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POSTERIOR REVERSIBLE ENCEPHALOPATHY SYNDROME AND OTHER SEVERE CENTRAL NERVOUS SYSTEM ADVERSE EVENTS IN THE NOPHO ALL2008 PROTOCOL:

CLINICAL AND RADIOLOGICAL FINDINGS, GENETIC RISK FACTORS, AND PROGNOSIS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family,
my friends, and my colleagues.

” Wherever the art of medicine is loved,
there is also a love of humanity”
Hippocrates, 460-370 BC

ABSTRACT

Background: The advances in the therapeutic protocols for pediatric acute lymphoblastic leukemia (ALL) have led to a current survival rate of more than 90% in developed countries. Treatment periods are, however, long and marked by complications and toxicity that may challenge treatment outcomes and quality of life for patients. Central nervous system (CNS) toxicity is common during pediatric ALL treatment and may implicate treatment postponement as well as long-term adverse effects. The aim of this thesis was to map CNS toxicities in pediatric ALL in patients treated according to the Nordic Society of Pediatric Hematology and Oncology (NOPHO) ALL2008 protocol.

Methods: Patients aged 1 to 17.9 years at diagnosis of B-cell-precursor and T-cell ALL who were treated according to the NOPHO ALL2008 protocol between 2008 and 2015 were included. Detailed data on CNS toxicity were collected from the NOPHO ALL2008 registry with seven participating countries and a complementary questionnaire addressing phenotypical and work-up details. Genome-wide association studies (GWAS) and candidate single nucleotide polymorphism (SNP) analyses were performed. A validation study of significant findings from GWAS and candidate SNP analyses was made in an independent Australian cohort of pediatric ALL patients (n=797) including patients with diverse CNS toxicities (n=103) and methotrexate-related CNS-toxicity (n=48). The role of minimal CNS leukemia in CNS toxicity risk was further examined by detecting leukemic blasts in cerebrospinal fluid (CSF) by flow cytometric immunophenotyping (FCI) in addition to cytomorphological analysis (CM), which is the CSF examination method specified in the NOPHO ALL2008 protocol.

Results: 1464 patients were included in the study of whom 52 (3.8%) had posterior reversible encephalopathy syndrome (PRES), and 135 (9.2%) had at least one form of CNS toxicity. Overall, 82/135 patients had at least one seizure episode (60.7%). PRES was the most common form of CNS toxicity in this cohort (38.5%). Older age, defined as each extra year of age and/or as patient group >10 years of age was a significant risk factor for PRES, seizures, and all CNS toxicities. T-cell immunophenotype was significant risk factor for PRES in univariate analysis and after adjustment for age. Leukemic blasts in CSF by CM were significantly related to PRES during induction and high-risk block treatment was related to PRES after induction. Minimal CNS leukemia, detected by FCI, was a significant risk factor for PRES, seizures, and all CNS toxicities in patients without CNS leukemia by CM in univariate analyses and for PRES and seizures after adjusting for induction therapy. Genome-wide association studies did not demonstrate any significant associations with CNS toxicities, but candidate SNP analyses showed that the *ATXN1*rs68082256 SNP, related to epilepsy, was associated with seizures in patients <10 years. *ATXN1*rs68082256 was replicated in the Australian cohort in the patient group with diverse CNS toxicities. At the last follow-up, 11.7% of survivors (12/103) who had displayed CNS toxicity were reported to have had an

epilepsy diagnosis. Clinical suspicion of neurocognitive impairment was reported for 10.9% of survivors (12/110) with CNS toxicity at their last follow-up, but neuropsychiatric testing was performed in only two cases.

Conclusion: Central nervous system toxicity was common during pediatric ALL treatment and PRES was the most common form of CNS toxicity in this cohort. Older patients had a greater risk of CNS toxicity as well as patients with minimal CNS leukemia. The role of *ATXN1*rs68082256 SNP in CNS toxicity warrants further studies. Epilepsy is rather common in ALL survivors, while the neurocognitive outcome warrants more systematic follow-up.

POPULAR SCIENCE SUMMARY OF THE THESIS

In the beginning of the 20th century, no one believed that acute lymphoblastic leukemia (ALL) would one day be a curable disease. Instead, the real case scenario was patients who filled up cancer wards and suffered for weeks or months before dying. Not only did the treatment attempts fail time after time, but patients also suffered from serious adverse effects from the treatments that were available, worsening their suffering during the time they had left. Nevertheless, here we are now, about 100 years later, witnessing pediatric ALL survival rates of around 90% with patients recovering and able to live full lives, though often with some treatment-related side effects. Thereby, the current challenge is 100% survival without any severe treatment-related toxicity.

It is not easy or uncomplicated to meet this challenge, however. We still need to understand the nature of treatment-related toxicities before we can diminish them. In this thesis, the focus is on toxicity in the central nervous system (CNS) during pediatric ALL treatment, according to the Nordic-Baltic treatment protocol NOPHO ALL2008, with main focus on the toxicity involving swelling of brain regions known as posterior reversible encephalopathy syndrome (PRES).

Data for this thesis were collected from a registry with collaboration of seven countries (Denmark, Finland, Iceland, Norway, Sweden, Estonia, and Lithuania) and a complementary questionnaire. Scientists working on statistical analyses of genetic data, known as bioinformaticians, performed advanced studies known as genome-wide association studies and candidate single nucleotide polymorphism (SNP) studies. Scientists with laboratory specialties analyzed the cerebrospinal fluid which circulates in the CNS to detect minimal leukemic cell counts. Collaboration with an independent study group in Australia was established in order to perform a validation study of the genetic findings.

Overall, 1464 patients were included in the study, of whom 52 were reported to have had PRES, and 135 were reported to have had at least one CNS toxicity including PRES. The most common symptom of CNS toxicities overall were seizures. Papers I-III demonstrated that older age was a significant risk factor for CNS toxicities. CNS leukemia was significantly related with PRES during the first five weeks of treatment, namely the induction period. Aggressive chemotherapy in patients with high-risk leukemia was significantly related to PRES after the induction period. Paper IV demonstrated that minimal CNS leukemia was a significant risk factor for PRES and seizures. Genome-wide association studies in Paper III did not demonstrate any significant associations to CNS toxicities, but candidate SNP analysis showed that the *ATXN1*rs68082256 SNP, related to epilepsy, was associated with seizures in patients <10 years. *ATXN1*rs68082256 was replicated in the Australian cohort in patients with diverse CNS toxicities. At their last follow-up, 12/103 survivors who had had CNS toxicity were reported to have had an epilepsy diagnosis.

Clinical suspicion of neurocognitive impairment was reported for 12/110 survivors with CNS toxicity at last follow-up, but neuropsychiatric testing was performed in only two cases.

Findings in this thesis do not answer all questions regarding CNS toxicity, but they shed some light on aspects like the role of age, individual genetic characteristics, leukemic cells in the CNS, and outcome including epilepsy and eventual neurocognitive impairment. Further studies and large international collaborations are warranted to further map out and defeat CNS toxicity in ALL. Hopefully, in the future people will remember the 21st century as the era that ALL was defeated completely.

LIST OF SCIENTIFIC PAPERS

I. **Posterior reversible encephalopathy syndrome in children with acute lymphoblastic leukemia: clinical characteristics, risk factors, course, and outcome of disease.** Anastasopoulou S, Eriksson MA, Heyman M, Wang C, Niinimäki R, Mikkel S, Vaitkevičienė GE, Johannsdottir IM, Myrberg IH, Jonsson OG, Als-Nielsen B, Schmiegelow K, Banerjee J, Harila-Saari A, Ranta S. Pediatr Blood Cancer. 2019 May;66(5):e27594. doi: 10.1002/pbc.27594.

II. **Seizures during treatment of childhood acute lymphoblastic leukemia: A population-based cohort study.** Anastasopoulou S, Heyman M, Eriksson MA, Niinimäki R, Taskinen M, Mikkel S, Vaitkeviciene GE, Johannsdottir IM, Myrberg IH, Jonsson OG, Als-Nielsen B, Schmiegelow K, Banerjee J, Ranta S, Harila-Saari A. Eur J Paediatr Neurol. 2020 Jul;27:72–77. doi: 10.1016/j.ejpn.2020.04.004.

III. **Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes.** Anastasopoulou S, Nielsen RL, Als-Nielsen B, Banerjee J, Eriksson MA, Helenius M, Heyman MM, Johannsdottir IM, Jonsson OG, MacGregor S, Mateos MK, Mayoh C, Mikkel S, Myrberg IH, Niinimäki R, Schmiegelow K, Taskinen M, Vaitkeviciene G, Warnqvist A, Wolthers B, Harila-Saari A, Ranta S. Haematologica. 2022 Oct 1;107(10):2318-2328. doi: 10.3324/haematol.2021.280016.

IV. **Does minimal central nervous system involvement in childhood acute lymphoblastic leukemia increase the risk for central nervous system toxicity?** Anastasopoulou S, Harila-Saari A, Als-Nielsen B, Eriksson MA, Heyman M, Johannsdottir IM, Marquart HV, Niinimäki R, Pronk CJ, Schmiegelow K, Vaitkeviciene G, Thastrup M, Ranta S. Pediatr Blood Cancer. 2022 Jul;69(7):e29745. doi: 10.1002/pbc.29745.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **Pharmacogenetics of the Central Nervous System-Toxicity and Relapse Affecting the CNS in Pediatric Acute Lymphoblastic Leukemia.** Sági JC, Gézsi A, Egyed B, Jakab Z, Benedek N, Attarbaschi A, Köhrer S, Sipek J, Winkowska L, Zaliova M, Anastasopoulou S, Wolthers BO, Ranta S, Szalai C, Kovács GT, Semsei ÁF, Erdélyi DJ. Cancers (Basel). 2021 May 12;13(10):2333. doi: 10.3390/cancers13102333.

CONTENTS

LIST OF ABBREVIATIONS

6MP	Mercaptopurine
ADHD	Attention deficit hyperactivity disorder
AED	Antiepileptic drug
ALL	Acute lymphoblastic leukemia
Ang II	Angiotensin II
Ara-C	Cytarabine
ASM	Anti-seizure medication
BBB	Blood-brain barrier
BCP	B-cell precursor
CI	Confidence interval
CNS	Central nervous system
CO	Carbon dioxide
CSVT	Cerebral sinus venous thrombosis
CT	Computed tomography
EEG	Electroencephalogram
ET1	Endothelin 1
FCI	Flow cytometric immunophenotyping
FDA	Food and drug administration
GWAS	Genome-wide association studies

GSEA	Gene set enrichment analysis
HR	Hazard ratio
ICAM-1	Intracellular adhesion molecule
ICH	Intracranial hemorrhage
ICU	Intensive care unit
ILAE	International league against epilepsy
JC	John Cunningham
LP	Lumbar puncture
MRI	Magnetic resonance imaging
NMDA	N-Methyl-d-Aspartate
NO	Nitric oxide
NOPHO	Nordic society for pediatric hematology and oncology
OR	Odds ratio
PdL	Ponte di Legno consortium
PGI2	Prostacyclin
PRES	Posterior reversible encephalopathy syndrome
PRS	Polygenic risk score
SCT	Stem cell transplantation
SLS	Stroke like syndrome
SNP	Single nucleotide polymorphism

TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

As pediatric neurologist, I have the chance to meet pediatric patients with various underlying diseases, presenting with acute or chronic neurological symptoms. Inevitably, I meet pediatric oncology patients with central nervous system (CNS) tumors, but also other malignancies with CNS symptoms, which are predominantly related to treatment complications.

My interest in posterior reversible encephalopathy syndrome (PRES) and other CNS toxicities during the treatment of acute lymphoblastic leukemia (ALL) comes directly from the clinic and a six-year-old girl with ALL who was admitted at the child neurology department due to treatment-related PRES. During the days I was responsible for the tiny patient I also met the oncologist in charge, who ended up being one of my PhD supervisors. We then discussed PRES in pediatric ALL patients, risk factors, and implications for ALL treatment, and I was impressed by the progress in ALL treatment, the frequent favorable outcome for patients, and the ambition to optimize treatment protocols and thereby outcomes for patients and their families. This was the beginning of my doctoral adventure. Soon after that, I met my main supervisor and my other two co-supervisors as well as many other competent scientists in Nordic countries and the rest of the world who helped me gain insights on pediatric ALL and its challenges with a focus on CNS toxicities, and the goal of uncomplicated treatment leaving survivors free of treatment-related sequelae. It also became clear to me that in order to eliminate treatment-related CNS toxicities, we first needed to understand their pathogenesis.

In this thesis, I have focused on studying PRES and other severe CNS toxicities, clinical presentation, risk factors and outcome in pediatric ALL patients. Thankfully, this work was supported by the Nordic Society of Pediatric Hematology and Oncology (NOPHO) group and the NOPHO ALL2008 registry, which facilitated data collection from seven countries.

2 LITERATURE REVIEW

2.1 HISTORICAL ASPECTS OF ALL IN CHILDREN AND ADOLESCENTS

The history of human cancer is more than 5,000 years old, according to evidence from ancient Egypt, but its recognition as distinct disease named "karkinos", later updated to the Latin "cancer", was coined about 2,500 years ago by the Greek physician Hippocrates (460–370 B. C.)[1,2]. It was, however, the 19th century that leukemia was first described, probably due to advances in microscopy and diagnostic methods[3]. The first case reports describe patients presenting with a lethal disease with spleen enlargement and purulent alterations of the blood and "pus and inflammation" were considered to be probable causes of disease[3,4]. The first case report that framed the distinct nature of leukemia for these patients came from Bennett in 1845, who referred to it as leucocythemia or 'white cell blood'[5]. The term "leukhemia" (white blood) was introduced a few years later by Virchow[6]. Eventually, Neumann's and Bizzozero's work demonstrated the involvement of bone marrow in leukemias, even if it took two decades before scientific society accepted their findings, and Erlich maintained that leukemia is a primary disease of the hematopoietic system[3]. Since then, discoveries contributed to better classification and subtyping of leukemias including ALL[3]. The first pediatric leukemia case was reported in 1850[3].

2.2 THERAPEUTIC ADVANTAGES

While diagnostic discoveries and classification of ALL advanced relatively fast, progress in treatment was not as rapid, and by the middle of the 20th century leukemia was still considered an incurable disease[3]. Early therapeutic strategies included quinine, arsenic, blood transfusions, and x-rays, some of which led to temporary remissions that unfortunately did not last. In the middle of the 20th century nitrogen mustards, alkylating agents, busulfan, folate antagonists, adrenal corticosteroids, and 6-mercaptopurine were identified as potent therapeutic agents targeting leukemia, especially pediatric leukemia, which catalyzed further therapeutic advantages[3,7].

The introduction of folate antagonists in 1947 and, following this, corticosteroids, by Farber and the introduction of combination chemotherapy in 1961 by Frei, and Freireich, et al. for the treatment of pediatric ALL leukemia started the new therapeutic era of chemotherapy for ALL[8]. Soon after that, Pinkel et al. proposed a four components therapy-strategy engaging: remission induction, CNS therapy, intensification (consolidation) therapy, and continuation treatment (maintenance) that is still the backbone of most ALL treatment protocols[9]. The parallel achievements in ALL biology and immunology eventually led to individual therapies and doses targeted to patient characteristics and genetic profiles, paving the way for ALL treatment to become a success story with current 5 years survival rates up to 90% in developed countries[7,10-13].

