhersagbarkeit für gutes bzw. schlechtes Outcome basierend auf der modifizierten Rankin-Skala (mRS) zu untersuchen. Darüber hinaus wurden Ergebnisse neuropsychologischer Untersuchungen als alternative Outcome Parameter untersucht.

Insgesamt sagte der NEOS-Score zuverlässig das mRS-basierte Outcome im ersten Jahr nach der Diagnose und darüber hinaus bis 16 Monate nach der Diagnose voraus. An die pädiatrische Kohorte adaptierte Grenzwerte für die fünf NEOS-Variablen verbesserten die Vorhersagekraft nicht signifikant. Neben den fünf Variablen beeinflussten jedoch weitere Merkmale wie der "HSE-Status" und "Alter bei Krankheitsbeginn" die Vorhersagbarkeit und könnten potenziell zur Definition von Risikogruppen nützlich sein. Zusätzlich konnte NEOS auch das kognitive Outcome und hier insbesondere persistierende Defizite bei Exekutivfunktion und Gedächtnis voraussagen.

Mit den Ergebnissen dieser Analyse konnten wir zeigen, dass der NEOS-Score auch für Kinder mit NMDARE ein zuverlässiges und praktikables Bewertungsinstrument für die Prognose des funktionellen Outcomes nach einem Jahr darstellt. Zusätzlich suggerieren unsere Daten, dass NEOS nicht nur funktionelle, sondern auch kognitive Beeinträchtigung vorhersagen kann. Daher könnte der Score dazu beitragen, Patienten zu identifizieren, die ein erhöhtes Risiko für schlechtes kognitives Outcome im Langzeitverlauf haben und somit helfen, nicht nur die optimale Akuttherapie für diese Patienten auszuwählen, sondern auch im Verlauf z.B. kognitive Rehabilitation spezifisch einzusetzen.

RESEARCH ARTICLE OPEN ACCESS

Retrospective Pediatric Cohort Study Validates NEOS Score and Demonstrates Applicability in Children With Anti-NMDAR Encephalitis

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Abstract

Background and Objectives

Anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) is the most common form of autoimmune encephalitis in children and adults. Although our understanding of the disease mechanisms has progressed, little is known about estimating patient outcomes. Therefore, the NEOS (anti-**N**MDAR **E**ncephalitis **O**ne-Year Functional **S**tatus) score was introduced as a tool to predict disease progression in NMDARE. Developed in a mixed-age cohort, it currently remains unclear whether NEOS can be optimized for pediatric NMDARE.

Methods

This retrospective observational study aimed to validate NEOS in a large pediatric-only cohort of 59 patients (median age of 8 years). We reconstructed the original score, adapted it, evaluated additional variables, and assessed its predictive power (median follow-up of 20 months). Generalized linear regression models were used to examine predictability of binary outcomes based on the modified Rankin Scale (mRS). In addition, neuropsychological test results were investigated as alternative cognitive outcome.

Results

The NEOS score reliably predicted poor clinical outcome (mRS ≥3) in children in the first year after diagnosis ($p = 0.0014$) and beyond ($p = 0.036$, 16 months after diagnosis). A score adapted to the pediatric cohort by adjusting the cutoffs of the 5 NEOS components did not improve predictive power. In addition to these 5 variables, further patient characteristics such as the "*Herpes simplex* virus encephalitis (HSE) status" and "age at disease onset" influenced predictability and could potentially be useful to define risk groups. NEOS also predicted cognitive outcome with higher scores associated with deficits of executive function ($p = 0.048$) and memory ($p = 0.043$).

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

Glossary

AIC = Akaike information criterion; **CSF** = cerebrospinal fluid; **GLM** = generalized linear model; **IV** = Intravenous; **mRS** = modified Rankin Scale; **NMDARE** = N-methyl-D-aspartate receptor encephalitis; **PLEX** = plasma exchange.

Discussion

Our data support the applicability of the NEOS score in children with NMDARE. Although not yet validated in prospective studies, NEOS also predicted cognitive impairment in our cohort. Consequently, the score could help identify patients at risk of poor overall clinical outcome and poor cognitive outcome and thus aid in selecting not only optimized initial therapies for these patients but also cognitive rehabilitation to improve long-term outcomes.

Anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) is the most common form of autoimmune encephalitis.[1] It occurs in all age groups, but most often affects young women and children. NMDARE is characterized by a combination of severe neuropsychiatric symptoms, seizures, and autonomic dysregulation.[2] IgG autoantibodies to NR1 subunits of NMDARs lead to receptor internalization and cause the disease.[3,4] Tumors, usually ovarian teratomas, are found in up to 50% of adult patients with NMDARE.[5] Children with NMDARE are less likely to have tumors, although ovarian teratomas are found in up to 30% of adolescents.[6,7] Pediatric patients present with seizures and movement disorders and only rarely develop autonomic dysfunction.[8,9] Overt psychosis is also less common, while subtle behavioral changes such as irritability, insomnia, or mutism may indicate NMDARE in infants.[10,11] Children respond well to immunotherapy, especially when initiated without delay.[6] Intravenous (IV) steroids, immunoglobulins, and plasma exchange (PLEX) represent first-line therapies, intensified in refractory cases by rituximab or cyclophosphamide as second-line and sometimes long-term treatment.[5] In contrast to the well-established diagnostic criteria for NMDARE[2] which allow rapid diagnosis and treatment, less is known about the clinical course and long-term prognosis of children with NMDARE. Functional neurologic outcome improves with treatment and is favorable in 80–90%, surpassing that of adults.[5,12,13] Yet, many patients endure unpredictable periods of failing treatment response or protracted recovery with cognitive deficits and 10–20% relapse.[5,13-15]

In recognition of this prognostic uncertainty, the NEOS (anti-**N**MDAR **E**ncephalitis **O**ne-Year Functional **S**tatus) score was developed,[16] a tool to predict the one-year outcome of NMDARE. It includes 5 independent predictors of poor functional status: (1) need for ICU admission, (2) treatment delay within the first 4 weeks after symptom onset, (3) lack of clinical improvement 4 weeks into treatment, (4) abnormal cranial MRI, and (5) cerebrospinal fluid (CSF) white blood cell count more than 20 cells/μL. While NEOS has been developed in a large mixed-age cohort[16] and was validated in adult patients,[17] there is only 1 brief report of assessing it in a small group of children.[18] In this study, we aimed to validate the NEOS score in a larger pediatric-only cohort and analyze its predictive value taking into account the particular characteristics of NMDARE in children.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The ethics committee of Charité-Universitätsmedizin Berlin approved this study (EA2/121/17). Patients' parents gave their written informed consent for the storage and use of samples and clinical information for research purposes. In this retrospective observational study, we contacted 23 sites in Germany and Europe and collected records of children with confirmed NMDARE from the following 12 sites, both university and district hospitals, between 2020 and 2021: In Berlin and surrounding areas of our hospital (Charité), Vivantes Klinikum Friedrichshain, St-Joseph Klinikum, and Klinikum Westbrandenburg Postdam; across Germany, from Aachen University Hospital, Augsburg University Hospital, Children's Hospital Datteln, University Witten/Herdecke, Göttingen University Hospital, Hamburg University Hospital, and Nordhessen Klinikum Kassel; and in Europe, from Medical University of Vienna (Austria) and Karolinska University Hospital, Stockholm (Sweden).

Inclusion Criteria

Patient data were accepted according to the following inclusion criteria: (1) Patients had to be younger than 18 years at the time of diagnosis; (2) patients had to be positive for anti-NMDAR autoantibodies in CSF and meet clinical criteria for autoimmune encephalitis (Graus criteria[2]); and (3) sufficient clinical information had to be available to complete at least the 5 items of NEOS at the time of diagnosis and the modified Rankin Scale (mRS)[19] after 1 year. There was one exception: Completed cases with full restitution or fatal outcome within the first 12 months were also included. In these cases, the mRS of the last available time point was taken as one-year mRS.