2.3 TREATMENT RELATED CNS TOXICITY

Along with improved ALL patients survival rates, reports on acute toxicities as well as late effects of therapy have also increased, including CNS toxicities[14]. The incidence of CNS toxicities during ALL treatment is 3.6–13% in various protocols and oncology centers, and they may result in significant morbidity and in some cases mortality[14-19]. The most frequently described of these toxicities are PRES, seizures, cerebral sinus venous thrombosis (CSVT), intracranial hemorrhage (ICH), methotrexate-related stroke-like syndrome (SLS), methotrexate-related leukoencephalopathy, and CNS infections, all of which commonly occur during the first months of treatment[14-16,19-26].

Additionally, ALL survivors with CNS toxicity during treatment are reported to suffer from long-term treatment-related sequelae such as neurocognitive impairment, motor difficulties, and epilepsy, and recent studies have shown that ALL survivors had decreased volume of grey and white matter in cortical and subcortical brain structures[16,20,27,28]. Of note, late sequelae affecting neurocognitive performance is described even in ALL survivors who did not experience CNS toxicities during treatment[27-29].

2.4 CNS TOXICITIES IN FOCUS FOR THIS THESIS

2.4.1 PRES

PRES was first described as a clinical entity in 1996 based on observations of patients receiving immunosuppressive therapy after transplantation or as treatment for aplastic anemia, hypertensive encephalopathy, eclampsia, and one patient with melanoma treated with interferon[30]. Most patients with PRES had hypertension, and about half of them had impaired renal function. Presenting symptoms were seizures, headache, cortical blindness, confusion, and motor symptoms[30]. Neuroimaging studies predominantly revealed edema in posterior cerebral regions but also of the brainstem and cerebellum. The symptoms regressed in all patients after antihypertensive treatment and withdrawal of immunosuppressive treatment[30]. Reports on PRES and its course, which is not allways favorable, in the presence of diverse conditions have accumulated over time and the syndrome is currently recognized as CNS toxicity in cancer patients, including patients with ALL[25,31,32].

The pathogenesis of PRES is believed to be related to discontinuation of the blood-brain barrier (BBB), mainly due to hypertension but also to toxic endothelial damage[31,32]. The BBB is formed by endothelial cells with tight junctions in cerebral vessels[32]. Vascular tone is regulated by vasodilators as nitric oxide, carbon dioxide, and prostacyclin as well as vasoconstrictors as thromboxane A2, endothelin 1, and angiotensin II[32]. It is suggested that increased systemic blood pressure exceeds the autoregulatory mechanisms of the cerebral vasculature leading to damage of the BBB, extravasation of plasma and vasogenic edema[31,32]. Posterior cerebral structures are more susceptible to impairment of cerebral autoregulation

due to poorer sympathetic innervation, which is compatible with edema mainly in posterior brain regions in PRES[32].

In some other cases, fluctuations in systemic blood pressure may implicate PRES, including patients with sepsis and hypotension[32]. In patients with inflammation, activation of lymphocytes and monocytes leads to the release of cytokines as interleukin 1, interferon, and tumor necrosis factors, which stimulate secretion of vasoactive factors including vascular endothelial growth factor (VEGF), intracellular adhesion molecule 1, and vascular cell adhesion protein 1, thereby increasing vascular permeability and leading to interstitial edema (Figure 1)[31,32]. Cytotoxic dugs, including immunosuppressive and chemotherapeutic agents, are suggested to cause PRES through direct endothelium damage or hypertension[31,32].

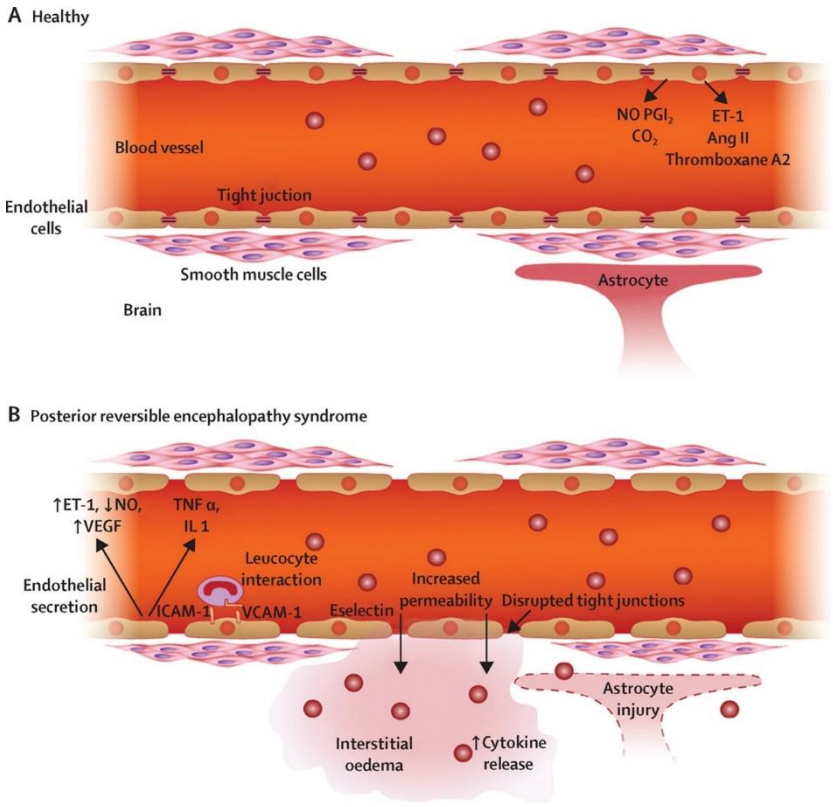

Figure 1. Endothelial function and pathophysiology of posterior reversible encephalopathy syndrome. (A) Physiological function of the blood-brain barrier. (B) Breakdown of the blood-brain barrier. NO: nitric oxide, CO_2: carbon dioxide, PGI2: prostacyclin, ET1: endothelin 1, Ang II: angiotensin II, VEGF: vascular endothelial growth factor, TNF: tumor necrosis factor, ICAM-1: intracellular adhesion molecule, VCAM-1: vascular cell adhesion protein 1. *Source: The Lancet, Vol 14, Fugate, J.E.; Rabinstein A.A. Posterior reversible encephalopathy syndrome: clinical and radiological manifestations, pathophysiology, and outstanding questions, p. 914–925., Copyright 2015, with permission from Elsevier.*

Posterior reversible encephalopathy syndrome in pediatric ALL is a clinical diagnosis supported by respective neuroimaging findings according to the international Ponte di Legno consortium (PdL) Delphi consensus on CNS toxicities in ALL published in 2016[25,33]. Abnormal electroencephalogram (EEG), hypertension and occurrence during the first months of ALL treatment also support PRES diagnosis[25]. The incidence of PRES in pediatric ALL

varies among diverse treatment protocols, from 1.6% to 4.5%[20,26,34,35]. Hypertension, constipation, and alkalization for over two weeks have been associated with higher risk of PRES in pediatric ALL patients, and epilepsy has been described as a common late sequelae among survivors with PRES during treatment[20]. However, comparative studies of risk factors and differences in PRES occurrence among various protocols are scarce, and therefore further studies of PRES in pediatric ALL patients treated according to different protocols are merited.

2.4.2 Seizures

A seizure is defined as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain"[36]. Clinical manifestations of seizures include non-motor and motor symptoms that might be focal or generalized, with or without impaired consciousness of the patient[36]. The pathophysiology of seizures builds on impairment of normal axonal transmission: either due to decreased inhibition, mediated by γ-amino-butyric acid neurotransmission; or increased excitation, mediated by glutamate neurotransmission[37]. Mutations of ion channels are involved in seizures due to prolonged action potentials[37]. Electrolyte disturbances such as hyponatremia (mostly)/hypernatremia, hypocalcemia (mostly)/hypercalcemia and hypomagnesemia, as well as metabolic disturbances as hypoglycemia (mostly)/hyperglycemia, decreased urea nitrogen, and increased creatinine can cause seizures[38-40]. Finally, yet importantly, drivers of toxicity, including medications and brain injuries can disturb normal neuronal activity and/or connectivity leading to seizures[40,41].

Seizures are intuitively linked to epilepsy. However, they may also constitute the symptom of an acute brain insult and thus be defined as acute symptomatic seizures: "events, occurring in close temporal relationship with an acute CNS insult, which may be metabolic, toxic, structural, infectious, or due to inflammation. The interval between the insult and the seizure may vary according to the underlying clinical condition"[38,40,41]. Both acute symptomatic seizures and epilepsy are closely related to cancer; predominately brain tumors and other cancers with brain metastases, but also cancer without brain involvement[41-43]. Brain tumors and metastases generate seizures due to disruption of cerebral connectivity, metabolism, neurotransmission, the BBB and increased vascular permeability[41]. Pathogenesis of seizures in cancer without brain involvement is commonly indirect, through treatment complications (infections, metabolic disorders, brain hemorrhages, stroke) or paraneoplastic syndromes, but also direct through pharmacotoxicity (L-asparaginase, cisplatin, methotrexate, chimeric antigen receptor T-cell therapies)[41-43].

Seizures in ALL are described as one of the common treatment-related toxicities by the PdL consortium[25]. They are often isolated events, due to direct pharmacotoxicity, but also occur as symptom of PRES, CSVT, encephalopathy, methotrexate-related SLS, infections and electrolyte disturbances[16,21,23-25]. Data on risk factors for seizures in pediatric ALL are limited[44]. Cognitive impairment and epilepsy have been reported as late sequelae in patients

with CNS toxicities displaying seizures, but data are also scarce[45,46]. Thus, there are reasons to further explore seizures in ALL.

2.4.3 Other CNS toxicities

Besides PRES and seizures, the latter commonly being a symptom rather than primary CNS toxicity, methotrexate-related SLS and leukoencephalopathy, CSVT, and CNS infections are examples of more treatment-related CNS toxicities that delay protocol treatment and affect outcomes[15,19,25]. Classification of CNS toxicities is actively being updated according to advances in understanding of ALL biology and diagnostic methods; for example CNS toxicity that previously was described as seizures with vision disturbances would probably be classified as PRES in many cases, and CNS toxicity that was described as transient paresis would probably be classified as methotrexate-related SLS currently[14-16,19,21,23-25,47,48].

Methotrexate-related SLS, methotrexate-related leukoencephalopathy, and CSVT that is mainly associated with asparaginase and steroids, are being studied particularly due to their well-defined phenotypes and association with chemotherapeutic agents[18,21,24,49].

Table 1 summarizes CNS toxicities, as described by the PdL Delfi consensus and in diverse literature according to current nomenclature[16,19,20,25,48,50].

Table 1. CNS toxicities in ALL.

	CNS toxicity
1.	Aseptic meningitis of cytarabine or methotrexate
2.	Brain abscess
3.	CSVT
4.	Encephalitis
5.	Encephalopathy (NOS)
6.	Depressed level of consciousness
7.	Intracerebral hemorrhage
8.	Ischemic injury
9.	Meningitis (including fungal)
10.	Methotrexate related chronic leukoencephalopathy
11.	Methotrexate-related SLS
12.	PRES
13.	Pseudotumor cerebri
14.	Seizures (alias: convulsions)
15.	Transient ischemic attack
16.	Transverse myelopathy

CNS: central nervous system, ALL: acute lymphoblastic leukemia, CSVT: cerebral sinus venous thrombosis, SLS: stroke like syndrome, PRES: posterior reversible encephalopathy syndrome, NOS: not otherwise specified.

2.5 ETIOLOGY OF CNS TOXICITY ACCORDING TO CURRENT KNOWLEDGE

2.5.1 Pharmacotoxicity

Some chemotherapeutic agents used in the treatment of pediatric ALL, which are summarized in this paragraph, are particularly related to CNS toxicity[51-53]. Their toxicity is commonly a consequence of their mechanism of action[53].

Methotrexate

Methotrexate is a hydrophobic antimetabolite which inhibits purine and thymidine synthesis by inhibition of dihydrofolate reductase[53,54]. Methotrexate's CNS toxicity is believed to be a result of disruption of folate homeostasis in the CNS and/or direct CNS damage[21,53-55]. High intravenous or intrathecal methotrexate doses implicate acute or chronic CNS toxicity, including acute encephalopathy, methotrexate-related SLS, chronic leukoencephalopathy, seizures, headache, aseptic meningitis, and PRES[21,25,53,54].

Vincristine

Vincristine, is a vinca alkaloid that acts mainly through disruption of mitotic spindle formation, which results in mitosis arrest at metaphase[53,56]. Vincristine's most common neurological toxicity is peripheral neuropathy, but CNS toxicity is also reported including PRES, coma, and dizziness[51,53,56].

Glucocorticoids

Glucocorticoids, including prednisolone and dexamethasone, have been used for the treatment of ALL since the early 1950s, and their role is essential for treatment of CNS leukemia[7,52,57-59]. Dexamethasone is more effective due to better CNS penetration, longer half-life, and anti-inflammatory implications, however it is also related to more severe toxicity[57]. The toxicity of glucocorticoids is mediated by the binding of glucocorticoid receptors that may eventually lead to inhibition of cytokine production, cell cycle arrest, apoptosis, and hypertension[57,60]. The use of glucocorticoids in ALL treatment is related to steroid psychosis, CSVT, and PRES and it has been suggested that they implicate late neurocognitive effects[57,58,61].

Doxorubicin

Doxorubicin is an antibiotic isolated from the bacterium Streptomyces peucetius, which has been used in cancer treatment since the 1960s and belongs to the anthracyclines category[62]. Doxorubicin inhibits DNA and RNA synthesis, damages DNA, and causes apoptosis[62]. Doxorubicin is related to cognitive impairment while a recent study indicates that it impairs synaptic processes associated with hippocampal neurotransmission[63,64].

Asparaginase

Asparaginase is an enzyme derived of Escherichia coli and Erwinia chrysanthemi, which catalyzes the conversion of asparagine to aspartic acid and ammonia, thereby reducing

circulating levels of asparagine[65,66]. Asparagine is a non-essential enzyme, necessary for DNA, RNA, and the protein synthesis mandatory for cellular growth and function[65,67]. Leukemic cells cannot synthesize asparagine, and by reducing the circulating asparagine levels asparaginase implicates leukemic cell death[65,66,68]. Asparaginase is reported to have been related to seizures, PRES, encephalopathy, and CSVT[24,65,66,68].

Mercaptopurine

Mercaptopurine (6MP) is a purine antagonist that inhibits DNA replication as well as RNA and protein synthesis; it requires conversion to 6-thioguanine nucleotides in the liver to be activated[69]. Mercaptopurine in not directly related to CNS toxicity, but may cause liver damage and hypoglycemia, which can lead to seizures[69,70].

Cytarabine

Cytarabine (Ara-C) is a nucleoside isolated from the sponge Cryptotethia crypta in the early 1950s, that is classified as antimetabolite and belongs to the anthracyclines drug category[71,72]. It is a pyrimidine analog that inhibits DNA replication and repair, and is metabolized by hepatic cytidine deaminase[72]. Cytarabine crosses the BBB, and CNS toxicity was suggested to depend on the absence of cytidine deaminase in the CNS[53,72]. Reports of cytarabine CNS toxicity include seizures, PRES, cerebellar dysfunction, and gait disturbances[53,71,72].

Fludarabine

Fludarabine is a purine analog which in its active form—2F-ara-ATP—is incorporated into nucleic acids and can inhibit DNA synthesis[73]. Fludarabine's toxicity is dose-related and includes seizures, encephalopathy, blindness, coma, and progressive multifocal leukoencephalopathy by activation of the John Cunningham (JC) virus[53,73].

Cyclophosphamide

Cyclophosphamide is a type of nitrogen mustard drug which acts through alkylation of DNA[74]. Central nervous system toxicity is not common, but several studies report PRES as complication during treatment with cyclophosphamide, however not all of these studies include ALL patients[74-78].

2.5.2 Genetic predisposition to CNS pathology

The occurrence of CNS toxicities in some ALL patients, despite the fact that treatment protocols are the same for patients who stratify to the same risk groups, has motivated research on individualized predisposition to pharmacotoxicity with a focus on polymorphisms related to toxicity or the efficacy of cytostatics[79,80]. Polymorphisms in *TPMT* and *NUDT15* genes are evidently associated with pharmacotoxicity (mainly myelosuppression) in ALL, through regulation of mercaptopurine metabolism, and thereby the food and drug administration agency in the United States (FDA) recommends reducing mercaptopurine doses in patients with reduced activity of the two genes in order to decrease the risk of liver toxicity[81].

There is currently not enough evidence on pharmacogenetic susceptibility to acute CNS toxicities but accumulating studies suggest this may be the case[17,21,80,82-86]. Regarding methotrexate, researchers have focused on polymorphisms in *MTHFR* that may inhibit folate metabolism and lead to increased methotrexate levels[80]. Genome-wide association studies (GWAS) have, so far, not shown any significant pharmacogenetic associations between methotrexate and its most common CNS toxicity, leukoencephalopathy[18,21].

In contrast, one study on neurocognitive outcomes in pediatric ALL survivors, applying a candidate single nucleotide polymorphism (SNP) approach, demonstrated that polymorphisms in *MS*, *GSTT1*, *GSTP1*, and *MAOA* genes were associated with specific neurocognitive deficits[87]. Even studies on pharmacogenetic associations with the relatively common vincristine peripheral neurotoxicity neuropathy have shown significant associations[82,86]. This suggests that studies of pharmacogenetics in larger patient cohorts are needed to demonstrate significant associations with CNS toxicities. Another aspect is the need of well-defined phenotypes of CNS toxicities for pharmacogenetic studies. Central nervous system toxicities are commonly studied as a coherent group of toxicities, probably to facilitate studies of larger patient groups and thereby increase their statistical power[14-16,19,53,83]. This is not necessarily misleading, but in order to demonstrate pharmacogenetic associations with CNS toxicity, concrete phenotypes are beneficial[88].

Introduction to genome-wide association studies

Genome-wide association studies analyze DNA sequence across the human genome to detect differences in allele frequencies that might relate to phenotypical traits and diseases in individuals with similar ancestry but different phenotypes. The most common genetic variants of interest in GWAS are single base-pair changes in the DNA sequence, also known as SNP that typically have two alleles. The reported SNP frequency is the one of the less-frequent allele, the "minor allele". One or more common alleles may be involved in complex diseases. Results of analyses typically report blocks of correlated SNP with significant associations with the studied phenotype called "genomic risk loci"[88,89].

Genome-wide associated studies can be performed for population-based cohorts, but also in families and isolated populations and biobanks; the study design can be case-control or quantitative. After having carefully defined the phenotype of interest and selected the study population, all individuals are genotyped with microarrays or next generation sequencing methods such as whole exome sequencing (WES) or whole genome sequencing (WGS) followed by quality control and imputation of untyped SNP. Subsequently, anonymized case numbers, gender, phenotypes, coded family relations between individuals, covariates, and genotype data are entered into files that proceed to testing for associations between SNP and phenotypes[88,89].

During GWAS analyses millions of associations between individual SNP and the studied phenotype are tested, necessitating a strict multiple-testing threshold to avoid false positive results. The international HapMap project suggest a Bonferroni testing threshold of $P < 5x10^{-8}$

(false discovery rate of $0.05/10^6$), but the appropriate threshold may vary according to study population. As GWAS test associations with complex diseases which may be determined by diverse genetic variants with small effects each, even significant findings can still be false positive: the so-called "winner's curse". Thereby, validation of GWAS findings is warranted in at least one completely-independent cohort. See an overview of GWAS steps in Figure 2. Of note, candidate gene or candidate SNP studies (also called replication studies) with a concrete hypothesis do not require correction to the above level since the number of effective, independent statistical tests is much lower than what is assumed for GWAS[88,89].

Although GWAS analyses can identify SNP with significant associations to phenotype traits, their effect is commonly small with limited predictive value, and some traits may be explained by evaluating the effects of several SNP simultaneously. Analyses of polygenic risk scores (PRS) have inevitably been introduced to estimate heritability and make an individual prognosis of phenotypes based on genetic profile. Polygenic risk scores are typically calculated as weighted sum scores of effect alleles, with weights based on the effect sizes as estimated by a GWAS on the phenotype in question[89,90].

Genome-wide association analyses are used in studies aiming to explore disease risk and underlying biology, heritability, drug development, and individualized treatment[88,89]. As with all genetic analyses, interpreting results is not always simple, and holistic evaluation of GWAS findings, clinical and laboratory evaluations, and neuroimaging studies are needed to arrive at the correct conclusions.

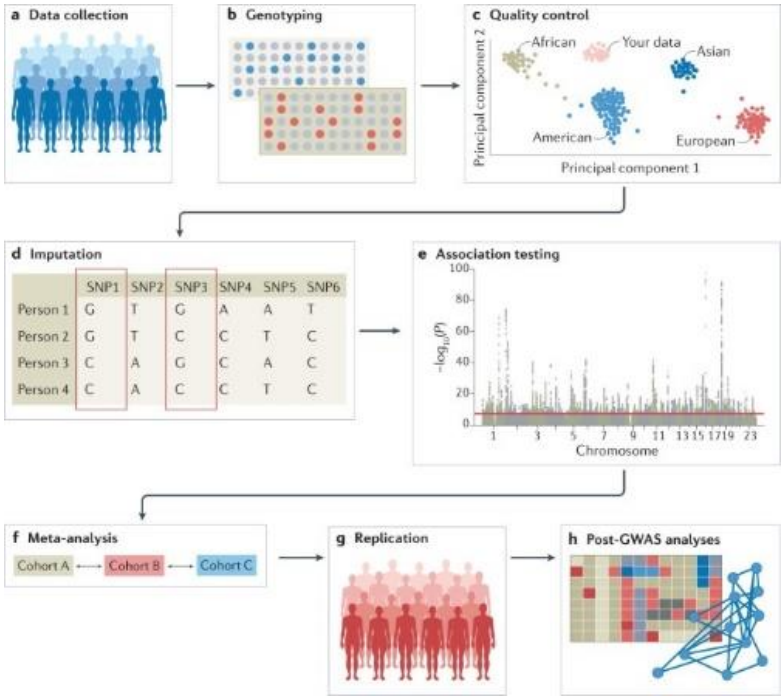

Figure 2. a) Data collection, **b**) Genotyping, **c)** Quality control, **d**) Imputation, **e**) Test of associations, **f**) meta-analysis, **g**) Replication studies,. **h**) post-GWAS analyses. GWAS: genome-wide association studies. *Source: Uffelmann, E., Huang, Q.Q., Munung, N.S. et al. Genome-wide association studies. Nat Rev Methods Primers **1**, 59 (2021). Permission was granted from Springer Nature.*

2.5.3 CNS leukemia

Leukemic cells in the CNS may predispose a patient to CNS toxicities both *per se* and indirectly due to more intensive intrathecal chemotherapy treatment[15,91,92]. The incidence of CNS involvement at diagnosis is currently estimated to be ≤5%[93,94]. The pathogenesis of CNS involvement in ALL is not fully understood. It has been suggested that possible routes of leukemic cell invasion of the CNS include the cranial marrow, osseous lesions, brain capillaries, growth along nerve roots into the subarachnoid room, solid brain tumors, local CNS hemorrhage in presence of blasts in the circulating blood, and during lumbar puncture (LP) (especially if it is traumatic)[95]. CNS leukemia is commonly classified as CNS1 with no leukemic cells present in the cerebrospinal fluid (CSF), CNS2 in the presence of <5 leukocytes/μL in the CSF with blasts, and CNS3 in the presence of >5 leukocytes/μL in the CSF with blasts, or other signs of CNS involvement such as solid leukemic mass or neurological symptoms[95,96]. The common clinical symptoms of CNS leukemia are cranial nerve palsies, headaches, vomiting, visual disturbances, impaired hearing, irritability, and seizures[94-96].

Besides the higher risk of CNS toxicity, CNS leukemia is related to relapse[95-99]. The CNS, despite the seemingly low incidence of leukemic infiltration at diagnosis, is considered to be a 'sanctuary' for leukemic cells, accounting for up to 60% of relapses prior to introduction of CNS-directed leukemia treatment, and for 30–40% of relapses after introduction of CNS treatment in ALL treatment protocols[95-97]. Patients with CNS involvement at diagnosis thus have a higher risk of both CNS relapse and CNS toxicities[15,98-100].

Introduction to diagnostics of CNS leukemia

Traditionally, the gold standard in detecting leukemic cells in the CNS has been CSF cytomorphology (CM) with cytospin[95,99,101]. Although CM is a simple and immediate method of detecting leukemic cells with high specificity, its sensitivity is limited, as it is depends on the time from CSF sample collection to analysis, the amount of leukemic blasts in the CSF, cytocentrifugation protocol, type of staining, and experience of the cytopathologist[95,101]. Thus, complementary diagnostic methods to detect leukemic cells in the CSF (with higher sensitivity) have been introduced, including flow cytometric immunophenotyping (FCI) and real time polymerase chain reaction (PCR)[95,99-103]. Flow cytometric immunophenotyping builds on staining CSF cells with fluorochrome-conjugated antibodies (against B-cells or T-cells) depending on the immunophenotype of the leukemia at diagnosis[99,101]. The sensitivity of FCI is higher compared to CM at detecting very low counts of leukemic cells[99-101]. Although the clinical significance of the detection of minimal CNS leukemia toward treatment outcome and stratification of patients at ALL diagnosis is uncertain, it has been suggested that FCI might be valuable as a diagnostic complement to CNS leukemia[99-101]. Real time PCR aiming to detect clonal rearrangements of immunoglobulin/T-cell receptor genes has also been shown to have high diagnostic value in detecting CNS leukemia and has been suggested as a complementary method of leukemic blast detection in the CSF[95,102,103].

2.5.4 Cranial irradiation

Cranial irradiation was introduced in pediatric ALL treatment in 1962 with the hope of eliminating residual leukemic cells in the CNS that are related to ALL relapse[9]. Prophylactic CNS radiotherapy was actually effective in preventing relapse, however it carried with it several severe adverse effects such as secondary malignancies and cognitive impairment[104,105]. Brain cell damage and death due to radiotherapy are related to myelination and cerebral maturation and children younger than five years are at higher risk of long-term sequelae[27]. Eventually, most current treatment protocols replaced prophylactic cranial irradiation with intrathecal administration of methotrexate, hydrocortisone, and cytarabine combined with more potent intravenous methotrexate doses that are as effective but less toxic than radiotherapy[7,27,104].

2.6 CLINICAL RISK FACTORS

Although the role of pharmacotoxicity of antileukemic drugs, radiotherapy, and assumed genetic risk factors has either been demonstrated or is currently being researched, not much is known about the clinical risk factors of CNS toxicity during ALL treatment. Central nervous system leukemia and age >10 years have recently been associated with CNS toxicities, but data are scarce[15,18]. Similarly, another study demonstrated that hypertension, constipation and alkalinization >14 days are associated with PRES; hypertension has an etiological relationship with PRES, which strengthens this finding, but more data are needed to explore the role of constipation and alkalinization in the pathogenesis of PRES[20,32]. High-risk treatment protocol arms, specific karyotypes, and T-cell leukemia seem related to CNS toxicities in univariate analyses, but not in multivariate analyses[15,20]. A previous study demonstrated that females have significantly higher risk of seizures, but this finding hasn't been replicated in subsequent reports[44].

2.7 NEUROIMAGING WORK-UP OF CNS TOXICITIES

Neuroimaging is essential in the work-up of CNS-toxicities to promptly make correct diagnoses and proceed to treatment. No diagnostic controversies are expected in the event of intracranial hemorrhage, but the differential diagnosis between PRES and methotrexate-related SLS might be challenging and thus the method of choice for neuroimaging when these conditions are suspected is a brain magnetic resonance imaging (MRI) even if computed tomography (CT) is often more accessible[31].

Brain lesions in patients with PRES show hyperintense signal in T2-weighted images, low-intense signal in T1-weighter images, normal diffusion weighted images (DWI) and enhanced apparent diffusion coefficient (ADC), and are visualized in fluid-attenuated inversion recovery (FLAIR) sequences (Figure 3)[31,106,107]. PRES lesions are located in posterior, anterior, central cerebral regions and in the brainstem and they cannot always be

visualized with brain CT[31,106,107]. Posterior reversible encephalopathy syndrome may be complicated by ICH, usually due to coagulopathy or treatment with anticoagulants[32,108]. Brain lesions in patients with methotrexate-related SLS show hyperintense signaling in DWI and restricted diffusion in ADC images; they are commonly located in the frontal and parietal cerebral regions[25,109].

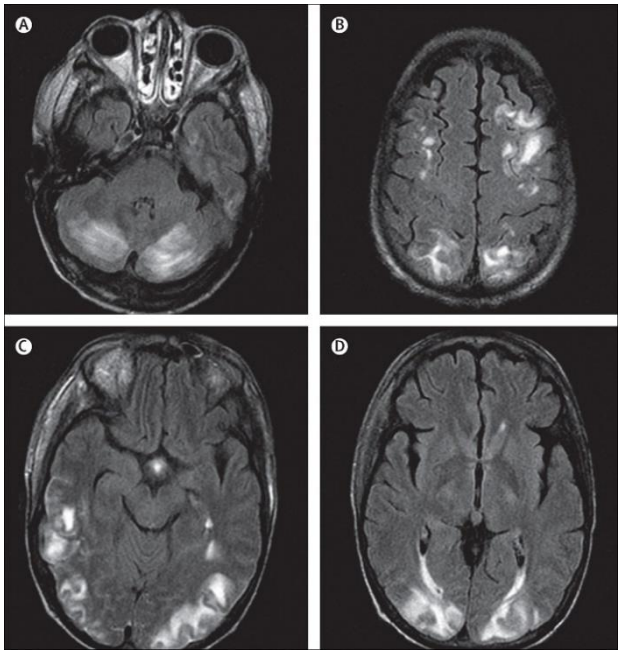

Figure 3. Typical MRI findings in PRES in axial T2 fluid-attenuated inversion recovery. MRI: magnetic resonance imaging, PRES: posterior reversible encephalopathy syndrome. *Source: The Lancet, Vol 14, Fugate, J.E.; Rabinstein A.A. Posterior reversible encephalopathy syndrome: clinical and radiological manifestations, pathophysiology, and outstanding questions, Pages No 914-925., Copyright 2015, with permission from Elsevier.*

2.8 LONG-TERM OUTCOME OF CNS TOXICITIES

One of the greatest current challenges in treatment of pediatric ALL is optimizing long-term outcomes of survivors. The most common reported late sequelae of ALL treatment are deficits in neurocognitive outcomes[27,28,110-113]. Data are not homogenous concerning which neurocognitive functions are affected, but deficits in attention, working memory, executive functions, and learning seem to be confirmed in several studies and neurocognitive follow-up of patients may be called for[28,110,111,113]. Treatment-related white matter abnormalities, decreased subcortical structures, and acquired brain damage have also been described, interestingly in combination with neurocognitive impairment[27,28,110]. Epilepsy has been reported as an outcome of CNS toxicities, but more studies are needed to map whether epilepsy is long-term effect of treatment[15,20,44].