Calculation of the NEOS Score and mRS

We used the original NEOS score[16] recomposed in a multivariable logistic regression model. The score includes 5 independent predictors of poor functional status (mRS ≥3): (1) need for ICU admission, (2) treatment delay within the first 4 weeks after symptom onset, (3) lack of clinical improvement 4 weeks into treatment, (4) abnormal cranial MRI, and (5) CSF white blood cell count more than 20 cells/μL. Each variable is scored with 1 point. The score ranges from 0 to 5 and is

calculated at bedside (eTable 1, links.lww.com/NXI/A813). For an adapted NEOS score tailored to our pediatric cohort, we defined cutoff points of the continuous variables following the original methodology[16] as the median of measures 1 year after diagnosis (between 6 and 18 months) in healthy/unaffected individuals (mRS = 0).

The mRS is a descriptive measure of global disability after stroke but is widely used to assess patients with autoimmune encephalitis. It comprises 6 categories of severity ranging from "no symptoms at all" to "severe disability" (grades 0 to 5), with the additional category "6" for death. The categories essentially cover activities of daily living and focus on motor function. The score was determined by physicians during physical examination at follow-up visits (eTable 1, links.lww.com/NXI/A813).

Analysis of the Clinical Variables and Evaluation of the NEOS Score

Each clinical record collected included demographic information, date of onset, age and clinical characteristics at admission, type of hospitalization, laboratory, electrophysiologic and radiologic findings, detailed information on treatment procedures, time from onset of symptoms to initiation of treatment, time from initiation of treatment to clinical improvement, and functional status during the course of disease. Data were collected from admission, first discharge, and up to 9 follow-up visits ranging between 1 and 52 months after diagnosis. Owing to the retrospective nature of the study, there was a wide variation in follow-up intervals. Individual follow-up visits clustered around 2, 5, 9, 12, and 16 months after diagnosis (eTable 2, links.lww.com/NXI/A813). To better reflect the time frame used in the original study[16] and consider as many patients as possible for the assessment of outcome after 1 year without pseudoreplication, we used data points from individuals recorded at follow-up visits between 6 and 18 months after diagnosis. If individuals were measured more than once during this period, only the visit closest to the 12-month mark after discharge was used. Status and outcome were quantified using the mRS. Cognitive test scores were collected at follow-up whenever possible.

Neuropsychological Assessment With Various Test Batteries

Data collected on cognitive tests were very heterogeneous, and the test batteries used in this retrospective study varied widely. Therefore, because of the general problem of comparability between these tests, we decided to divide the various quantitative results, including percentile ranks and numerical subscale scores, into a binary measure of "normal" and "pathologic" findings and to broadly assign them to the main categories of neuropsychological assessment: intelligence, memory (including working and episodic memory), language, executive function (including attention span, concentration, processing speed), and visuospatial perception. Given the encephalopathy symptoms of most patients, we included behavior as an additional category, including fatigue, emotional instability, aggression, and hyperactivity. These items were used as an alternative outcome reference to examine the predictive power of NEOS.

Statistical Analysis

All statistical analyses, including the generation of figures, were performed in the statistical programming environment R, version R 3.5.3.[20] Mean differences were assessed using permutative, nonparametric Wilcoxon tests with 10^6 permutations as implemented in the R package *coin*.[21] Differences in frequencies and distribution of variables between this cohort and that in the original study[16] were assessed using Fisher exact tests. The predictability of binary outcomes (mRS ≥3) was assessed using generalized linear models with a binomial error structure and a "clog-log" link function as implemented in MASS.[22] The nonsymmetric complementary log-log link function (clog-log) was chosen because we found in most cases an unbalanced distribution between positive and negative outcomes in the target variables, which is significantly better represented by this link function as compared with *logit*.[23] Models were built individually for each tested covariate and selected to minimize the Akaike information criterion (AIC) and to achieve significant improvement over the less complex null model (mRS ≥ 3∼NEOS). This was achieved by step-wise model selection, testing extended models (i.e., mRS ≥ 3∼NEOS + covariate; mRS ≥ 3∼NEOS + covariate + NEOS:covariate) against the null model, using likelihood ratio tests and AIC calculation, to select the best and most parsimonious model in this comparison and to reduce the potential of overfitting through the inclusion of noninformative variables. Models that were too heterogeneous in fit, had residual patterns, or were too unbalanced or sparse were excluded from further analyses. If applicable, *p*-values were adjusted for multiple testing using FDR/Benjamini-Hochberg correction.[24]

Data Availability

All data are provided in this article and are available in anonymous form on request.

Results

Demographic and Clinical Characteristics of the Pediatric Cohort

We included 59 pediatric patients with confirmed NMDARE from 63 collected records (n = 1 exclusion because of unclear diagnosis, n = 3 exclusions because of incomplete follow-up data). The minimum follow-up period was 12 months. Ten cases (17%) with shorter follow-up time because of early complete restitution (n = 9/10) or fatal outcome (n = 1/10) were included (see inclusion criteria). The median follow-up time was 20 months (12–52 months). Age at disease onset was 8 years (median, 9 months–17 years) and showed a bimodal distribution with maxima at 2 and 16 years, respectively. 44 patients (75%) were female (Table 1, eFigure 1, links.lww.com/NXI/A812). Two cases had preexisting autoimmune comorbidities (Hashimoto thyroiditis, type 1 diabetes). Tumors were found in 3

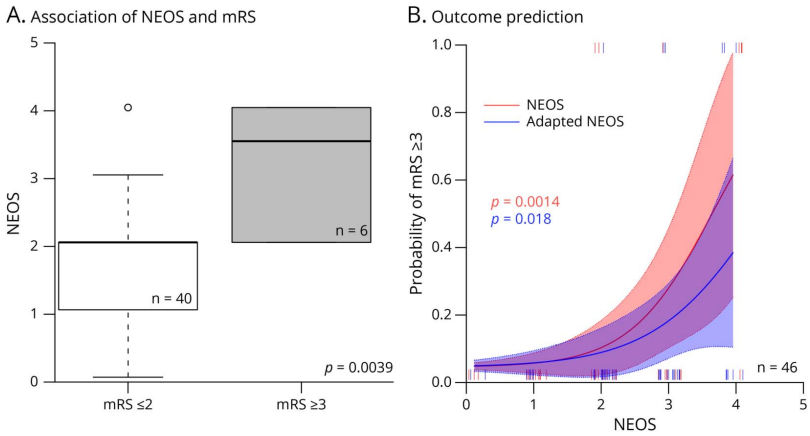
Table 1 Demographic and Epidemiologic Characteristics of Our Pediatric Cohort in Comparison With the Original Cohort[16]