3 RESEARCH AIMS

The goal of this thesis was to explore CNS toxicity in pediatric patients with ALL treated according to the Nordic protocol NOPHO ALL2008 in order to better understand the underlying mechanisms, risk factors, and outcomes and thus contribute to the ultimate aim of optimizing ALL treatment.

The specific aims of the thesis were to:

1) Describe the incidence, clinical course, risk factors and outcome of PRES in pediatric patients with ALL treated with the NOPHO ALL2008 protocol (Paper I).

2) Describe the incidence, clinical course, risk factors and outcome of seizures in pediatric patients treated with the NOPHO ALL2008 protocol (Paper II).

3) Describe all acute CNS toxicities in pediatric patients treated with the NOPHO ALL2008 protocol and explore potential genetic risk factors (Paper III).

4) Study the role of CNS involvement at diagnosis of pediatric ALL, detected by FCI, as risk factor for CNS toxicities (Paper IV).

4 MATERIALS AND METHODS

4.1 NOPHO ALL2008 PROTOCOL AND REGISTRY

The NOPHO ALL2008 protocol is a Nordic-Baltic protocol that included patients from five Nordic countries: Denmark, Finland, Iceland, Norway, Sweden and two Baltic countries: Estonia and Lithuania (Figure 4). The NOPHO ALL2008 registry is an online registration system created for the NOPHO ALL2008 protocol to meet the European Clinical Trials Directive of 2004[114]. In order to acquire information from all relevant toxicities a unique on-line toxicity registration system was created, where 18 different toxicity domains (including those relevant to this study) were registered for all patients every three months. Compliance to this registration was > 95%[114]. All four Papers are grounded in the NOPHO protocol and registry (Figure 5).

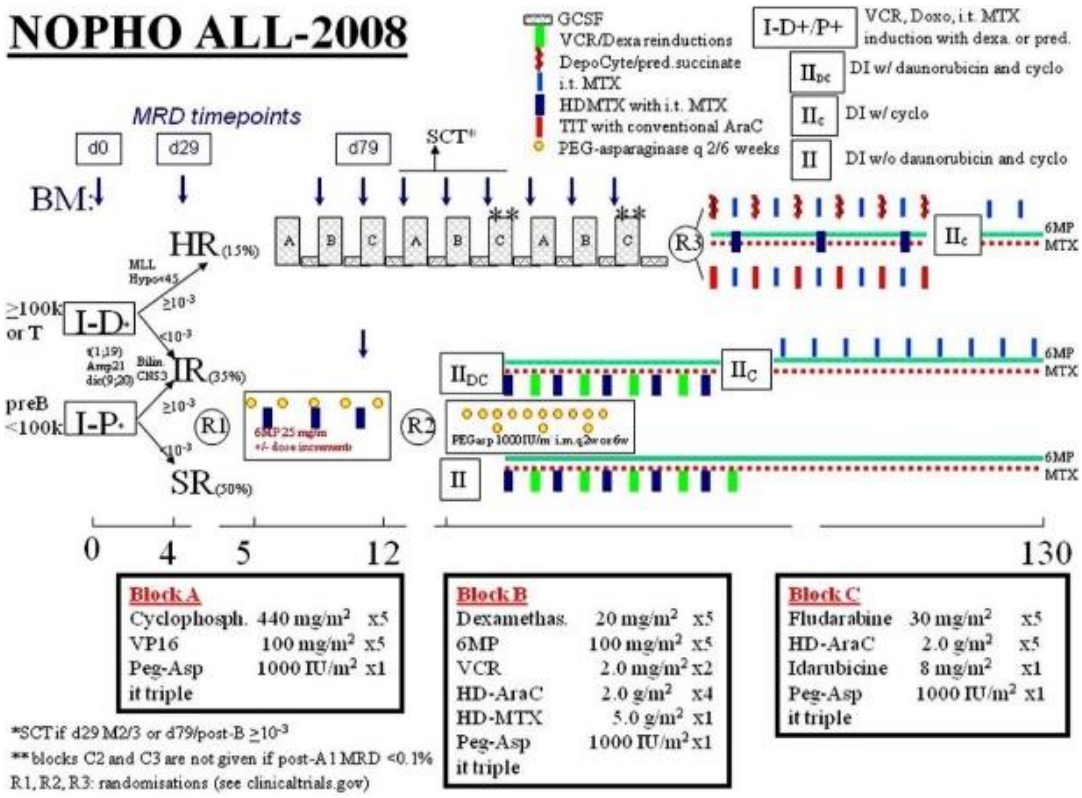

Figure 4. The NOPHO AL 2008 protocol with stratifications and treatments. NOPHO: Nordic society of Pediatric Hematology and Oncology, ALL: acute lymphoblastic leukemia, AraC: cytarabine, MTX: Methotrexate, HD: high dose, HDM: High Dose Methotrexate, 6MP: 6-MercaptoPurine, Peg-Asp: Peg-asparaginase, VCR: Vincristine, SCT: stem cell transplantation, TIT: triple intrathecal Treatment with conventional cytarabine, prednisolone and methotrexate, GCSF: Granulocyte Colony Stimulating Factor. *Source: European Journal of Cancer, Frandsen, T.L. et al. Complying with the European Clinical Trials directive while surviving the administrative pressure - an alternative approach to toxicity registration in a cancer trial. Eur J Cancer. 2014 Jan;50(2):251–9. Permission was granted from the journal.*

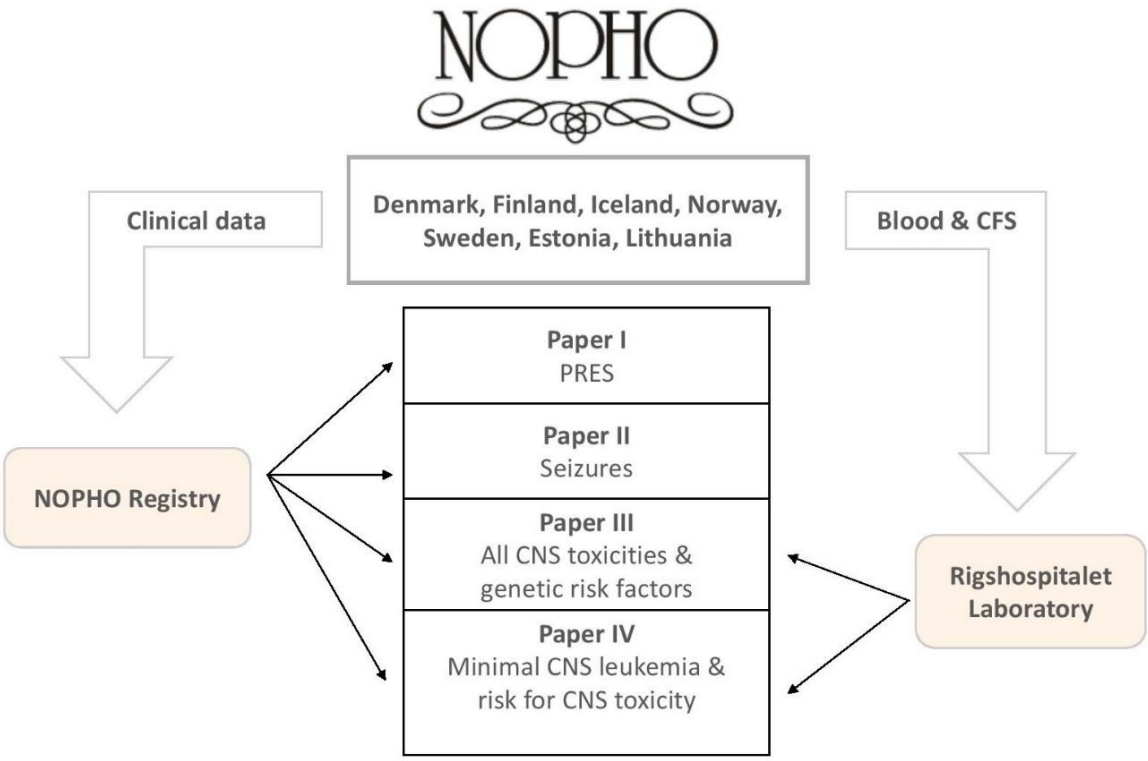

Figure 5. Overview of the Thesis plan. NOPHO: Nordic society of Pediatric Hematology and Oncology, CNS: central nervous system, PRES: posterior reversible encephalopathy syndrome.

4.2 SUBJECTS

Patients aged 1–17.9 years with ALL diagnosis between the 1st of July 2008 and the 31st of December 2015 who were treated according to the NOPHO ALL2008 protocol were included. Patients with CNS toxicity or who were suspected of CNS toxicity in the seven countries were identified through the registry. To verify the toxicities, a complementary detailed questionnaire addressing phenotypical variables, work-up, and outcomes was sent to all pediatric clinics in the NOPHO ALL2008 protocol countries. Two pediatric neurologists and two pediatric oncologists have also assessed answers and phenotypes.

Paper I

Patients with robust PRES phenotypes were tested against controls with no CNS toxicity. Cases with possible PRES phenotypes but insufficient data were excluded from analyses to avoid bias.

Paper II

Patients who displayed seizures were tested against patients without seizures.

Paper III

Patients who displayed CNS toxicities were tested against controls with no CNS toxicity.

Paper IV

Patients with CSF data by both CM and FCI with PRES, seizures, or any CNS toxicity were tested against controls with the same CSF data and no CNS toxicity.

4.3 GENOME-WIDE ASSOCIATION AND CANDIADATE SNP ANALYSES

About 90% of patients treated with the NOPHO ALL2008 protocol have agreed to participate in genetic analyses to identify genetic associations to pharmacotoxicity and ALL. Patients' blood samples were sent to Copenhagen and genomic analyses were performed there. In Paper III, we combined our phenotype data with patients' genomic data to test associations with GWAS and perform candidate SNP analyses with the help of bioinformatics specialists.

Genome-wide associations were analyzed on three phenotype groups: all CNS toxicities, PRES, and seizures. The most significant SNP were annotated using the variant effect predictor (GRCh37.p13); Ensembl GRCh37 and GeneCards genetic databases were used to check gene functions and related disorders[115-117]. Genes were further tested for functional enrichment by gene set overlap analysis (GSEA)[118].

For the candidate SNP analyses, we selected 22 SNP that have previously been associated with epilepsy or methotrexate-related CNS toxicity (Table 2)[18,119]. Single nucleotide polymorphisms reaching statistical significance for association with seizures ($P<0.05$) before corrections were subsequently tested separately for children with seizures <10 years and ≥10 years of age. Two PRS were estimated for risk for seizures based on all candidate SNP (unweighted) and SNP associated with methotrexate-related CNS toxicity (weighted).

A validation study of the most significant findings of GWAS and candidate SNP analyses was made in an independent Australian cohort of pediatric ALL patients (n=797) with diverse CNS toxicities (n=103) and methotrexate-related CNS-toxicity (n=48), (Figure 6).

Table 2. SNP associated with epilepsy and methotrexate-related CNS toxicity.

SNP	***Chr***	***Gene***	***Minor allele***	***MAF***	***P****	***Phenotype***
rs4671319	2	*FANCL, BCL11A*	G	0.44	8.1E–09	All epilepsy
rs6432877	2	*SCN3A, SCN2A, TTC21B SCN1A*	G	0.26	1.7E–13	All epilepsy
rs4638568	16	*HEATR3, BRD7*	A	0.06	4.0E–08	All epilepsy
rs2212656	2	*SCN3A, SCN2A, TTC21B SCN1A*	A	0.26	7.3E–09	Focal epilepsy
*rs4665630***	2	None**	C	0.13	4.3E–08	Generalized epilepsy
rs1402398	2	*FANCL, BCL11A*	G	0.36	1.2E–11	Generalized epilepsy
rs11890028	2	*SCN3A SCN2A, TTC21B SCN1A*	G	0.27	4.7E–08	Generalized epilepsy
rs887696	2	*STAT4*	C	0.34	3.0E–08	Generalized epilepsy
rs1044352	4	*PCDH7*	T	0.42	2.2E-09	Generalized epilepsy
rs11943905	4	*GABRA2*	T	0.27	3.9E–08	Generalized epilepsy
rs4596374	5	*KCNN2*	C	0.45	7.2E–10	Generalized epilepsy
rs68082256	6	*ATXN1*	A	0.20	1.7E–09	Generalized epilepsy
*rs13200150****	6	None***	G	0.30	5.9E–09	Generalized epilepsy
rs4794333	17	*PNPO*	C	0.38	6.8E–09	Generalized epilepsy
rs2833098	21	*GRIK1*	G	0.38	1.7E–08	Generalized epilepsy
rs4712462	6	*MBOAT1*	A	0.35	2.54E–07	MTX-related CNS toxicity
rs2241357	19	*GIPC1*	A	0.2	3.60E–07	MTX -related CNS toxicity
rs1106479	3	*ZDHHC19*	T	0.16	4.08E–07	MTX -related CNS toxicity
rs35307996	17	*NXN*	CC	0.2	5.70E–07	MTX -related CNS toxicity
rs74956940	19	*PKN1*	G	0.23	6.19E–07	MTX -related CNS toxicity
rs62576054	9	*HMGB1P37*	G	0.18	7.50E–07	MTX -related CNS toxicity
rs9590003	13	None	A	0.11	9.73E–07	MTX -related CNS toxicity

SNP: single nucleotide polymorphism, CNS: central nervous system, Chr: chromosome, MAF: minor allele frequency. *P value according to previously published studies, ** rs4665630: updated data show that the SNP is located in KLHL29 gene, *** rs13200150: updated data show that the SNP is located in PTPRK gene. *Source: Anastasopoulou S. et al. Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes. Haematologica. 2022 Oct 1 (supplementary appendix). Permission has been granted.*

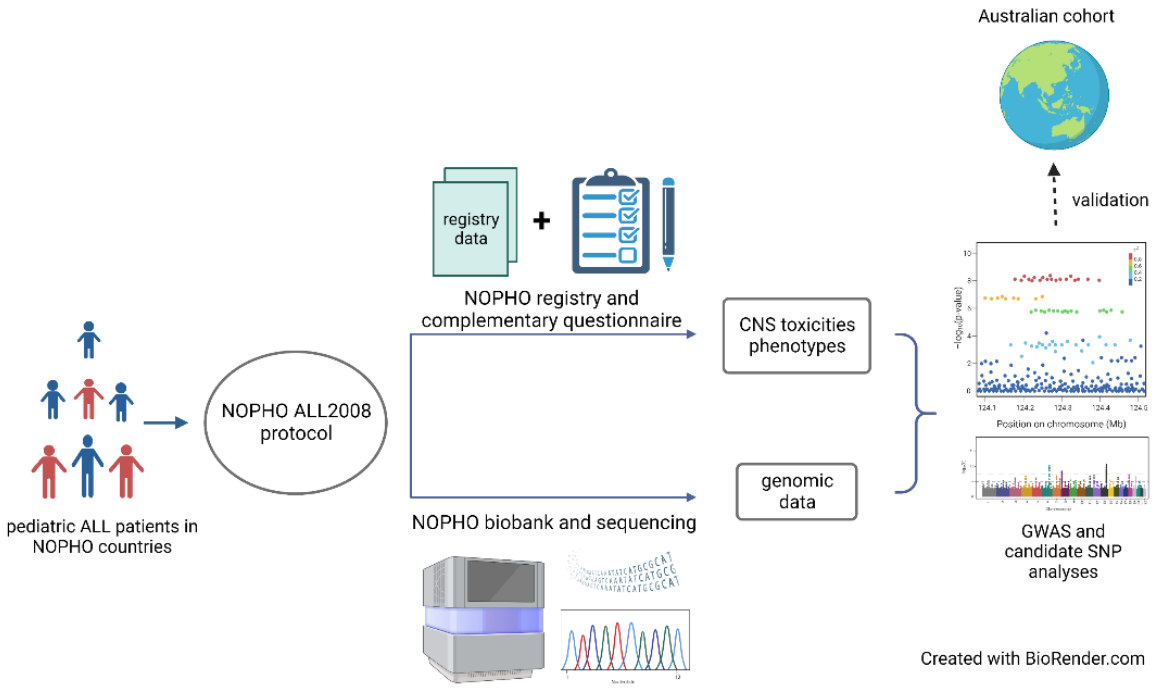

Figure 6. Overview of the study plan of genomic analyses. NOPHO: Nordic society of Pediatric Hematology and Oncology, GWAS: genome-wide association studies, SNP: single nucleotide polymorphism.