	Pediatric	Original[16]	Comparison
Cohort	**% (N)**	**% (N)**	**Fisher and #Wilcoxon test (FDR adjusted)**
N	59	382	—
Follow-up time [mo]	20	24	—
Sex			
Female	75% (44)	82% (315)	0.3052
Male	25% (15)	18% (67)	
Age [median, range]	8 (9 mo–17 y)	21 (8 mo–85 y)	**#4.7059e-9**
Tumor	5% (3)	42% (159)	**4.1746e-4**
HSV encephalitis before NMDARE	17% (10)	n/a	—
Main symptoms			
Behavior	95% (56)	96% (386)	0.9002
Memory	83% (49)	76% (284)	0.8026
Seizures/therapy refractory	80% (47)/19% (11)	72% (273)	0.8395 (refractory: **7.3860e-4**)
Consciousness	73% (43)	63% (239)	0.7872
Sleep	73% (43)	52% (136)	**0.0072**
Speech	71% (42)	76% (283)	0.9622
Movement	68% (40)	78% (297)	0.8026
Autonomic function	31% (18)	46% (177)	0.3052
Hypoventilation	2% (1)	36% (136)	**1.2821e-4**
NEOS items			
Admission to ICU	59% (35)	77% (291)	0.5271
Disease onset to treatment ≥4 wk	36% (21)	38% (145)	0.9220
Treatment to first improvement ≥4 wk	30% (18)	44% (163)	0.5162
Pathologic MRI findings	47% (28)	31% (112)	0.2179
CSF cell count >20/μL	44% (26)	51% (166)	1.0000
Second-line therapy (rituximab or cyclophosphamide)	41% (24)	27% (102)	0.2936
Outcome after 1 y			
mRS ≤2	88% (49)	74% (281)	0.0991
mRS ≥3	12% (7)	26% (101)	

Cohorts were compared based on the relative frequencies or median differences in characteristics, which were assessed using the Fisher exact test and one-sample Wilcoxon rank test, respectively.
Abbreviations: CSF = cerebral spinal fluid; FDR = false discovery rate; HSV = *Herpes simplex* virus; ICU = intensive care unit; mRS = modified Rankin Scale.

patients (n = 2 ovarian teratomas, n = 1 brain tumor). A subgroup of 17% (n = 10/59) had *Herpes simplex* virus encephalitis (HSE) before NMDARE. Symptoms on admission and NEOS components are listed in Table 1. Almost all patients, 97% (n = 57/59), had 3 or more of these symptoms; 81% (n = 48/59) had at least 5; and 24% (n = 14/59) had all symptoms. Thus, our cohort included a large proportion of severe cases, showing the full picture of NMDARE. Thirty-nine percent (n = 23/59) of patients had pathologic findings in CSF as well as EEG and MRI (83% in CSF, 80% in EEG, and 47% in MRI); 59% (n = 35/59) required ICU treatment; 51% (n = 30/59) were treated with PLEX or immunoadsorption in addition to IV steroids; and 41% (n = 24/59) received second-line therapy (rituximab, additional cyclophosphamide in 3 cases). Most patients showed continuous improvement on therapy; a fluctuating

Figure 1 Validation of the NEOS Score in Children



(A) Association of the original NEOS score with mRS-based outcomes (good outcome mRS ≤ 2, poor outcome mRS ≥ 3) at 1 year after diagnosis. Box plots represent IQR, solid lines mark the median, whiskers display range (upper/lower quartile ± 1.5*IQR), and circles show outliers. "n" indicates the number of subjects included at each time point. (B) Predictability analysis of mRS-based clinical outcomes by the NEOS score with binomial generalized linear models (GLMs). Line plots show association of the original (red curve) and adapted (blue curve) NEOS scores with poor clinical outcome (mRS ≥ 3) at 1 year after diagnosis. Solid lines represent best fit and shadows indicate confidence intervals. tick marks on the upper and lower x axes indicate the number of subjects (also written next to every graph) with each score with a good or poor mRS-based clinical outcome. A small random jitter was added to spread ticks around the discrete NEOS score values, to discriminate single data points. The *p* values were adjusted for multiple testing. NEOS 0: n = 5, NEOS 1: n = 12, NEOS 2: n = 16, NEOS 3: n = 9, NEOS 4: n = 4, NEOS 5: n = 0. For further results (comparison of original and adapted NEOS score, analysis over time), see eFigure 2 (links.lww.com/NXI/A812) and eTable 2 (links.lww.com/NXI/A813).

course with transient deterioration was observed in 22% (n = 13/59); and only 4 children relapsed.

The original NEOS cohort[16] consisted of 382 individuals, of whom 35% (n = 132/382) were younger than 18 years. Our pediatric-only cohort, in comparison (Table 1), more closely represented the spectrum of NMDARE in children. This included a lower tumor prevalence, lower rate of autonomic dysfunction, more frequent use of second-line therapy and, accordingly, shorter time to improvement, and a lower proportion of poor outcomes (mRS ≥3) at 1 year. Otherwise, there were no confounding differences in either group, such as initial disease severity. Our cohort was also comparable with that of the previous pediatric study[18] which reported a test of NEOS in 30 children and whose patients differed only by a higher rate of second-line therapy (70%, n = 21/30).[20]

Validation of the NEOS Score in Pediatric Patients

To examine the association between NEOS and clinical outcomes in our pediatric cohort, we first calculated the score for all patients, dichotomized their functional status by mRS, associated each assessed variable with good (mRS ≤2) or poor (mRS ≥3) status, and rederived the NEOS score based on the original characteristics and cutoff values[16] (eTable 3, links.lww.com/NXI/A813).

To validate NEOS, we grouped all score values to the mRS-based outcome and found that patients with poor functional status (mRS ≥3) consistently had higher NEOS scores than patients with good functional status (mRS ≤2) ($p = 0.0039$, Figure 1A). Using binomial generalized linear models (GLMs), we assessed the relationship between mRS-based outcomes and NEOS, confirming the correlation between the NEOS score and the risk of poor clinical outcome at 1 year after diagnosis ($p = 0.0014$, Figure 1B). In an extended analysis with the multiple follow-up data of readmitted or re-examined patients over time (eTable 3, links.lww.com/NXI/A813), we found robust predictability of mRS even beyond 1 year ($p = 0.036$, 16 months after diagnosis, eFigure 2, links.lww.com/NXI/A812 *p*-values adjusted for multiple testing).

Questioning whether the NEOS score could be further developed to optimize predictive power in children with NMDARE, we recomposed the score by adjusting the population-specific cutoffs of the 5 NEOS components (Table 2). Both scores, the original and the adapted, correlated strongly with each other (eFigure 2, A–D, links.lww.com/NXI/A812); the association between higher adapted NEOS scores and patients with poor status (mRS ≥3) was significant ($p = 0.032$, eFigure 2A, links.lww.com/NXI/A812); and prediction by GLM analysis confirmed this, again up to 16 months after diagnosis ($p = 0.026$, eFigure 2B, links.lww.com/NXI/A812), showing that the adapted NEOS score did not perform better.

To investigate whether additional factors, not included in the 5 NEOS components, might influence the score and

Table 2 Adapted NEOS Score Compiled by Adjusting the Population-Specific Cutoff Median Values of the 5 NEOS Items

NEOS	Original	Adapted
Disease onset to treatment	>28 d	>16 d
Treatment to improvement	>28 d	>15 d
Admission to ICU	Yes/No	Yes/No
MRT pathology	Yes/No	Yes/No
CSF cell count	>20/μL	>13/μL

improve prediction of long-term outcomes, we systematically examined patient characteristics recorded between initial admission and discharge. Of the many factors found (eTable 4, links.lww.com/NXI/A813), 2 patient characteristics seemed of clinical relevance and could be useful to define risk groups: (1) "HSE status", as within the subgroup of individuals with HSE before NMDARE, NEOS predicted an increased risk of poor clinical outcome (mRS ≥3) beyond 1 year after diagnosis and (2) "age at disease onset", as younger individuals showed a persistently higher risk of poor clinical outcome (mRS ≥3) already at lower NEOS scores indicating the effect of age (eFigure 3, links.lww.com/NXI/A812, eTable 4, links.lww.com/NXI/A813).

Overall, our retrospective study confirms that the NEOS score performed very well in children with NMDARE, predicting clinical outcome during the first year and beyond. Adapting the NEOS components did not improve predictive power. Additional items influence the score, but have limited clinical relevance. Yet, HSE status and age at disease onset could complement NEOS and improve its prediction of long-term outcomes.

Correlation of the NEOS Score With Neuropsychological Test Results

To further evaluate the potential of the NEOS score, we examined cognitive test scores from our patient data sets as an alternative outcome measure to mRS. Quantitative data were grouped into a binary measure (normal vs pathologic) and distributed among categories of neuropsychological assessments: intelligence, memory, language, executive function, and visuospatial function.[25] Taking into consideration the encephalopathy in most patients, we added behavior. A total of 33 children underwent neuropsychological assessments at some time during follow-up. Seventy percent (n = 23/33) of them were tested at 1 year after diagnosis or later. Most patients were tested multiple times. Unfortunately, in one-third of the cases, available data were incomplete. For those complete, 78% (n = 18/23) of the early assessments showed pathologic results in at least one category (Figure 2A). One year after diagnosis, deficits remained in 62% (n = 13/21) of retested patients. Persistent pathologic results frequently concerned executive function and memory (Figure 2B).

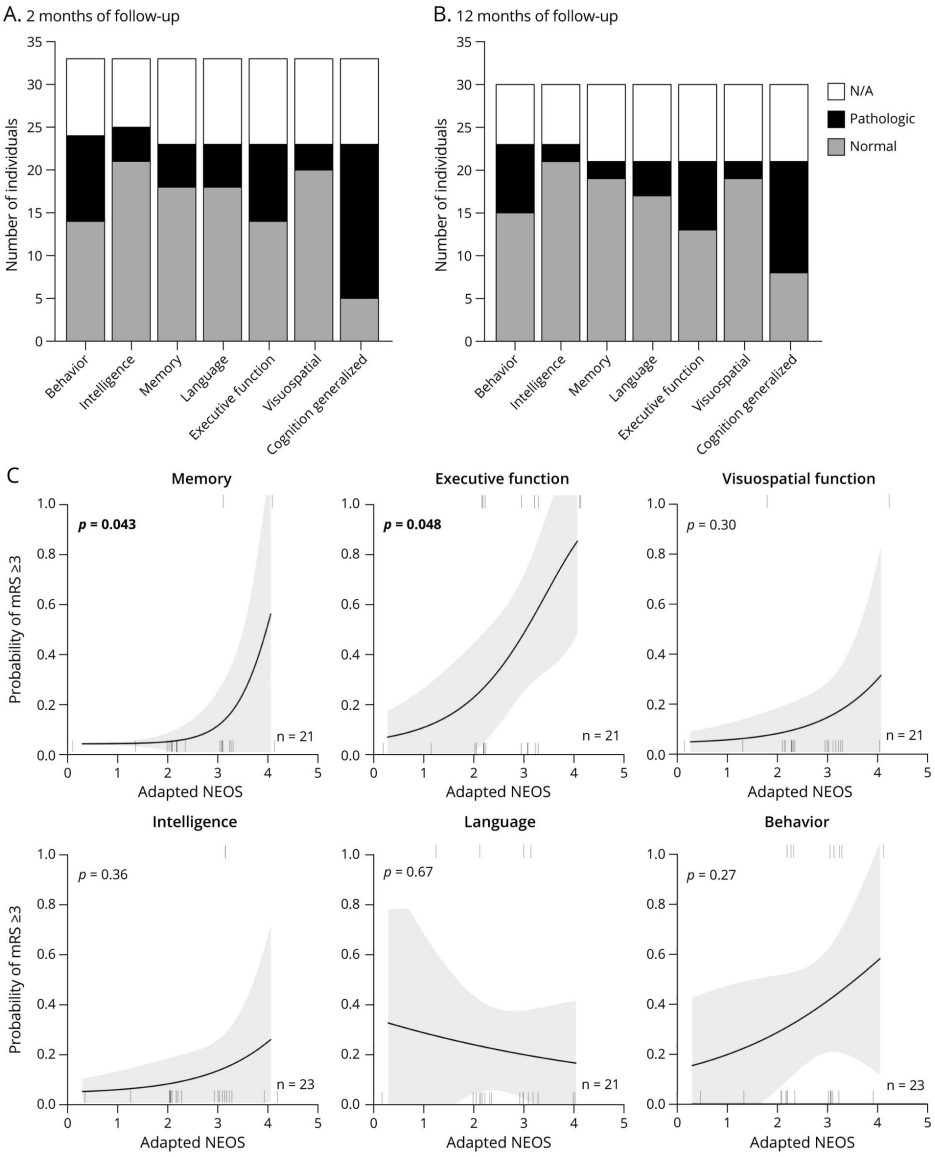
These results were assigned to each patient's NEOS score value. GLM predictive analysis using this cognition-based outcome reference revealed higher NEOS scores in patients with cognitive impairment and particularly an association to deficits in executive function ($p = 0.048$) and memory ($p = 0.043$). This was comparable for both the original and adapted NEOS scores (Figure 2C, eTable 5, links.lww.com/NXI/A813). No significant associations were found with intelligence, behavior, and visuospatial function. In conclusion, these data provide preliminary evidence of the predictive power of NEOS also for cognitive outcomes in children with NMDARE.

Discussion

To provide a predictive tool in NMDARE, the NEOS score was introduced.[16] This score facilitates the estimation of outcome in NMDARE, is expected to identify subgroups with poor prognosis, and can help assign the optimal treatment regimen to the right patient. The score was developed in a mixed-age cohort, which suggested its applicability also to children with NMDARE.[16] A brief evaluation in pediatric patients[18] was promising, although the results were limited by the relatively small sample size and the lack of severe cases. In this study, we present an in-depth analysis of NEOS in a comparatively large pediatric-only cohort. We demonstrate that the NEOS score performs well in children, reliably predicting the mRS-based outcome at 1 year and at least up to 16 months after diagnosis. Beyond, the predictive power gradually decreases. This time dependence is explained in part (1) by sparse data because of the decreasing number of study subjects with increasing follow-up time and selection bias but also (2) by rapid recovery in our pediatric-only cohort—despite severe disease of most patients at baseline, only 12% showed poor functional status (mRS ≥3) at 1 year, compared with 26% in the original mixed-age cohort.[16] Therefore, at least in this study, the NEOS score could not reliably predict long-term outcome.

Adapting the 5 existing NEOS components to the pediatric cohort did not improve the score. Adding further items influenced its performance. Of clinical relevance here, NEOS predicted a worse outcome beyond 1 year after diagnosis in children with NMDARE after HSE. The entity of NMDARE after HSE[26] was still unknown in the original cohort[16] and was not included in the previous pediatric study[18] This finding is consistent with the clinical course of these patients, whose long-term outcomes remain poor despite complete recovery from NMDARE because of persistent brain damage from viral infection.[27,28] Therefore the HSE status of patients should be considered when applying the NEOS score. Age at disease onset was another clinically relevant patient characteristic associated with NEOS. It is already known as an independent predictor of outcome in children, and within a pediatric cohort, children younger than 12 years tend to recover more slowly than older ones.[29] Consistent with this observation, we found that the NEOS score predicted poorer long-term outcome in children of a younger age. This element, although discussed in

Figure 2 Association of the NEOS Score With Cognitive Outcome



(A) Bar plots display the frequency of pathologic test scores from neuropsychologic assessments early (2 months) in follow-up and 1 year after diagnosis. The categories intelligence, memory, language, executive function, visuospatial function, and behavior contain raw scores of various test batteries grouped into a binary measure—normal (gray bars) vs pathologic (black bars). White bars (N/A) indicate cases with incomplete data. (B) Predictability analysis using binomial generalized linear models (GLMs) with a now cognition-based outcome reference. After assigning cognitive test scores instead of mRS values to each patient's NEOS score, models revealed associations of a poor outcome (mRS ≥ 3) with deficits in executive function and in memory at 1 year after diagnosis, here shown for the adapted NEOS score. No associations were found with intelligence, behavior, language, and visuospatial function. Solid lines represent best fit and shadows indicate confidence intervals. Tick marks on the upper and lower X axes indicate the number of subjects included, also written next to each line plot. A small random jitter was added to spread ticks around the discrete NEOS score values, to discriminate single data points. NEOS 0: n = 5, NEOS 1: n = 12, NEOS 2: n = 16, NEOS 3: n = 9, NEOS 4: n = 4, NEOS 5: n = 0. For further results (original score, analysis over time), see eTable 5 (links.lww.com/NXI/A813).

the original study[16] was not included as a component in the original NEOS score, but may be of greater importance in a pediatric-only cohort. However, both variables HSE status and age were derived from a smaller cohort than in the original study[16] and would have a priori resulted in lower overall validity of a NEOS score modified by them. Future large-cohort studies are needed to evaluate whether these additional patient characteristics should be included in a pediatric NEOS score to further improve predictive power for long-term clinical outcome in children.