4.4 CEREBROSPINAL FLUID ANALYSES WITH FLOW CYTOMETRIC IMMUNOPHENOTYPING

The standard analysis of CSF in the NOPHO ALL2008 is by CM. A parallel NOPHO project explored CNS involvement with FCI between the 27th September 2012 and 31st December 2017 in patients treated according to NOPHO ALL2008 protocol with participation of Denmark, Finland, Lithuania, Norway, and Sweden. Cerebrospinal fluid samples were collected at diagnostic LP and until treatment day 15 (optional)[98]. Flow cytometric immunophenotyping analyses were performed at the immunology laboratory in Rigshospitalet. In Paper IV, we combined our phenotypical data including data on classification by CM with data on CSF analyzed by FCI (Figure 7). We studied the role of minimal leukemia detected by FCI analysis, but not by CM, in patients with CNS1 that did not receive enhanced CNS directed treatment ($CNS1_{flow+}$). We have also studied the role of CNS leukemia by FCI regardless stratification by CM (CNS_{flow+}).

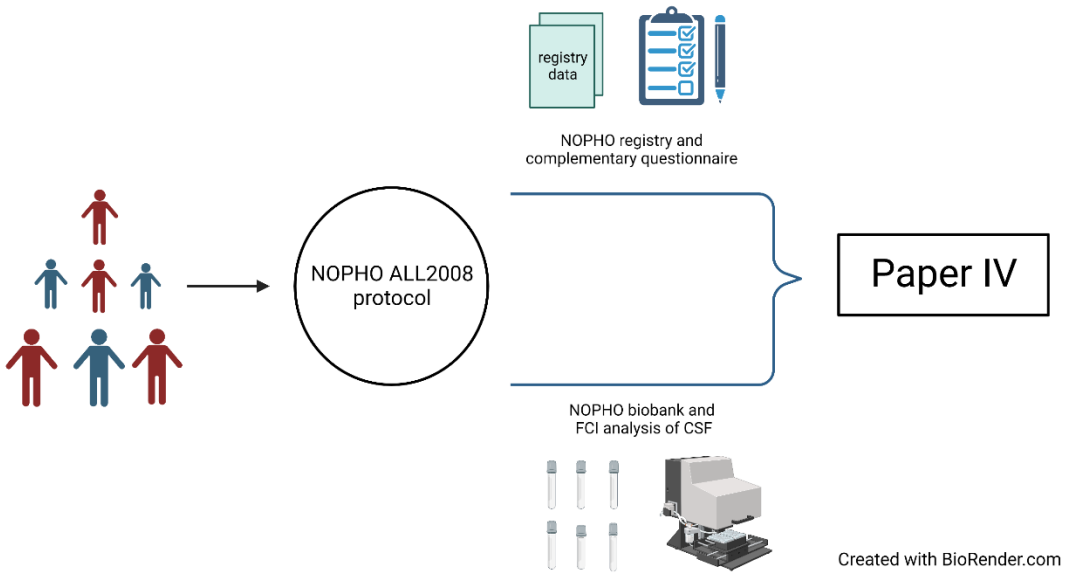

Figure 7. Overview of the study plan for evaluating the role of minimal CNS leukemia in the risk of CNS toxicity. CNS: central nervous system, NOPHO: Nordic society of Pediatric Hematology and Oncology, ALL: acute lymphoblastic leukemia, FCI: flow cytometric immunophenotyping, CSF: cerebrospinal fluid.

4.5 STATISTICAL ANALYSES

Paper I

Statistical analyses were made using SPSS. The follow-up period began at the day of ALL diagnosis and continued until relapse, stem cell transplantation (SCT), secondary malignancy, death, or last follow-up date, whichever occurred first. Time to PRES was defined as days from ALL diagnosis to the day PRES occured, with censoring for death, relapse, SCT, secondary malignancy, or the end of follow-up, whichever came first. The cumulative incidence of CNS toxicity was calculated using Kaplan-Meier analyses. Cox proportional hazard models estimating hazard ratios (HR) were used to evaluate the association of risk factors and PRES in univariate and multivariate analyses. PRES cases per person-year in a certain treatment phase were calculated by dividing the number of PRES cases during the treatment phase by the total risk-time for all patients during the specific treatment phase. The association between risk factors and early PRES during induction (defined as the first five weeks from treatment start) was evaluated with univariable logistic regression, estimating odds ratios (OR). The role of high-risk and standard/intermediate-risk treatments was evaluated after the end of the induction period with Cox regression. Two-sided P values below 0.05 were considered significant.

Paper II

Statistical analyses were made using SPSS and R. Follow-up period, time to seizures and evaluation of risk factors by HR were calculated as described in Paper I. The cumulative

incidence of seizures was calculated using the Gray method. Two-sided P values below 0.05 were considered significant.

Paper III

Statistical analyses of clinical risk factors were made using SPSS and R. Follow-up period, time to CNS toxicity and evaluation of risk factors by HR were calculated as described in Paper I. The cumulative incidence of CNS toxicity was calculated using the Gray method. Two-sided P values below 0.05 were considered significant.

Genome-wide association analyses were performed in PLINK2 using logistic regression. A suggested threshold of $P<5X10^{-6}$ and a Bonferroni-corrected $P<2X10^{-8}$, which were regarded as significant, were used to explore top findings by GWAS. For the weighted methotrexate-related CNS toxicity SNP PRS, each SNP was weighted by the log-transformed OR from Mateos et al[18].

Paper IV

Statistical analyses were performed using SPSS. Follow-up period, time to CNS toxicity and evaluation of risk factors by HR were calculated as described in Paper I. The association between $CNS1_{flow+}$ and the risk of early CNS toxicities during induction period was evaluated using OR. The association between $CNS1_{flow+}$ and the risk of late CNS toxicities after the induction period was evaluated using HR. Group differences were assessed by the Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. Two-sided P values below 0.05 were considered significant.

Of note: The CNS toxicities included in the analyses were the ones that occurred for the first time during the course of treatment. Recurrent CNS toxicities in the same patients were not included in analyses.

4.6 ETHICAL CONSIDERATIONS

There were no risks for patients related to the studies in the thesis. All patients and their parents have received information on NOPHO registry studies and parents and older patients (when appropriate) have given written consent of participation. The consent addresses multiple uses, including future use, in approved studies to help other children with ALL and also spare patients struggling with long and complicated treatment from exposure to many requests for consent. Additional information on the parallel studies of genetic predisposition to different toxicities, and mapping of the consequences of CNS leukemia diagnosed by FCI and CM (as well as comparison of the two diagnostic methods) was provided to patients and their parents. Written consent of participation to both studies was obtained from parents and

older patients. Collection of blood and CSF samples was made at the same time as other planned collection of blood/CSF samples according to the treatment protocol.

Ethical approvals were obtained in all participating countries. Swedish ethical approvals were granted from the Central Ethical Review Boards in Gothenburg and Lund.

- Reference number of approval of main application regarding treatment with NOPHO ALL2008 protocol and the ALL registry: 458-08; approved 18-08-2008.
- Reference number of approval of application regarding genomic analyses and phenotype data: 731-10; approved 28-12-2010.
- Reference number of approval of application regarding CNS leukemia and diagnostic methods: 2013/10; approved 29-10-2013.

5 RESULTS

The cohort included 1464 ALL patients treated according to the NOPHO ALL2008 protocol (Figure 8).

PAPER I

Fifty-two patients had PRES before censoring. Selection of controls was strict, excluding patients who displayed CNS toxicities other than PRES and three patients with missing data, leaving 1326 controls (Figure 8).

PAPER II

Eighty-one patients were reported to have had seizures as isolated event or as a symptom of other underlying CNS toxicities before censoring. Patients without seizures served as controls, adding up to 1383 (Figure 8).

PAPER III

One-hundred thirty-five patients had diverse CNS toxicities before censoring. Controls were patients with no CNS toxicity, for a total of 1329. Genome-wide association analyses were performed for 109 patients with CNS toxicities, of whom 67 had seizures, and 1057 controls with available blood samples in Copenhagen; 46 of these 109 patients with CNS toxicity had PRES, but this group showed signs of genomic inflation and were excluded from further analyses. Nineteen of the 22 selected candidate SNP qualified for analyses after imputation (13 SNP associated to epilepsy and six SNP associated to methotrexate-related CNS toxicity). Candidate SNP analyses were performed for 67 patients with seizures and 1057 controls (Figure 8). The most significant findings were further evaluated through a validation study in the independent Australian cohort.

PAPER IV

Cerebrospinal fluid analyses by FCI were performed in 370 cohort patients. Of them, 320 patients were classified as CNS1 by CM including 256 without ($CNS1_{flow-}$) and 64 with ($CNS1_{flow+}$) blasts in CSF by FCI (Figure 8).

Main analyses were made regarding the 320 patients with CNS1, of whom 33 displayed CNS toxicity including seizures (18) and PRES (16). Controls were 287 patients that did not have any CNS toxicity during treatment. Additional analyses were made for all 370 patients with available FCI data. In this group 38 patents displayed CNS toxicity, including 22 with seizures and 18 with PRES. Controls were 332 patients that did not have any CNS toxicity during treatment.

Of note: In results, the denominator is not always equal to number of cases with CNS toxicity. In these cases, the denominator reflects available data.

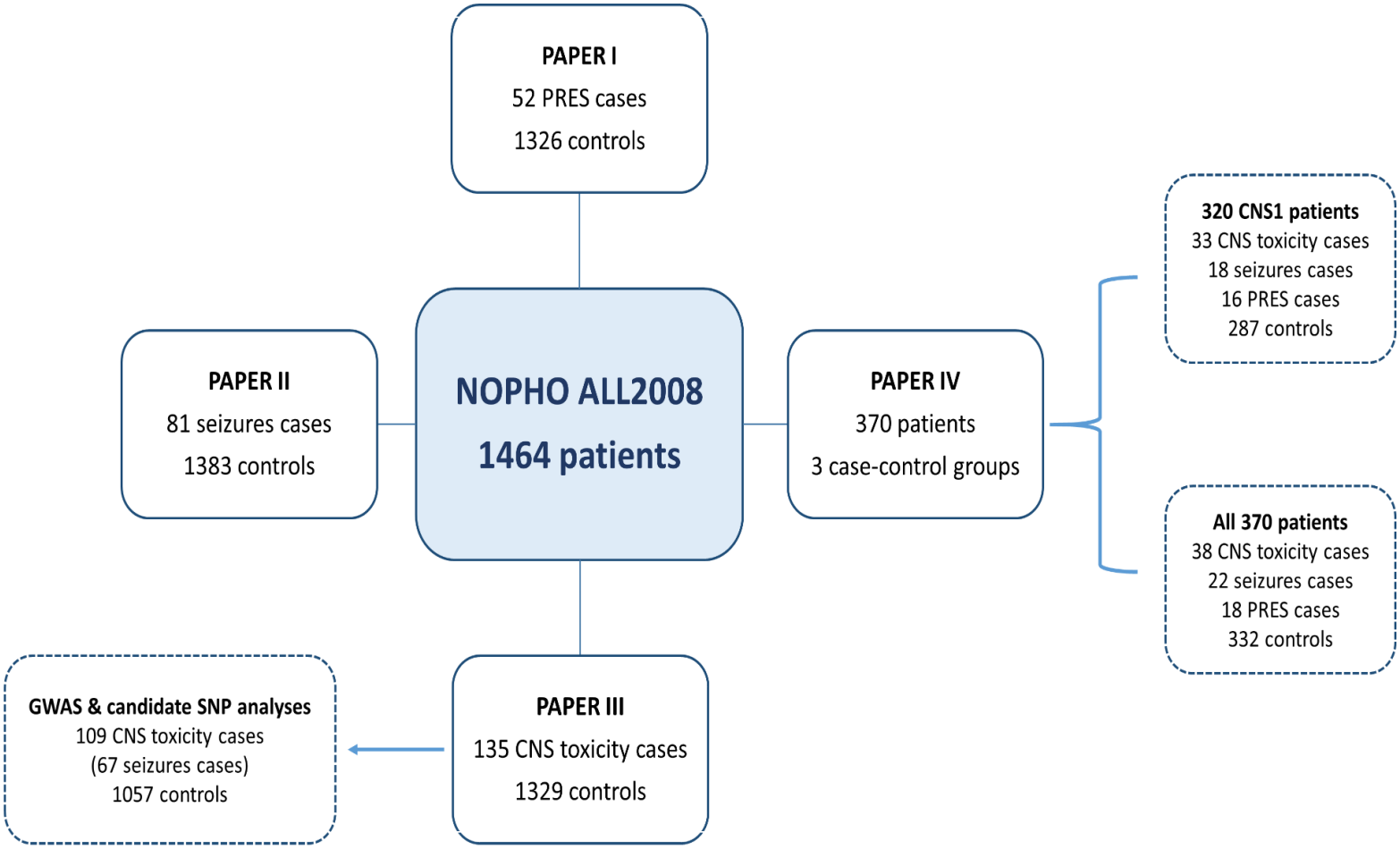

Figure 8. Cohort and all papers populations. NOPHO: Nordic society of Pediatric Hematology and Oncology, ALL: acute lymphoblastic leukemia, PRES: posterior reversible encephalopathy syndrome, CNS: central nervous system.

5.1 PAPER I

5.1.1 Incidence of PRES

The overall incidence of PRES was 3.8% (52/1378). PRES was most common during induction (28/52), followed by consolidation (10/52) and high-risk block treatment (6/52) (Figure 9).