The original cohort[16] consisted of patients diagnosed 10–15 years ago, and much has changed in the field of autoimmune encephalitis since then. The frequency of NMDARE is higher than initially suspected and, in children, exceeds that of viral encephalitis.[30] Increasing awareness combined with established diagnostic criteria[2] has led to a rise in anti-NMDAR antibody testing,[31] faster diagnosis, and earlier treatment initiation.[32] Research into disease etiology has resulted in, e.g., rigorous tumor screening to exclude ovarian teratomas and the discovery of HSE-induced NMDARE.[26] Overall, growing clinical experience with NMDARE generated data on treatment response and relapse rates[12,13] and led to rapid treatment escalation, increasing the use of second-line therapy,[33] and most recently, a consensus recommendation for therapy of pediatric NMDARE.[15] Remarkably, NEOS predicted clinical outcome both in patients diagnosed 10–15 years ago[16] and in our current cohort. In this light, our results show considerable robustness of the NEOS score not only across age groups but also over the years.

Cognitive dysfunction is a major cause of long-term morbidity in pediatric and adult NMDARE,[34] and the contrast between good functional neurologic outcomes and persistent severe cognitive impairment has been repeatedly shown.[35,36] While motor function improves rapidly in most patients with NMDARE, cognitive recovery is still incomplete, and deficits in episodic and working memory, executive function,[29,37,38] attention,[35] language,[29,39] or visuospatial function[40] may persist for years, affecting academic performance, social behavior, and overall quality of life (QoL).[35] In our cohort, two-thirds of the patients assessed 1 year after diagnosis had cognitive deficits, despite already showing good functional neurologic outcome (mRS ≤2). Similarly, in most adults with NMDARE persistent cognitive impairment was found more than 2 years after disease onset, while improvement was observed after up to 5 years of follow-up, highlighting the opportunity for cognitive rehabilitation.[36] Most of our patients suffered from memory impairment and executive dysfunction. This reflects impairment in frontal lobe and hippocampal function and is in line with data from adult patients.[36,38,41] In children, fatigue was identified as an additional factor that particularly affects school performance and QoL.[35]

Despite this, assessment of outcomes in NMDARE is still based on the mRS, a score originally developed to evaluate patients with stroke and monitor their recovery. Focusing mainly on walking ability,[19] the mRS was not intended to be a comprehensive assessment that would take into account the wide range of symptoms seen in NMDARE.[5,42] Therefore, we investigated cognitive function as an alternative outcome. Using the cognitive test scores from our cohort instead of mRS, we found an association between persistent deficits in executive function and memory and a poor clinical outcome predicted by NEOS. These preliminary findings extend on previous results on the outcome of NMDARE in children and adults.[29,36-38] Although it remains to be validated by prospective studies, our data suggest that NEOS may also predict cognitive outcome, which is more important in the long term for most pediatric patients with NMDARE.

This study has several limitations, most of which are related to its retrospective design. First, we included data from 12 centers, both university and district hospitals, which differ in size, resources, and expertise. Therefore, selection bias is less of a concern than differences in treatment approaches and monitoring strategies. This resulted in individual follow-up intervals, differing responses in cases of deterioration or relapse, and inconsistencies in the selection of cognitive tests. Second, follow-up data were sometimes incomplete or scattered across follow-up time points and could be susceptible to recall bias. In particular, the neuropsychological assessment protocol was not standardized and raw scores of the various test batteries could not be directly compared with each other. In addition, most cognitive test scores were obtained within a year and a half of diagnosis and, therefore, could not fully reflect persistent deficits in long-term outcome. Third, both our cohort and the original cohort[16] were comparable because there were no confounding differences in clinical characteristics or initial disease severity. However, we were unable to provide a control group to validate the results of additional variables for a pediatric NEOS score. Furthermore, our group was not large enough to be split for cross-validation.

In conclusion, our data demonstrate the applicability of NEOS in children with NMDARE. This score, which can be easily calculated at bedside, could help estimate the clinical course also in children, thereby supporting their families, physicians, and therapists and identifying pediatric patients at risk who could benefit from intensified therapy and novel treatment strategies including individualized cognitive rehabilitation to improve long-term outcome.

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Continued

Appendix *(continued)*

Name	Location	Contribution
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Retrospective Pediatric Cohort Study Validates NEOS Score and Demonstrates Applicability in Children With Anti-NMDAR Encephalitis

Marc Nikolaus, Philipp Rausch, Kevin Rostásy, et al.

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2.5 Publikation 5

Seit Entdeckung der NMDARE durch Dalmau et al. wurden in den letzten Jahren kontinuierlich neue antineuronale Autoantikörper identifiziert, was zu einer Neubewertung vieler ungeklärter neurologischer oder neuropsychiatrischer Fälle mit Verdacht auf neuroimmunologische Genese geführt hat (Dalmau et al., 2019). Dies legt nahe, dass es noch weitere unidentifizierte Autoantikörper gibt und somit die Gruppe der sogenannten Antikörper-negativen Autoimmunenzephalitiden, wie von Graus et al. definiert, nach wie vor relevant ist. In einer weiteren Arbeit haben wir uns daher mit der Prävalenz dieser Gruppe der Antikörper-negativen Autoimmunenzephalitiden unter ungeklärten pädiatrisch-neurologischen Erkrankungen beschäftigt und untersucht, wie häufig antineuronale Autoantikörper bei Kindern ohne eindeutige Enzephalitis gefunden werden und wie man solchen Verdachtsfällen diagnostisch begegnen kann.

Nikolaus M, Meisel C, Kreye J, Prüss H, Reindl M, Kaindl AM, Schuelke M, Knierim E. Presence of anti-neuronal antibodies in children with neurological disorders beyond encephalitis. ***European Journal of Paediatric Neurology*****. 2020. https://doi.org/10.1016/j.ejpn.2020.07.004 (IF=3,7)**

Antineuronale Autoantikörper können bei verschiedenen neurologischen Störungen jenseits des klinischen Bildes einer Enzephalitis pathogenetisch relevant sein. Bisherige Daten basieren jedoch hauptsächlich auf Serumuntersuchungen, deren Aussagekraft begrenzt ist.

In der vorliegenden Arbeit analysierten wir daher retrospektiv den Liquor von 254 Kindern mit verschiedenen neurologischen Erkrankungen jenseits von Enzephalitis, um das Vorhandensein bislang nicht identifizierter Autoantikörper zu untersuchen. Liquorreaktivität gegen neuronale Oberflächenantigene wurde mittels indirekter Immunfluoreszenz auf unfixierten murinen Hirnschnitten (*tissue-based assay*, TBA) und kommerzieller Testung (*cell-based assay*, CBA) bestimmt. Danach klassifizierten wir die Ergebnisse anhand eines semi-quantitativen Fluoreszenz-Scores und verglichen die klinischen Daten und Krankheitsverläufe aller Screening-positiven Patienten.

Unsere Daten zeigten, dass auch nach Abzug aller nachträglich mittels kommerzieller Testung identifizierter Positivbefunde immer noch eine Gruppe von 10 pädiatrischen Patienten (4%, n = 10/254) mit ungeklärten neurologischen Störungen verblieb, die in unserer Untersuchung eine hoch-positive antineuronale IgG-Immunreaktivität unbekannter Antigenspezifität im Liquor zeigte. Fast alle diese Patienten wiesen Zeichen eines entzündlichen Liquorsyndroms auf und die meisten erholten klinisch sich entweder nicht oder nur teilweise. Fünf Screening-positive

Patienten präsentierten eine Kombination aus Kopfschmerzen und Sehstörung aufgrund einer Optikusatrophie. Die Ergebnisse legen nahe, eine Antikörper-negativen Autoimmunenzephalitis insbesondere bei Fällen ohne definitive Diagnosen in Betracht zu ziehen. Hierbei erweist sich ein gewebsbasiertes Screening auf unbekannte antineuronale Autoantikörper als hilfreich. Wir schlagen daher vor, diese Methode in einem zweiten Schritt als Ergänzung durchzuführen, wenn kommerzielle Antikörpertests ein negatives Ergebnis liefern.

In einem nächsten Schritt wird es notwendig sein, solche noch nicht identifizierte Antikörper zu charakterisieren, ihre pathogenetische Relevanz zu bewerten und schließlich die Frage zu beantworten, ob positive Neuroreaktivität im Liquor immuntherapeutische Ansätze bei V.a. Antikörper-negative Autoimmunenzephalitis rechtfertigen könnte.

Publikation 5 ist keine open access Veröffentlichung. Daher wird an dieser Stelle nur der Literaturhinweis veröffentlicht:

Nikolaus, Marc, Christian Meisel, Jakob Kreye, Harald Prüss, Markus Reindl, Angela M. Kaindl, Markus Schuelke, and Ellen Knierim. "Presence of Anti-Neuronal Antibodies in Children with Neurological Disorders beyond Encephalitis." *European Journal of Paediatric Neurology* 28 (2020): 159–66. https://doi.org/10.1016/j.ejpn.2020.07.004.

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3. DISKUSSION

3.1 Autoimmunenzephalitis im Kindesalter – *from bedside to bench, to bedside*

In meiner Forschung schlage ich einen Bogen von der Klinik in die Grundlagenforschung, *from bedside to bench*, und kehre von dort immer wieder zur klinischen Fragestellung zurück.

In den ersten drei in dieser Habilitationsschrift vorgestellten Studien habe ich primär grundlagenwissenschaftliche Untersuchungen zur Bedeutung und Pathogenese seltener Autoantikörper-assoziierter ZNS-Erkrankungen im Kindes- und Jugendalter beschrieben. Die beiden letzten Studien zeigen hingegen den klinischen und unmittelbar translationalen Ansatz meiner Forschung.

So konnten wir in der ersten Arbeit den Zusammenhang zwischen Autoimmunenzephalitis und Tumor, konkret zwischen Ophelia-Syndrom und Hodgkin-Lymphom, genauer beleuchten. Während frühere Arbeiten auf die Identifizierung von $mGluR_5$ als neuronalem Autoantigen bei Ophelia-Syndrom fokussiert waren, blieb die Frage unbeantwortet, worin die pathogenetische Verbindung zum Hodgkin-Lymphom besteht. Ziel unserer Studie war es daher, nach $mGluR_5$-Expression in Tumorzellen des Hodgkin-Lymphoms zu suchen, um so vielleicht ein besseres Verständnis für die Assoziation zwischen Tumorwachstum und Autoimmunität zu gewinnen. Tatsächlich konnten wir erstmals zeigen, dass $mGluR_5$ in den Tumorzellen des Hodgkin-Lymphoms exprimiert wird und die Hochregulation dieses Rezeptors zu Aktivierung von Signalwegen führt, die eine Rolle bei Proliferation und Migration von Tumorzellen spielen und an Tumorprogression im Hodgkin Lymphom beteiligt sind. Und insgesamt legen diese Ergebnisse nahe, dass $mGluR_5$-Expression nicht nur zu Tumorprogression im Hodgkin-Lymphom führt, sondern gleichzeitig eine anti-$mGluR_5$-Enzephalitis auslösen kann. Daraus leiteten wir die Empfehlung ab, in Fällen von anti-$mGluR_5$-Enzephalitis noch engmaschigere Nachuntersuchung und Monitoring bzgl. der Entwicklung eines Hodgkin-Lymphoms durchzuführen. Gleichzeitig könnte eine reguläre Untersuchung der $mGluR_5$-Expression auf Hodgkin-Zellen als prognostischer Marker dienen und weitere Erkenntnisse über den Krankheitsverlauf eines Lymphoms liefern.

In einer zweiten pathomechanistischen Arbeit untersuchten wir den Zusammenhang zwischen Autoimmunenzephalitis und Hirntumoren. Basierend auf Daten, die zeigten, dass Ovarialteratome von NMDARE-Patienten häufig neuronales Gewebe enthalten und atypische NMDAR-Expression aufweisen, stellten wir die Frage, ob Hirntumore als Auslöser einer

Autoimmunenzephalitis fungieren könnten. Wir untersuchten daher Hirntumorgewebe einer NMDARE-Patientin und fanden atypische NR1-spezifische NMDAR-Expression in dysmorphen Neuronen des Tumors, die von den im Patientenliquor enthaltenen Anti-NMDAR-Antikörpern gebunden wurden. Ähnliche Expressionsmuster wurden auch in entzündetem Gewebe von Patienten mit HSE nicht jedoch in anderen Tumorentitäten beobachtet. Dies legt nahe, dass dysmorphe Neuronen in Tumorgewebe oder entzündetem Gewebe eine Rolle bei der Entstehung von NMDARE spielen können. Mit den Ergebnissen dieser Studie weisen wir darauf hin, dass bestimmte glioneuronale Hirntumore mit NMDARE assoziiert sein können und dass atypische NMDAR-Expression auf dysmorphen Neuronen zur Entwicklung einer autoimmunen Enzephalitis führen kann. Aus diesen Erkenntnissen entwickelten wir ein pathophysiologisches Konzept, das neben der HSE auch die immunogenen Eigenschaften dysmorpher Neuronen als Trigger für die NMDARE benennt. Zusätzlich schlussfolgern wir *from bench to bedside*, dass bei Kindern mit NDMARE verstärkt nach Hirntumoren und umgekehrt bei glioneuronalen Tumoren nach Autoantikörpern gesucht werden sollte.

In der dritten hier vorgestellten grundlagenwissenschaftlichen Studie untersuchten wir antineuronale Reaktivität und Antikörpereigenschaften im Liquor von Patienten mit $GABA_AR$-Enzephalitis. Aus früheren Studien war bekannt, dass bei Autoimmunenzephalitis im Liquor eines Patienten in der Regel verschiedene antineuronale Autoantikörper unterschiedlicher Spezifität gefunden werden. Auch unsere Ergebnisse aus vergleichenden immunhistochemischen Untersuchungen zwischen Liquorreaktivität und der Anti-$GABA_AR$-Bindung kommerzieller monoklonaler Antikörper zeigten signifikante Unterschiede in der Kolokalisation, auf regionaler, zellulärer und subzellulärer Ebene. Unsere Ergebnisse legten nahe, dass Patientenliquor eine polyklonale Immunantwort aus Autoantikörpern gegen verschiedene Untereinheiten des $GABA_AR$ und zusätzlich gegen noch weitere Autoantigene enthält. Mit dieser Heterogenität der individuellen Antikörperzusammensetzung im Liquor eines Patienten bieten wir eine Erklärung für die aus unserer Erfahrung sehr unterschiedlichen klinischen Präsentationen bei $GABA_AR$ Enzephalitis (Nikolaus et al., 2018b) und Autoimmunenzephalitis Patienten im Allgemeinen.