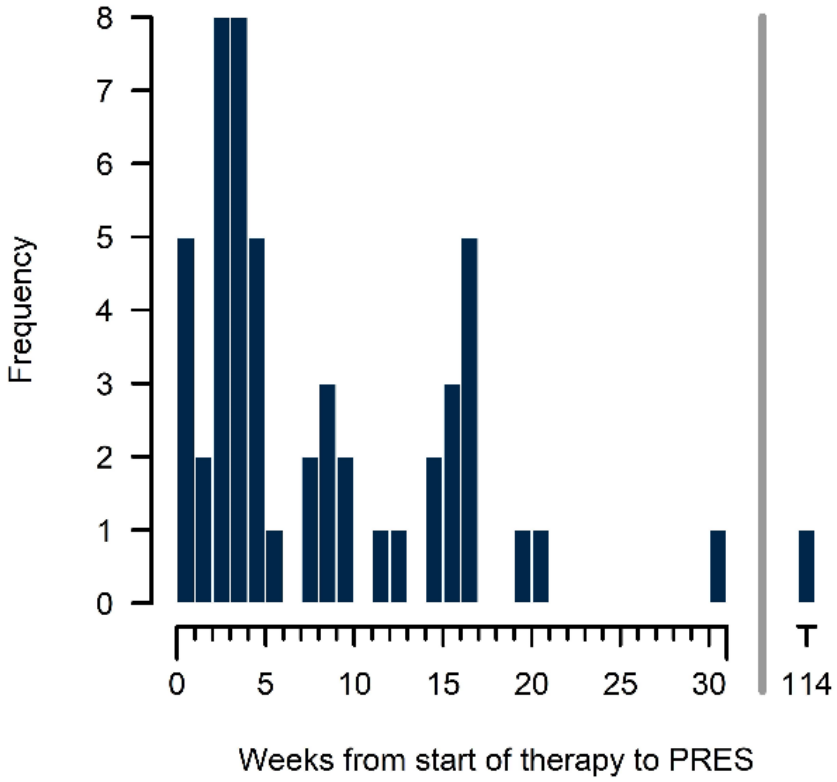

Figure 9. Distribution of PRES cases over treatment weeks. *Source: Anastasopoulou, S. et al. Posterior reversible encephalopathy syndrome in children with acute lymphoblastic leukemia: Clinical characteristics, risk factors, course, and outcome of disease. Pediatr Blood Cancer. 2019 May. Permission has been granted.*

5.1.2 Risk factors of PRES

Older age as both continuous (every extra year in age) or age group 10–17 years, induction with dexamethasone, and T-cell immunophenotype were significant risk factors for PRES in univariate analyses (Table 3). T-cell immunophenotype was a significant risk factor for PRES after adjusting for age (HR =2.4; 95% CI: 1.3–4.1; P =0.006) as well as for early PRES (OR =2.9; 95% CI: 1.2–6.6; P =0.014). High-risk treatment protocol with block treatment was significantly associated with PRES after induction (HR =2.6; 95% CI: 1.1–6.4; P =0.033). Central nervous system involvement (defined as CNS2 or CNS3 status) was related to significantly higher risk of early PRES as compared with no CNS involvement (OR =2.8; 95%; CI: 1.2–6.5; P =0.015).

Table 3. Clinical significant risk factors for PRES in univariate analyses

Patients (n)	**Controls (n=1326)**	**PRES (n=52)**	**HR (95% CI; p)**
Age in years (median and range)	4.4 (1.0-18.0)	8.5 (1.8-14.8)	1.09 (1.04-1.15; 0.001)
Age group (%)			
1-9 years	1077 (81.2%)	36 (69.2%)	1 (ref)
10-17 years	249 (18.8%)	16 (30.8%)	1.91 (1.06–3.44; 0.032)
***Induction therapy (%)**			
Prednisolone	1079 (81.9%)	34 (65.4%)	1 (ref)
Dexamethasone	235 (17.8%)	18 (34.6%)	2.40 (1.35–4.24; 0.003)
Immunophenotype (%)			
BCP ALL	1167 (88.0%)	37 (71.2%)	1 (ref)
T-cell ALL	159 (12.0%)	15 (28.8%)	2.92 (1.60–5.32; 0.001)

Hazard ratio for a one-year increase in age. PRES: posterior reversible encephalopathy syndrome, HR: hazard ratio, BCP: B-cell precursor. *3 controls received other induction, data missing for 9 controls. *Source: Modified from Anastasopoulou, S. et al. Posterior reversible encephalopathy syndrome in children with acute lymphoblastic leukemia: Clinical characteristics, risk factors, course, and outcome of disease. Pediatr Blood Cancer. 2019 May. Permission has been granted.*

5.1.3 Clinical symptoms and signs

The most common neurological symptoms of PRES were seizures and encephalopathy (Figure 10). Constipation and abdominal pain were reported in more than half of PRES patients. The majority of patients had hypertension (41/52). Simultaneous infection was common (22/49). Four patients had pancreatitis (4/36), and one had ileus (1/36) at the time they had PRES.

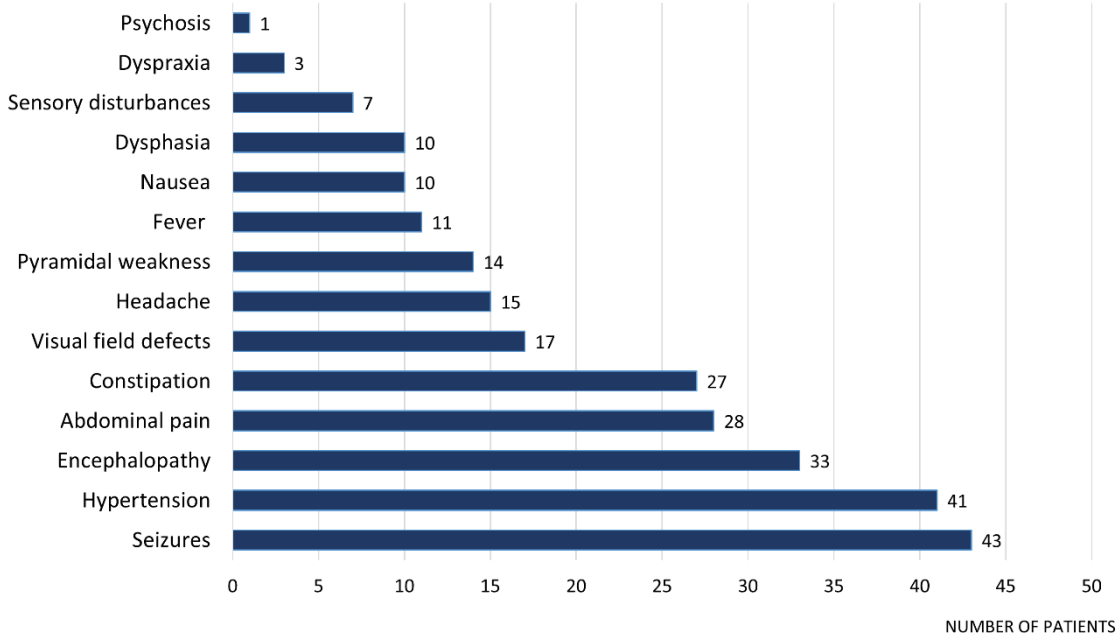

Figure 10. Clinical symptoms of PRES. PRES: posterior reversible encephalopathy syndrome.

5.1.4 Work-up

Brain MRI was performed in 48/52 cases and brain CT in 30/52 cases. All MRI findings were conclusive of PRES diagnosis (Figure 11). Findings were normal or inconclusive in 7 and 19 CT evaluations, respectively. Abnormal changes were localized in the parietal, occipital, frontal, and temporal lobes as well as in the cerebellum, basal ganglia, and brainstem. Electroencephalogram was performed in 31/51 patients and was abnormal in 30 cases. Common laboratory findings included hyponatremia (31/44), hypocalcemia (18/43), and abnormal glucose levels that could be increased or decreased (15/42).

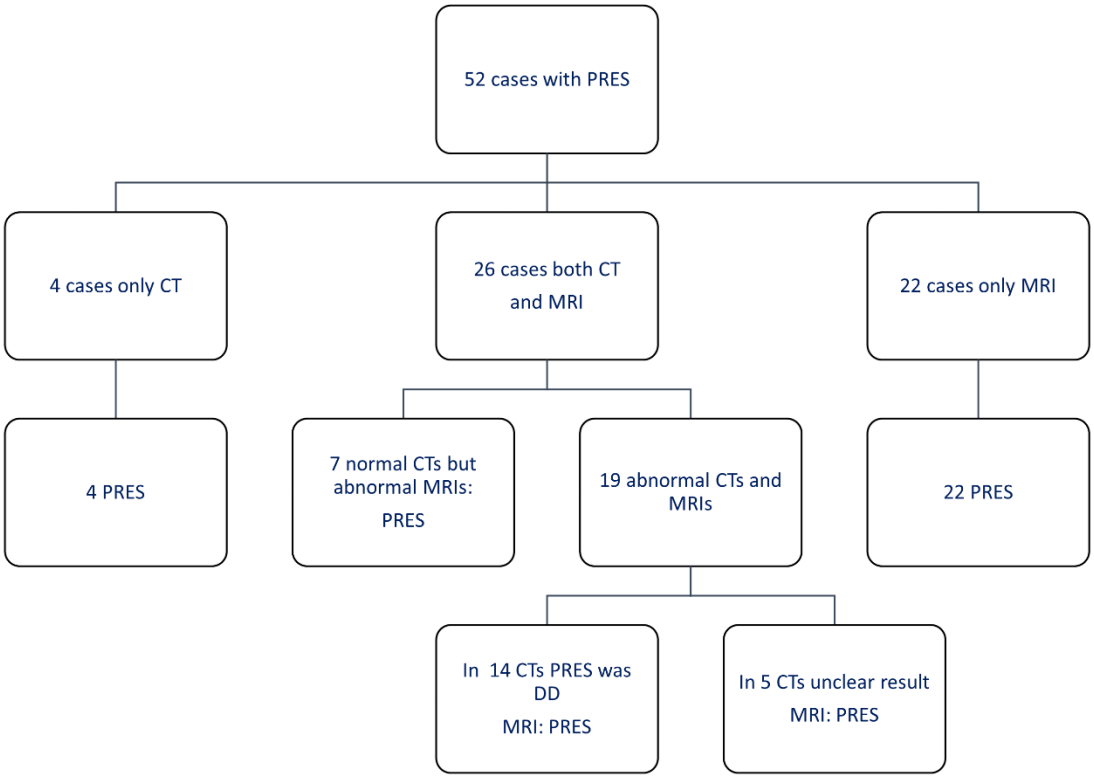

Figure 11. Diagnostic neuroimaging flow. DD: differential diagnosis, PRES: posterior reversible encephalopathy syndrome, CT: computed tomography, MRI: magnetic resonance imaging. *Source: Anastasopoulou, S. et al. Posterior reversible encephalopathy syndrome in children with acute lymphoblastic leukemia: Clinical characteristics, risk factors, course, and outcome of disease. Pediatr Blood Cancer. 2019 May. Permission has been granted.*

5.1.5 Treatment

The treatment was symptomatic including antiseizure medications (ASM) (36/48 patients) and antihypertensives (33/49 patients), as well as treatment of underlying infection or other condition. Thirty-three of 51 patients were admitted to intensive care unit (ICU) and fourteen patients needed mechanical ventilation. Temporary changes to the chemotherapy plan were made in 32/51 cases.

5.1.6 Outcome

Median time for last follow-up after PRES was 5.8 years (range: 4.5–8.8 years). Seven patients were reported to have had an epilepsy diagnosis at last follow-up. Seven patients were reported to be having neurocognitive sequelae according to clinical suspicions, but neuropsychiatric testing was performed only in one case with a pre-existing suspicion of attention deficit hyperactivity disorder (ADHD); one of these patients was among the ones who were reported to have had epilepsy diagnosis at last follow-up.

5.2 PAPER II

5.2.1 Incidence of seizures

The overall incidence of seizures was 5.5% (81/1464). Most seizures occurred during the first 20 weeks of treatment (65/81). In 15 cases, seizures were reported as isolated events without any other underlying pathologic condition. In the remaining 66 cases seizures were the symptom of other CNS or systemic events (Figure 12). Other symptoms in patients displaying seizures were encephalopathy, headache, paresis, nausea, dysphasia, dyspraxia, visual field defects, and/or sensory disturbances depending on the underlying pathology.

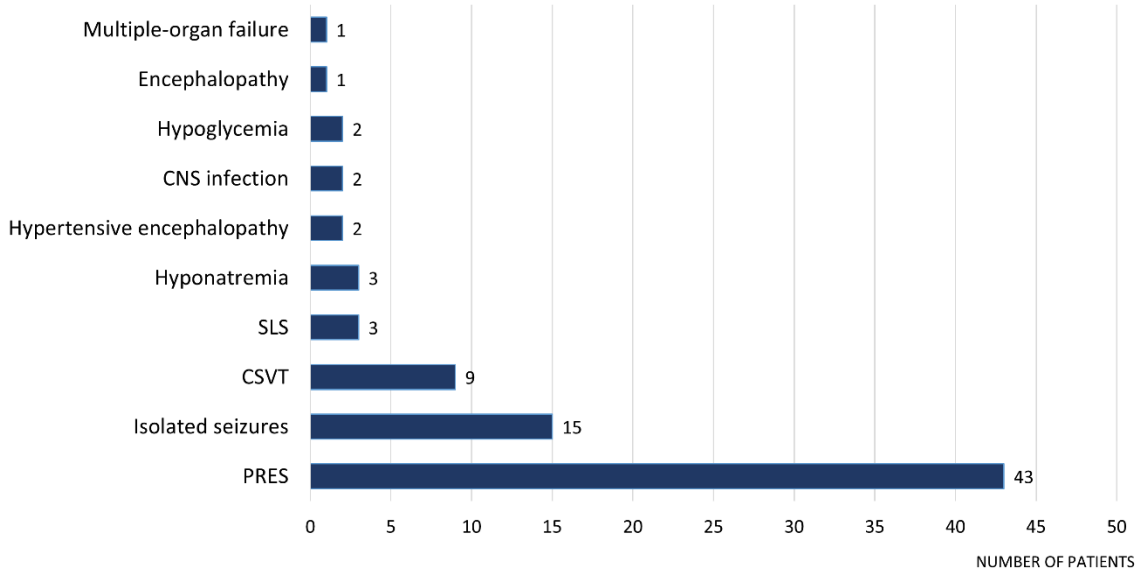

Figure 12. Pathological conditions underlying seizures. CNS: central nervous system, SLS: methotrexate-related stroke-like syndrome, CSVT: cerebral sinus venous thrombosis, PRES: posterior reversible encephalopathy syndrome.

5.2.2 Risk factors

Older age as both continuous (every extra year in age) or age group 10–17 years, T-cell immunophenotype, CNS leukemia, and induction with dexamethasone were significant risk factors for PRES in univariate analyses (Table 4). Age was a significant risk factor for seizures after multivariate analysis including all evaluated risk factors (Table 4).

Table 4. Clinical characteristics of patients with seizures and risk factors for seizures.