Neben diesen pathophysiologischen Fragestellungen beschäftigen mich in meinem klinischen Alltag insbesondere die Verläufe von Kindern mit Autoimmunenzephalitis, ihr initiales Therapieansprechen und ihre langfristige Prognose. In der hier vorgelegten multizentrischen Studie über die Analyse des NEOS-Scores betrachteten wir daher klinische Verläufe und Outcome von Kindern mit NMDARE mit dem Ziel, dieses neue Bewertungsinstrument zur Vorhersage des Outcomes von Patienten mit NMDARE in einer möglichst großen Kohorte auch für Kinder zu validieren. Die Ergebnisse zeigten, dass der NEOS-Score das Outcome bei Kindern bis zu 16 Monaten nach der Diagnose zuverlässig vorhersagt. Darüber hinaus konnten wir

nachweisen, dass Faktoren wie Alter oder eine vorausgegangene HSE den NEOS-Score ebenfalls beeinflussen. Dass kognitive Beeinträchtigungen bei Kindern und Erwachsenen mit NMDAR-Enzephalitis langfristig zu einem Großteil der Morbidität beitragen, ist seit kurzem bekannt – auch in unserer Studie wiesen die meisten Kinder trotz allgemein gutem neurologischem Outcome langfristige kognitive Defizite insbesondere bei Gedächtnis und exekutiver Funktion auf. Wir konnten jedoch erstmals zeigen, dass der NEOS-Score dieses kognitive Outcome ebenfalls abschätzen kann. Insgesamt zeigt diese Studie somit die Anwendbarkeit des NEOS-Scores bei Kindern mit NMDAR-Enzephalitis und betont die Bedeutung einer frühzeitigen Identifizierung von Risikopatienten, die von intensivierter Therapie und individualisierter kognitiver Rehabilitation profitieren könnten.

Den translationalen Ansatz meiner Forschung habe ich in der letzten hier vorgestellten Arbeit versucht deutlich zu machen. In dieser retrospektiven Analyse an Liquorproben von 254 Kindern mit verschiedenen neurologischen Störungen jenseits von Enzephalitis konnten wir bei 4% der Patienten nicht-identifizierte Neuroreaktivität nachweisen, was auf das Vorhandensein von unbekannten antineuronalen Autoantikörpern hindeutet. Die Kombination von TBA und CBA könnte der bevorzugte diagnostische Ansatz sein, um diese Autoantikörper zu identifizieren. Weitere Untersuchungen sind erforderlich, um Zielantigene zu identifizieren, die Pathogenität zu bewerten und die Frage zu beantworten, wann positive Liquor-Neuroreaktivität eine Immuntherapie rechtfertigt. Zusätzlich kann ein erweiterter Algorithmus im klinischen Alltag helfen, indem er Patienten mit ZNS-Entzündungen und negativen Antikörpertests einbezieht. Ein solcher multimodaler diagnostischer Algorithmus, der TBA, CBA und z.B. molekulargenetische Methoden kombiniert, könnte die klinische Entscheidungsfindung unterstützen und dazu beitragen, Behandlungsoptionen für Patienten mit nicht identifizierter ZNS-Neuroreaktivität zu optimieren. Nicht zuletzt aber zeigen wir mit diesen Daten, dass auch weiterhin nach neuronalen Zielantigenen gesucht werden muss und Forschungsarbeiten notwendig sind, um die Relevanz und zugrunde liegende Pathomechanismen sogenannter Antikörper-negativer Autoimmunenzephalitiden besser zu verstehen.

3.2 Der translationale Ansatz – Bedeutung unseres immunologischen Liquorscreenings

Die Ergebnisse unseres oben vorgestellten Liquorscreenings haben unmittelbare Auswirkungen auf die Patientenversorgung an unserer Klinik. So führen wir regelmäßig gewebebasierte Assays mit Patientenliquor durch, wenn der Verdacht auf Autoimmunenzephalitis besteht und kommerzielle Antikörper-Assays negativ ausfallen. Im Vergleich zur Routinediagnostik bietet ein TBA mehrere Vorteile, wie z.B. hohe Sensitivität, die Erfassung unbekannter Neuroreaktivität und

die Möglichkeit der Untersuchung eines gesamten Hirnschnitts ohne Limitation auf bestimmte Hirnareale. Nachteile bleiben jedoch eine geringe Spezifität, die deskriptive, untersucherabhängige Methode und die in vielen Fällen bislang unklare Relevanz solcher positiver Signale.

Seit Veröffentlichung der oben genannten Studie konnten so bis heute über 1200 Liquorpoben von Kindern mit nicht geklärter neurologischer Symptomatik und V.a. autoimmune Genese zusätzlich zur kommerziellen Liquordiagnostik mittels TBA analysiert werden. Wir detektieren auch weiterhin in ca. 5% dieser Fälle Signale unbekannter Neuroreaktivität, die wir näher untersuchen.

Ein konkretes Beispiel für die Lösung klinischer Fälle mithilfe dieses Liquorscreenings war ein Patient mit komplexer Symptomatik bestehend aus Myoklonien, Hyperhidrosis, Schmerzen und Schlafstörung. Ausgehend von einem auffälligen Befund im gewebebasierten Assay, konnten wir Antikörper gegen CASPR2 und LGI1 nachweisen, was nicht nur zu dem Beginn einer schließlich erfolgreichen immunsuppressiven Therapie führte, sondern auch zur erstmalig publizierten Diagnosestellung des Morvan-Syndroms bei einem Jugendlichen (Nikolaus et al., 2018a).

Mit diesem translationalen Ansatz bearbeiten wir inzwischen Anfragen bei V.a. Autoimmunenzephalitis und erhalten Probenzuweisungen aus Kliniken deutschlandweit. Aufgrund der Ergebnisse und unserer Erfahrung (noch unveröffentlichte Daten) stellen wir bei einer Kombination aus auffälligem Liquorscreening und dem klinischen V.a. Antikörper-negative Autoimmunenzephalitis inzwischen regelmäßig die Indikation zu immunsuppressiver Therapie.

3.3 Der nächste Schritt – Identifikation noch unbekannter Antikörper

Die Identifikation von noch unbekannten antineuronalen Autoantikörpern bzw. ihren Zielantigenen ist der entscheidende Schritt zum Verständnis der Gruppe Antikörper-negativer Autoimmunenzephalitiden. Verschiedene Ansätze und Methoden werden derzeit verfolgt, um diese Lücke zu schließen.

Eine etablierte Methode um Antikörperspezifität aufzuklären, ist die Kombination aus Immunpräzipitation und Massenspektrometrie. Mit Hilfe der Immunpräzipitation werden die Autoantikörper aus Serum oder Liquor eines Patienten durch spezifische Antigen-Antikörper-Interaktion immobilisiert und so gezielt aus der Probe isoliert. Diese Zielantigene werden anschließend mithilfe von Massenspektrometrie analysiert. Seit langem etabliert, hat diese Methode bereits zur Identifizierung bekannter Autoantikörper wie Anti-LGI1 (Irani et al., 2010) oder später Anti-$GABA_AR$ beigetragen (Ohkawa et al., 2014). Sie wird jedoch auch weiterhin

genutzt, um neue Autoantikörper zu entdecken und zu charakterisieren. So wurde in einer jüngeren Arbeit (Scharf et al., 2018) mit Hilfe einer Kombination aus Histo-Immunpräzipitation, Massenspektrometrie und rekombinanter zellbasierter Immunfluoreszenz gleich eine ganze Reihe potenziell relevanter neuronaler Autoantigenen entdeckt und für weitere Untersuchungen bzgl. ihrer pathomechanistischen Rolle bei Autoimmunenzephalitis nutzbar gemacht.

Ein anderer, vielversprechender Ansatz zur Identifikation unbekannter Autoantikörper bei Autoimmunenzephalitis verwendet sog. Bakteriophagen-Bibliotheken, *Phage display* genannt. Diese Methode beruht auf der Präsentation von kurzen Peptiden oder Peptidfragmenten auf der Oberfläche von Bakteriophagen. Ziel ist es, das Peptid auf der Oberfläche desjenigen Phagen zu identifizieren, der spezifisch durch Autoantikörperbindung immobilisiert wird. Damit ist es möglich „rückwärts" ausgehend von der Antikörperbindung den richtigen Phagen, über diesen Phagen die DNA-Information des von ihm exprimierten Peptids und über diese wieder das gesamte Antigen rückzuschließen. Somit wird es schließlich möglich, die gesuchte Zielstruktur zu identifizieren, gegen die der Autoantikörper ursprünglich gerichtet ist. In der Vorarbeit zum *phage display* werden sogenannte Phagen-Bibliotheken hergestellt, in denen unzählige Phagen je ein zufällig über Oligonukleotidsynthese generiertes und auf ihrer Oberfläche exprimiertes Peptidfragment tragen. Eine solche Phagen-Bibliothek kann damit theoretisch das gesamte humane Peptidom repräsentieren. Wird ein solches Gemisch aus Phagen-Peptid-Komplexen nun mit Serum oder Liquor eines Patienten inkubiert, binden Autoantikörper darin spezifisch an diejenigen präsentierten Peptide, welche in Sequenz und Struktur Bestandteilen des Zielantigens gleichen. Die gebundenen Phagen werden isoliert und ihre DNA-Sequenzen analysiert, um die Peptid-sequenz zu bestimmen und damit auf die Sequenz des Zielantigens rückzuschließen. Diese Methode führte u.a. zur Entdeckung neuer Autoantikörper gegen Kelch-like Protein 11 (KLHL11) bei Patienten mit Seminom-assozierter paraneoplastischer Enzephalitis (Mandel-Brehm et al., 2019).

Darüber hinaus bieten moderne Technologien wie Hochdurchsatzsequenzierung *(Next Generation Sequencing, NGS)* und die Einzelzell-Analyse von B-Zell-Repertoires weitere Möglichkeiten, die Antikörper-Vielfalt sowie Zusammensetzung und spezifische Bindungseigenschaften einzelner Antikörper in einer Patientenprobe zu untersuchen. Durch Sequenzierung und Identifizierung der variablen Regionen einzelner Antikörper sowie ganzer B-Zell-Repertoires können spezifische Antikörperklone nicht nur identifiziert, sondern auch in ihrer klonalen Entwicklung verfolgt werden. So können Antikörperproduktion und Bindungseigenschaften in Patientenproben z.B. aus verschiedenen Zeitpunkten charakterisiert werden. Dies erlaubt die detaillierte Untersuchung einer spezifischen Antikörperantwort auf bestimmte Autoantigene – und durch einen Vergleich z.B. der B-Zell-Repertoires von

Autoimmunenzephalitis Patienten mit denen gesunder Kontrollen können so potenzielle Autoantikörperkandidaten identifiziert werden. Mit dieser Methodik konnten z.B. die B-Zell-Repertoires im Liquor von Patienten mit NMDARE erstmals hochauflösend bis auf Einzelzellebene untersucht, sowohl Mono- und polyklonale Antikörper gegen NMDAR identifiziert und der endgültige Nachweis ihrer Pathogenität erbracht werden (Kreye et al., 2016). Diese und ähnliche Ergebnisse verdeutlichen das Potenzial dieser Techniken zur weiteren Aufklärung der zugrunde liegenden Mechanismen von Autoimmunenzephalitis.
In aktuellen Forschungsvorhaben konzentrieren auch wir uns daher auf die Anwendung und Weiterentwicklung solcher Methoden, um bei Verdachtsfällen von Antikörper-negativer Autoimmunenzephalitis im oben erläuterten Liquorscreening nach nicht-identifizierten Autoantikörpern zu suchen.

Durch ein tieferes Verständnis der zugrunde liegenden Immunmechanismen und der spezifischen Autoantigene können neue diagnostische und therapeutische Ansätze entwickelt werden, um die Behandlungsergebnisse für Patienten mit Autoimmunenzephalitis zu verbessern.

4. ZUSAMMENFASSUNG

Die vorliegende Habilitationsschrift mit dem Thema „Untersuchungen zur Bedeutung und Pathogenese seltener Autoantikörper-assoziierter ZNS-Erkrankungen im Kindes- und Jugendalter“ enthält eine repräsentative Auswahl meiner grundlagenwissenschaftlichen und klinischen Arbeiten über Autoimmunenzephalitis und verdeutlicht die Bandbreite meiner Forschung zu diesem Thema. Zwei Studien zur Pathogenese von Autoimmunenzephalitiden beleuchten den Zusammenhang zwischen Enzephalitis und Tumorerkrankungen. So konnten wir für das seltene Ophelia-Syndrom, die Kombination aus anti-mGluR$_5$-Enzephalitis und Hodgkin-Lymphom, zeigen, dass mGluR$_5$ auf Tumorzellen exprimiert wird und hierüber sowohl eine Rolle bei der Tumorprogression, als auch der Entwicklung dieser Autoimmunenzephalitis spielt. Für die NMDARE postulierten wir, dass neben dem Ovarialteratom und der HSE auch Hirntumoren als weiterer Auslöser fungieren können und über atypische NMDAR-Expression auf dysmorphen Neuronen die Entstehung der Enzephalitis triggern. In einer Arbeit über die Charakterisierung neuer Autoantikörper fanden wir im Liquor von Patienten mit GABA$_A$R Enzephalitis Hinweise für eine polyklonale Immunantwort aus Autoantikörpern gegen verschiedene Untereinheiten des GABA$_A$R, womit das sehr heterogene klinische Bild dieser Patienten erklärt würde.

In einer multizentrischen klinischen Studie konnten wir den NEOS-Score für Kinder mit NMDARE validieren und zeigen, dass dieser nicht nur das motorisch-funktionelle Outcome dieser Patienten zuverlässig vorhersagt, sondern auch eine valide Abschätzung der langfristigen kognitiven Defizite geben kann, was für eine frühzeitige Identifizierung von Risikopatienten möglicherweise entscheidend ist. Schließlich gelang es uns im Rahmen eines immunologischen Liquorscreenings bei Kindern mit unklarer neurologischer Symptomatik und V.a. autoimmune Genese, nicht-identifizierte Neuroreaktivität nachzuweisen und damit sowohl einen diagnostischen Ansatz für Verdachtsfälle von Antikörper-negativer Autoimmunenzephalitis zu etablieren als auch eine wissenschaftliche Grundlage für die Identifikation noch unentdeckter antineuronaler Autoantikörper zu schaffen.

Insgesamt verdeutlichen diese Arbeiten die Relevanz erweiterter Diagnostik für die Entdeckung Antikörper-negativer Autoimmunenzephalitiden, eine frühzeitige Identifizierung von Risikopatienten und bessere Therapieoptionen. Durch den translationalen Ansatz, tragen die Ergebnisse nicht nur zu einem besseren Verständnis der Pathogenese, sondern auch von Klinik und Outcomes seltener Autoantikörper-assoziierter ZNS-Erkrankungen bei Kindern und Jugendlichen bei.

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ERKLÄRUNG

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