	Controls (n =1383)	**Seizure (n =81)**	**Univariable HR (95% CI; P)**	**Multivariable HR (95% CI; P)**
Age in years (median, IQR, range)	4.5 (2.8–8.3; 1.0-18.0)	7.8 (4.4–11.3; 1.7–17.0)	1.09 (1.04–1.13; <0.001)	-
Age group, n (%)				
1-9 years	1111 (80.3)	53 (65.4)	Ref	Ref
10-17 years, n (%)	272 (19.7)	28 (34.6)	2.15 (1.36–3.41; 0.001)	1.95 (1.21–3.13; 0.01)
Sex, n (%)				
Female	632 (45.7)	40 (49.4)	Ref	Ref
Male	751 (54.3)	41 (50.6)	0.86 (0.56–1.33; 0.50)	0.78 (0.50–1.22; 0.28)
Immunophenotype, n (%)				
BCP ALL	1213 (87.7)	61 (75.3)	Ref	Ref
T cell ALL	170 (12.3)	20 (24.7)	2.35 (1.42–3.90; <0.001)	1.60 (0.64–4.05; 0.32)
***CNS status, n (%)**				
CNS 1	1205 (87.1)	64 (79.0)	Ref	Ref
CNS 2 or 3	174 (12.6)	17 (21.0)	1.83 (1.07–3.13; 0.03)	1.62 (0.93–2.78; 0.09)
****Induction therapy, n (%)**				
Prednisolone	1120 (81.0)	56 (69.1)	Ref	Ref
Dexamethasone	251 (18.1)	25 (30.9)	2.00 (1.25–3.21; <0.001)	1.09 (0.45–2.65; 0.85)

HR =hazard ratio, CI =confidence interval, IQR =interquartile range, CNS =central nervous system. *Data missing for 4 controls, **3 controls received other induction, data missing for 9 controls. *Source: Anastasopoulou, S. et al. Seizures during treatment of childhood acute lymphoblastic leukemia: A population-based cohort study. Eur J Paediatr Neurol. 2020 Jul. Permission has been granted.*

5.2.3 Work-up

Electroencephalogram was performed in 52/77 patients with seizures and was abnormal in 43/51 cases. A neuroimaging study was performed at least once on 75 patients: brain MRI was performed on 66 patients, brain CT was performed on 44 patients, and 37 patients were examined by both brain MRI and CT.

5.2.4 Treatment

The treatment was symptomatic including ASM (57/73 patients), antihypertensives (34/73 patients), intravenous immunoglobulin (2/73 patients patients), aminophylline (1/67 patients), and magnesium (1/68 patients). Forty-two of 78 patients were admitted to ICU.

5.2.5 Outcome

Median time for last follow-up after seizures was 5.1 years (range: 1.7–9.2 years). Seven patients had repeated seizures after the first episode of CNS toxicity with seizures. Eleven patients were reported to have had an epilepsy diagnosis at their last follow-up. Three of them had ongoing treatment for epilepsy at their last follow-up, while treatment had been successfully withdrawn in eight patients 18–40 months after their last seizure (median: 24 months).

5.3 PAPER III

5.3.1 Incidence of acute CNS toxicities

The overall incidence of CNS toxicities was 9.2% (135/1464). The most common CNS toxicity in the NOPHO cohort was PRES (52/135) (Table 5). The most common CNS toxicity in the Australian cohort was methotrexate-related SLS (Table 5). Most CNS toxicities in the NOPHO cohort occurred within the first six months of treatment (110/135).

Table 5. CNS toxicities in the NOPHO and the Australian cohorts.

CNS toxicity	NOPHO ALL2008 n	Australian cohort n
PRES	52	4
CSVT	28	0
Isolated seizures	16	14
Hypertensive encephalopathy	8	0
SLS	6	28
Encephalopathy NOS	4	8
CNS infection	4	0
Other/unclear symptoms	3	7
ICH	3	3
Seizures secondary to hyponatremia	3	0
Aseptic meningitis	2	1
Seizures secondary to hypoglycemia	2	0
Steroid psychosis	1	0
Anoxic brain injury secondary to cardiac arrest	1	0
Seizures secondary to multiorgan failure	1	0
Pontine myelinolysis	1	0
Possible SLS	0	17
Motor deficits	0	11
Leukoencephalopathy	0	10
Total	**135**	**103**

CNS: central nervous system, NOPHO: Nordic society of pediatric hematology and oncology, PRES: posterior reversible encephalopathy syndrome, CSVT: cerebral sinus venous thrombosis, SLS: methotrexate-related stroke-like syndrome, NOS: not otherwise specified, ICH: intracranial hemorrhage. *Source: Modified from Anastasopoulou S. et al. Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes. Haematologica. 2022 Oct 1. Permission has been granted.*

5.3.2 Risk factors for acute CNS toxicities in the NOPHO cohort

Older age group 10–17 years, T-cell immunophenotype, CNS leukemia, and induction with dexamethasone were all significant risk factors for PRES in univariate analyses (Table 6). Age group 10-17 years was a significant risk factor for CNS toxicity after multivariate analysis including all evaluated risk factors.

Table 6. Clinical characteristics of patients with acute CNS toxicities and risk factors for CNS toxicities

	Controls (n =1 329)	CNS toxicities (n =135)	Univariable HR (95% CI; P)	Multivariable HR (95% CI; P)*
Age group, n (%)				
1-9 years	1 078 (81.1)	86 (63.7)	Ref	Ref
10-17 years, n (%)	251 (18.9)	49 (36.3)	2.38 (1.68–3.38; <0.001)	2.22 (1.55–3.19; <0.001)
Sex, n (%)				
Male	726 (54.6)	66 (48.9)	Ref	Ref
Female	603 (45.4)	69 (51.1)	1.25 (0.89–1.75; 0.19)	1.37 (0.97–1.93; 0.07)
Immunophenotype, n (%)				
BCP ALL	1 168 (87.9)	106 (78.5)	Ref	Ref
T–cell	161 (12.1)	29 (21.5)	1.99 (1.32–3.00; 0.001)	1.33 (0.65–2.72; 0.43)
***CNS status, n (%)**				
CNS 1	1 159 (87.2)	110 (81.5)	Ref	Ref
CNS 2 or 3	166 (12.5)	25 (18.5)	1.59 (1.03–2.46; 0.04)	1.42 (0.91–2.22; 0.12)
****Induction therapy, n (%)**				
Prednisolone	1 080 (81.3)	96 (71.1)	Ref	Ref
Dexamethasone	237 (17.8)	39 (28.9)	1.84 (1.27–2.67; 0.001)	1.26 (0.66–2.40; 0.48)

CNS: central nervous system, HR: hazard ratio, CI: confidence interval, BCP =B-cell precursor. *data missing for 4 controls, **3 controls received other induction, data missing for 9 controls. *Source: Anastasopoulou S. et al. Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes. Haematologica. 2022 Oct 1. Permission has been granted.*

5.3.3 Recurrent CNS toxicity

Twelve of 135 patients with CNS toxicity (8.9%) had recurrent CNS toxicity after re-exposure to treatment: six patients with initially PRES subsequently displayed: seizures (3), PRES (1), seizures and hemiplegia (1), methotrexate-related encephalopathy (1); four patients with initially isolated seizures subsequently displayed: seizures (suspect in one case) (2), encephalopathy (1), SLS (1); one patient with initially encephalopathy subsequently displayed a new episode of encephalopathy; one patient with aseptic meningitis subsequently displayed a new episode aseptic meningitis.

5.3.4 Outcome

Median time for last follow-up after seizures was 4.4 years (range: 0.1–9.2 years). Twelve of 103 survivors (11.7%) with CNS toxicity were reported to have had epilepsy at last follow-up (seven with PRES, three with seizures, one with hypertensive encephalopathy and one with CNS infection). Clinical suspicion of impaired cognition was reported for 12/110 survivors (10.9%) with CNS toxicity, but only two had been evaluated systematically (four with PRES, three with CSVT, two with isolated seizures, one with CNS infection, one with aseptic meningitis, and one with symptoms affecting cognition); two of these patients were among the ones who were reported to have had epilepsy at last follow-up. One patient with ICH had spastic tetraparesis and one patient with PRES had right-sided hemiplegia.

5.3.5 Genome-wide association studies

One-hundred nine of 135 patients with CNS toxicities, of whom 67 had seizures, and 1057 were controls participated in GWAS studies. All cases with CNS toxicities and cases with seizures were tested separately. In the group of all CNS toxicities, five SNP passed the suggestive but not the Bonferroni-corrected threshold. In the group of seizures, 12 SNP passed the suggestive but not the Bonferroni-corrected threshold (Table 7). Overall, 18/50 of the most important SNP in the all CNS toxicities groups and 13/50 most important SNP in the seizures group were mapped to genes with neurological or neuropsychological and developmental disorders. Functional enrichment testing of genes in which SNP related to all CNS toxicities or seizures were located showed no significant gene set overlaps.

Table 7. Top SNP identified by GWAS related to all CNS toxicities and seizures with significance level P<5e-06.

	SNP	chr	Gene (BP distance from gene)	Effect allele (minor)	Reference allele (major)	MAF	OR	P
All CNS toxicities								
	rs72798143	2	*AC068490.2*	A	C	0.08	2.88	1.11e-06
	rs79459815	4	-	A	G	0.01	11.5	2.29e-06
	rs13407218	2	*CTNNA2*	T	C	0.02	5.61	3.50e-06
	rs35916740	7	-	G	T	0.09	2.63	3.71e-06
	rs62325077	4	-	C	A	0.11	2.43	4.89e-06
Seizures								
	rs75487096	3	*KIAA0226*	C	T	0.02	7.01	2.11e-06
	rs16936423	9	-	G	A	0.03	4.68	2.27e-06
	rs116011797	5	-	T	C	0.02	7.36	2.46e-06
	rs114884102	6	-	T	C	0.01	9.24	2.78e-06
	rs79566233	6	-	G	A	0.01	9.23	2.81e-06
	rs78682412	8	-	A	G	0.05	3.62	2.97e-06
	rs17641985	13	*AL355390.1 LINC00381* (2394)	C	T	0.01	8.03	3.48e-06
	rs16936230	9	*RP11-443B9.1 pseudogene* (*3432*)	G	A	0.03	4.48	4.09e-06
	rs1528779	2	-	C	T	0.48	0.39	4.14e-06
	rs353999	19	*SUMO1P4 pseudogene* (3944)	A	G	0.29	2.32	4.24e-06
	rs10478527	5	*RP11-510I6.2 pseudogene* (*1545*)	G	A	0.32	2.35	4.87e-06
	rs12340816	9	-	G	T	0.03	4.41	4.91e-06

SNP: single nucleotide polymorphism, chr: chromosome, GWAS: Genome-wide association studies, CNS=central nervous system, MAF=minor allele frequency, BP=base pair, OR=odds ratio. *Source: Modified from Anastasopoulou S. et al. Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes. Haematologica. 2022 Oct 1. Permission has been granted.*

5.3.6 Candidate SNP analyses

Two SNP, *GRIK1*rs2833098 and *ATXN1*rs68082256, were both associated with generalized epilepsy and had significant associations with seizures. However, the statistical significance of the associations did not survive after adjusting for multiple testing by Benjamini-Hochberg (Table 8). Stratification by age group showed significant association with seizures for *ATXN1*rs68082256 (P =0.01) in patients <10 years and trend of significant association with seizures in this patient group for *GRIK1*rs2833098 (P =0.06).

Table 8. Candidate SNP with association to seizures before FDR.

SNP	Gene (chr)	Effect allele	Reference allele	MAF (minor allele)	OR	P- value	FDR
rs2833098	*GRIK1* (21)	A	G	0.37 (G)	0.71	0.04	0.36
rs68082256	*ATXN1* (6)	A	G	0.19 (A)	0.47	0.01	0.13

SNP=single nucleotide polymorphism, chr: chromosome, MAF=minor allele frequency, OR=odds ratio, FDR=false discovery rate. *Source: Modified from Anastasopoulou S. et al. Acute central nervous system toxicity during treatment of pediatric acute lymphoblastic leukemia: phenotypes, risk factors and genotypes. Haematologica. 2022 Oct 1. Permission has been granted.*

5.3.7 Validation study

The most significant SNP from GWAS, 12 related to seizures and 5 related to all CNS toxicities, as well as the two candidate SNP *ATXN1*rs68082256 and *GRIK1*rs2833098 related to epilepsy were included. *ATXN1*rs6802256 was associated with diverse CNS toxicities (P =0.04), but not with methotrexate-related CNS toxicity.

5.4 PAPER IV

5.4.1 Incidence of CNS toxicities

Thirty-three of 320 patients (10.3%) in the CNS1 group had at least one CNS toxicity including 18 with seizures and 16 with PRES (of whom 14 had seizures). Thirty-eight of 370 patients (10.3%) in the whole group had at least one CNS toxicity, including 22 with seizures and 18 with PRES (of whom 16 had seizures).

5.4.2 Clinical characteristics of patients with CNS1 with and without leukemic cells in CSF by FCI

Leukemic cells by FCI were significantly more common in patients stratified as high-risk at diagnosis (white blood cell count [WBC] ≥100 x 10^9/L and/or T-cell immunophenotype) who received induction with dexamethasone (Table 9).

Table 9. Comparison of clinical characteristics in patients with ALL and CNS1 status with and without leukemic cells in the cerebrospinal fluid by flow cytometric immunophenotyping.

Patients	ALL	$CNS1_{flow-}$	$CNS1_{flow+}$	P*
Total (n) **Age median and range (years)**	320 4.0 (1.0-17.0)	256 4.0 (1.0–17.0)	64 4.0 (1.0-16.0)	 0.647
Sex				
Male (%)	173 (54.1)	145 (56.6)	28 (43.8)	
Female (%)	147 (45.9)	111 (43.4)	36 (56.3)	0.064
WBC				
<100x10^9/L (%)	291 (90.9)	244 (95.3)	47 (73.4)	
>100x10^9/L (%)	29 (9.1)	12 (4.7)	17 (26.6)	<0.001
Immunophenotype				
BCP (%)	286 (89.4)	238 (93.0)	48 (75.0)	
T-cell (%)	34 (10.6)	18 (7.0)	16 (25.0)	<0.001
Induction therapy**				
Prednisolone (%)	266 (84.7)	226 (89.7)	40 (64.5)	
Dexamethasone (%)	48 (15.3)	26 (10.3)	22 (35.5)	<0.001
Stratification into block treatment at the end of induction				
Non-block-treatment (%)	272 (85.0)	220 (85.9)	52 (81.3)	
Block-treatment (%)	48 (15.0)	36 (14.1)	12 (18.8)	0.348

*P calculated by Mann-Whitney U test for age and WBC and by Chi-square for sex, immunophenotype, induction therapy, and stratification into block-treatment at the end of induction. **Missing values for 6 patients. ALL =acute lymphoblastic leukemia, CNS=central nervous system, CNS1 =patients without CNS leukemia by cytomorphology, CNS1flow- =patients with CNS1 and negative flow cytometric immunophenotyping, $CNS1_{flow+}$ =patients with CNS1 and positive flow cytometric immunophenotyping, WBC =white blood cells, BCP =B-cell precursor. *Source: Anastasopoulou S. et al. Does minimal central nervous system involvement in childhood acute lymphoblastic leukemia increase the risk for central nervous system toxicity? Pediatr Blood Cancer. 2022 Jul.* Permission has been granted.

5.4.3 The role of minimal CNS leukemia in CNS1 patients

Minimal CNS leukemia ($CNS1_{flow+}$) was a significant risk factor for CNS toxicities in all three groups (PRES, seizures, all CNS toxicities) in univariate analyses and for seizures and PRES also after adjusting for induction therapy (Table 10). Minimal CNS leukemia remained a significant risk factor for all three groups after adjusting separately for age (Table 10). Minimal CNS leukemia remained a significant risk factor for PRES after induction after adjusting for stratification to block-treatment, but the cohort was too small for confident conclusions (HR =6.21; 95% CI: 1.75–22.03; P=0.005). Minimal CNS leukemia was associated with seizures during induction compared to those with no minimal CNS leukemia ($CNS1_{flow-}$), but the number of cases was too small for confident conclusions (seizures, n =5: OR =6.34; 95% CI: 1.035–38.844; P =0.046).

Table 10. Risk of CNS toxicity in cases with CNS1 and leukemic cells in the cerebrospinal fluid by flow cytometric immunophenotyping.

	Controls n	**Cases n**	**Univariate HR (95% CI; P)**	**Multivariate HR (95% CI; P)***	**Multivariate HR (95% CI; P)****
$CNS1_{flow+}$ vs $CNS1_{flow-}$					
All CNS toxicities	287	33			
$CNS1_{flow-}$, n (%)	234 (81.5)	22 (66.7)	Ref	Ref	Ref
$CNS1_{flow+}$, n (%)	53 (18.5)	11 (33.3)	2.08 (1.01–4.28; 0.048)	2.35 (1.13–4.87; 0.022)	1.90 (0.88–4.10; 0.101)
Seizures	287	18			
$CNS1_{flow-}$, n (%)	234 (81.5)	10 (55.6)	Ref	Ref	Ref
$CNS1_{flow+}$, n (%)	53 (18.5)	8 (44.4)	3.34 (1.32-8.47; 0.011)	3.83 (1.50–9.76; 0.005)	3.33 (1.26–8.82; 0.016)
PRES	287	16			
$CNS1_{flow-}$, n (%)	234 (81.5)	7 (43.8)	Ref		Ref
$CNS1_{flow+}$, n (%)	53 (18.5)	9 (56.3)	5.30 (1.98-14.24; <0.001)	6.13 (2.26–16.64; <0.001)	4.85 (1.71–13.75; 0.003)

*Adjusted for age. **Adjusted for induction therapy. High-risk induction with dexamethasone included 3 weeks dexamethasone 10mg/m2 as opposed to non-high–risk induction with four weeks of prednisolone 60mg/m2, otherwise there were no differences in systemic treatment. CNS =central nervous system, CNS1=patients without CNS leukemia by cytomorphology, $CNS1_{flow+}$ =patients with CNS1 and positive flow cytometric immunophenotyping, $CNS1_{flow-}$ =patients with CNS1 and negative flow cytometric immunophenotyping, HR =hazard ratio, CI =confidence interval, PRES= posterior reversible encephalopathy syndrome. *Source: Anastasopoulou S. et al. Does minimal central nervous system involvement in childhood acute lymphoblastic leukemia increase the risk for central nervous system toxicity? Pediatr Blood Cancer. 2022 Jul. Permission has been granted.*

5.4.4 The role of minimal CNS leukemia by FCI regardless of CM classification

Leukemic cells in the CSF by FCI regardless of CM classifications increased the risk of seizures and PRES in univariate analyses and after adjusting for age compared to not having leukemic cells in the CSF by CSF. Leukemic cells in the CSF by FCI remained a significant risk factor for PRES after adjusting for type of induction therapy (Table 11).

Table 11. Risk of CNS toxicity in cases with and without CNS involvement by flow cytometric immunophenotyping (CNS_{flow+} vs CNS_{flow-}).

	Controls n	***Cases n***	***Univariate HR (95 % CI; P)***	***Multivariate HR (95 % CI; P)****	***Multivariate HR (95 % CI; P)*****
CNS_{flow+} vs CNS_{flow-}					
All CNS toxicities	332	38			
CNS_{flow-}, n (%)	246 (74.1)	24 (63.2)	Ref	Ref	Ref
CNS_{flow+}, n (%)	86 (25.9)	14 (36.8)	1.60 (0.83-3.10; 0.161)	1.86 (0.95-3.63; 0.068)	1.38 (0.69-2.78; 0.368)
Seizures	332	22			
CNS_{flow-}, n (%)	246 (74.1)	11 (50)	Ref	Ref	Ref
CNS_{flow+}, n (%)	86 (25.9)	11 (50)	2.72 (1.18-6.28; 0.019)	3.24 (1.39-7.55; 0.007)	2.39 (0.99-5.78; 0.053)
PRES	332	18			
CNS_{flow-}, n (%)	246 (74.1)	8 (44.4)	Ref	Ref	Ref
CNS_{flow+}, n (%)	86 (25.9)	10 (55.6)	3.42 (1.35-8.66; 0.010)	4.05 (1.57-10.42; 0.004)	3.01 (1.31-8.01; 0.027)

*Adjusted for age. **Adjusted for induction therapy. CNS=central nervous system, CNS_{flow+}=patients with CNS involvement by flow cytometric immunophenotyping, CNS_{flow-}=patients without CNS involvement by flow cytometric immunophenotyping, HR=hazard ratio, CI=confidence interval, PRES=posterior reversible encephalopathy syndrome. *Source: Anastasopoulou S. et al. Does minimal central nervous system involvement in childhood acute lymphoblastic leukemia increase the risk for central nervous system toxicity? Pediatr Blood Cancer. 2022 Jul (supplementary appendix). Permission has been granted.*

6 DISCUSSION

6.1 ALL REPORTED CNS TOXICITIES DURING THE TREATMENT OF PEDIATRIC ALL

Acute neurotoxicity during pediatric ALL treatment is common and most cases occurred within the first six months of treatment. The incidence of all acute CNS toxicities in the NOPHO ALL2008 protocol (9.2%) is within the reported interval in literature. Posterior reversible encephalopathy syndrome was clearly the most common CNS toxicity, as was also reported in another Finnish study[15]. However, in other cohorts, for example the Australian cohort in Paper III, PRES is not as frequent or not described at all, which could possibly reflect different protocols having different doses and schedules of neurotoxic medications, or less access to MRI diagnostics at the acute phase, difficulties in differential diagnoses of MRI images or less awareness of the syndrome[25]. The second-most frequent CNS toxicity was CSVT, which in many other studies is not counted as CNS toxicity but is considered to be thromboembolic event—including in the PdL classification[25]. Here, we included CSVT in the list of CNS toxicities, as symptoms during the event are predominantly neurological.

Age group 10-17 years, T-cell immunophenotype, induction therapy with dexamethasone and CNS leukemia were significant risk factors for CNS toxicity in univariate analyses. Older age group remained a significant risk factor for CNS toxicity in multivariable analyses, which may reflect the fact that older children more often have high-risk leukemia and receive more aggressive treatment as well as age-dependent pharmacokinetics[120-122].

One-hundred and nine patients with diverse CNS toxicities participated in GWAS analyses that did not show any significant associations with CNS toxicities as a group. We did not proceed to candidate SNP analyses for the group of patients with diverse CNS toxicities in the NOPHO cohort because they did not reflect a homogenous phenotype.

6.2 PRES DURING THE TREATMENT OF PEDIATRIC ALL

Posterior reversible encephalopathy syndrome was the most common form of CNS toxicity during the treatment of pediatric ALL. It is a neurologic emergency, which requires acute symptomatic treatment with ASM, antihypertensives, eventually antibiotics or antiviral medications and ICU support in severe cases.

The typical edema-associated changes may be located in various cerebral regions, not only the posterior ones, and therefore various neurological deficits may be expected. The gold standard in neuroimaging for PRES diagnoses is a brain MRI. Brain CT is commonly a more accessible investigation but may fail to identify PRES-related changes. The most common symptom of PRES were seizures, and the most common sign was hypertension, both of which were in line with results previously reported in the literature.

Older age, both as each extra year of age and as age group >10 years, was a significant risk factor in the univariate analysis for PRES. T-cell immunophenotype and induction therapy with dexamethasone were significant risk factors for PRES in univariate analyses. T-cell immunophenotype is one of the criteria to stratify ALL patients to high-risk induction with dexamethasone at diagnosis, but it is not clear to which extent each factor alone or the combination of both increase the risk of PRES. Unfortunately, the PRES group in Paper I was small and simultaneous adjustments with all risk factors would not be reliable. We were, however, able to perform multivariate analysis for two factors each time that showed that T-cell immunophenotype was still significant risk factor for PRES after adjusting for age.

Interestingly, while CNS leukemia by CM was shown to be a significant risk factor only for early PRES in Paper I, when we explored minimal CNS leukemia by FCI in Paper IV patients with $CNS1_{flow+}$ had a higher risk for PRES overall. This finding suggests that CNS leukemia, even without enhanced intrathecal treatment, is associated with PRES. The reason is yet unknown, but possible mechanisms may include higher concentrations of methotrexate in the CNS related to CNS leukemia and increased cerebrovascular permeability mediated of increased levels of VEGF produced by leukemic cells[123-126].

Unfortunately, we could not explore potential SNP associations to PRES in Paper III since the number of cases was inadequate. Larger patient groups with PRES may help illuminate this aspect.

6.3 SEIZURES DURING THE TREATMENT OF PEDIATRIC ALL

Seizures were the most common CNS symptom during treatment of pediatric ALL in our study. The most common underlying pathology for seizures was PRES, followed by isolated seizures and CSVT. Neuroimaging evaluations, preferably with brain MRI that was shown to be a more sensitive examination for PRES, are essential for work-up of seizures along with EEG and standard laboratory analyses. Treatment is symptomatic, including ASM as appropriate, treatment of underlying pathology and ICU support when needed.

Older age, T-cell immunophenotype, CNS leukemia, and induction therapy with dexamethasone were significant risk factors for seizures in univariate analysis. Older age remained a significant risk factor for seizures after multivariate analyses.

Sixty-seven patients with seizures participated in GWAS and candidate SNP analyses. No significant associations were demonstrated by GWAS but *ATXN1*rs68082256, a SNP previously associated to epilepsy, was associated to seizures in patients younger than ten years[119]. Furthermore, *ATXN1*rs68082256 was replicated in the Australian cohort in the patient group with diverse CNS toxicities (data on patients with seizures in this group were not available), but not in the patient group with methotrexate-related CNS toxicity. The role of *ATXN1* gene encoding ataxin-1 protein, which is the gene underlying spinocerebellar ataxia type 1 and is implicated in epilepsy, merits further investigations[119,127].

6.4 MINIMAL CNS LEUKEMIA AND RISK FOR CNS TOXICITIES

The detection of leukemic cells in the NOPHO ALL2008 protocol is made by CM. In Papers I–III CNS leukemia by CM (CNS2 or CNS3) was a risk factor for early PRES, seizures, and CNS toxicities respectively in univariate analyses. As patients with CNS leukemia by CM receive enhanced CNS-directed treatment, it is unclear whether CNS leukemia, *per se*, the chemotherapeutic agents, or the combination of the two factors increase the risk of CNS toxicities.

In Paper IV, minimal CNS leukemia by FCI in patients without CNS leukemia by CM, and thus without enhanced CNS directed treatment ($CNS1_{flow+}$), was alone a significant risk factor for PRES, seizures, and all CNS toxicities in univariate analyses and for PRES and seizures after adjusting for induction therapy with dexamethasone.

This finding, together with previous results demonstrating that minimal leukemia is related to relapse, advocates the use of advanced diagnostic techniques for detection of CNS leukemia as well as discussions about how CNS-directed treatment should be tailored to these patients[98].

6.5 OUTCOME

Twelve patients were reported to have had epilepsy diagnosis at last follow up. This could be speculated to reflect a possible underlying genetic susceptibility for epilepsy among ALL patients, especially with the knowledge that at least one previously related to epilepsy SNP may be associated with seizures in pediatric ALL patients[119]. However, as we do not know the local criteria of epilepsy diagnosis at each center or when attempts to stop ASM medication were performed, we cannot make any firm conclusions.

As many patients, two of whom were among the ones reported to have had epilepsy, were suspected to have some cognitive difficulties, but systematic evaluation was made only in two cases. There are several studies indicating the same thing, but survivors are still not monitored for neurocognitive impairment after treatment[27,28,110-113]. The neurocognitive follow-up of patients is an ongoing discussion among child oncologists and will hopefully be integrated into protocols in the future.

Two patients were reported to have severe neurological impairment at last follow-up. One of them had right-sided hemiplegia after having PRES, and another had spastic tetraparesis following ICH. Ongoing efforts to optimize treatment protocols have as goal to minimize risks of severe sequelae for survivors.

6.6 LIMITATIONS

6.6.1 Phenotypes and reported toxicities

The occurrence of the CNS toxicities was reported prospectively in the toxicity registration system, but detailed data collection from patient files occurred retrospectively, in many cases several years later, which may impair data quality and is one limitation of the study. Both study nurses and pediatricians in the participating centers supplied the registry and the complementary questionnaire data, which may have contributed to different interpretations of the questions or the events and under-reporting of subtle CNS toxicities, for example mild seizures or short periods of encephalopathy. Two child neurologists and two child oncologists evaluated all data for patients with CNS toxicities according to the PdL classification, and current nomenclature criteria prior to final classification for this thesis. When we assessed the questionnaires and registry data to identify patients with seizures for Paper II, 81 patients were reported to have had seizures. However, when we assessed all CNS toxicities for Paper III there was a minor comment in one patient reported as having SLS as first CNS toxicity, which marked that the patient had an episode with seizures prior to SLS. Thereby we re-classified this patient as having seizures as the first CNS toxicity episode in Paper III.

6.6.2 Epilepsy diagnosis

Local routines in continuation of ASM of patients who displayed seizures varies between centers, which may affect the epilepsy diagnosis.

6.6.3 Neuroimaging studies

We did not have access to images of the CNS, instead the results of the neuroimaging examinations in this thesis are based on reports from each center. Moreover, we report real-world data, where all patients were not systemically examined by neuroimaging. Also, the symptoms of the different CNS toxicities overlap. Therefore, we cannot be sure that some cases with isolated seizures or hypertensive encephalopathy did not have PRES or methotrexate-related SLS, especially with missing imaging studies.

6.6.4 Size of the cohort and groups with CNS toxicities

The size of the study, especially the group of patients with PRES was not sufficient to allow multivariate analyses in Paper I and get conclusive results in GWAS studies in Paper III. Likewise, the size of the cohort in Paper IV was small and did not allow confident conclusion for some findings.

7 CONCLUSION

Posterior reversible encephalopathy syndrome is the most common form of CNS toxicity during treatment of pediatric ALL, according to NOPHO ALL2008 protocol, and seizures are its most common neurological symptom. Seizures are, moreover, the most common symptom of CNS toxicity during treatment of pediatric ALL. Brain MRI is the gold standard of neuroimaging in work-up of PRES and seizures. Older age is independent significant risk factor for PRES, seizure, and all CNS toxicities. Leukemic cells in the CNS are a risk factor for PRES and seizures, even at a low level. The role of the *ATXN1* gene and *ATXN1*rs68082256 SNP in seizures in ALL patients needs to be further examined. Survivors of pediatric ALL may require follow-up over long period of time, primarily due to epilepsy, neurocognitive impairments, or neurologic deficits due to permanent brain injury following CNS toxicity.

8 POINTS OF PERSPECTIVE

In an era with favorable pediatric ALL outcomes, CNS toxicity is still a clinical challenge. Clinicians should be aware of the wide spectrum of CNS toxicities in pediatric patients with ALL so they can promptly proceed to differential diagnoses and appropriate treatment. Brain MRI should be prioritized in the work-up of CNS toxicity as brain CTs may be inconclusive.

Advantages in genetic science and precision medicine will hopefully facilitate personalized treatments with high effectiveness. Studies of larger cohorts through international collaborations should investigate the genetic or pharmacogenetic background of CNS toxicities in ALL to contribute to personalized treatment with a minimal risk of complications.

The suspicion of neurocognitive deficits among survivors should motivate further studies to map it. Institutes should start exploring the inclusion of neurocognitive screening of all pediatric ALL survivors, or at least the ones for whom clinical suspicion of neurocognitive impairment is strong, to recognize deficits and initiate supporting measures in a timely fashion.

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